The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Righetti, Karima Maria (2011) Study of Rsm/Gac post-transcriptional regulation by quorum sensing, extracellular and intracellular signals in Pseugomonas aeruginosa. PhD thesis, University of Nottingham.

Access from the University of Nottingham repository:
http://eprints.nottingham.ac.uk/13853/1/546517.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

- Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners.
- To the extent reasonable and practicable the material made available in Nottingham ePrints has been checked for eligibility before being made available.
- Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.
- Quotations or similar reproductions must be sufficiently acknowledged.

Please see our full end user licence at:
http://eprints.nottingham.ac.uk/end_user_agreement.pdf

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

10 0659625 1

The University of Nottingham

Study of Rsm/Gac post-transcriptional regulation by Quorum sensing, extracellular and intracellular signals in *Pseudomonas aeruginosa*

Karima Maria Righetti

MEDICAL LIBRARY
QUEENS MEDICAL CENTRE

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

January 2011

THESIS CONTAINS

CD

BEST COPY

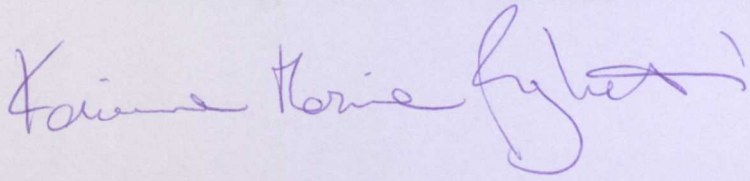
AVAILABLE

Some text bound close to the spine.
Some images distorted

DECLARATION

Unless otherwise acknowledged, the work presented in this thesis is my own. No part has been submitted for another degree at the University of Nottingham or any other institute of learning.

Karima M. Righetti



ACKNOWLEDGMENTS

First of all, I would like to express my gratitude towards Prof. Paul Williams and Prof. Miguel Cámara for offering me the possibility to carry out a PhD-project, then supervising and supporting me during the course of this PhD.
Many thanks to Dr. Stephan Heeb for his useful help and advice.
Thanks to my internal examiner, Prof. Nigel Minton, and to my external examiner, Dr. Christophe Bordi.

I would also like to thank everybody in the *Pseudomonas* Research group for support, especially my colleagues and friends Marco Messina and Christian Pustelny.
Thanks to everybody from the Marie Curie Antibiotarget group, in particular to the Marseille group, Dr. Christophe Bordi and Romé Voulhoux for taking care of me while I was visiting the CNRS institute.

Special thanks to:

Teratakia, two lines are not enough to thank you for the time you spent helping me. You have been noz only the first Cosmologist reading a Molecular Biology thesis, buz a great guru. If there is something I will miss about the writing up period is the time we spent looking for the "unknown phage fragment" and the "missing commas"!

Sister-Nara, Iruru-Pistacchietto and Asereje', you were always there girls!
Emanuela, Giordano, Marcolino.
Da General, Fernandito-Miao and Alkistoula, you made "number eeeeight" a home!
Pashou's Limoncello, you cheered me up in the darkest moments
Brontola for taking me to the lab in the worse weather conditions

Finally, thanks to all my Faunistic friends!!!

to Baboomba

ABSTRACT

Study of Rsm/Gac post-transcriptional regulation by Quorum sensing, extracellular and intracellular signals in *Pseudomonas aeruginosa*

Bacteria have evolved ways to sense and respond to changes in their population density through quorum sensing (QS) systems, and to adapt to changes in the extracellular environment through two component systems (TCS). In *Pseudomonas aeruginosa*, QS and the GacS/GacA TCS are global regulatory systems that modulate the expression of virulence genes at the transcriptional and post-transcriptional level, respectively. Although in *P. aeruginosa* the QS network has been extensively characterized, the way the Gac/Rsm global regulatory system is regulated is still unclear. The study of QS and Gac/Rsm networks is crucial for the development of new drugs able to interfere with these regulatory systems.

This thesis is dedicated to the study of the Gac/Rsm global regulatory system and its interaction with the QS network. An introduction to these systems is presented in **Chapter 1**.

The materials and methods used in this study are described in **Chapter 2**.

In **Chapter 3** the methods to detect and identify the extracellular signals modulating Gac/Rsm system are investigated. This analysis led to the identification of the Pseudomonas Quinolone Signal (PQS) molecule, which is responsible for the activation of the gene coding for the small RNA RsmZ. RsmZ (in synergy with RsmY) antagonises by titration the effects of the global post-transcriptional regulator RsmA, a small RNA-binding protein which targets specific mRNAs.

Since the discovery of QS, there have been many studies showing the importance of this type of regulatory mechanism in the global transcriptional control of gene expression. However, there has been no clear evidence to attribute to QS a key role in post-transcriptional regulation of terminal gene targets. In **Chapter 4**, the importance of PQS in the control of *lecA* is demonstrated. *lecA* encodes for the PA-I galactophilic lectin protein whose translation rates is modulated by the activity of the regulatory small RNA *rsmZ* in concert with RsmA. These results demonstrate that QS not only controls terminal target gene expression at the transcriptional level, but also at the translational level.

Using a genetic bank, a transposon mutagenesis and a promoter pull-down approach, new regulators were identified together with regulatory networks involved in the modulation of the global Gac/Rsm system. These results are described in **Chapter 5**.

Chapter 6 is focused on the effect of a library of compounds available in our laboratory, for their QS-inhibiting potential. The conclusions and future directions are presented in **Chapter 7**.

INDEX

ABBREVIATIONS

µl	Microlitre
3O-C12-HSL	N-(3-Oxo-dodecanoyl)-L-homoserine lactone
AHL	*N*-Acyl-L-Homoserine lactone
Ap^R	Ampicillin resistant
APS	Ammonium persulfate
AQ	2-Alkyl-4-quinolones
BIO	Biotynilated
Bp	Base Pairs
BSA	Bovine serum albumin
C4-HSL	N-Butanoyl homoserine lactone
$CaCl_2$	Calcium chloride
Cm^R	Chloramphenicol resistant
Csr	Carbon Storage Regulator
CTAB	Cetyl trimethylammonium bromide
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide triphosphate
DTT	Dithiothreitrol
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol

$FeCl_3$	Ferric chloride
Gac	Global activator of secondary metabolism
Gm^R	Gentamicin resistant
H	Hour(s)
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HHQ	2-heptyl-4-quinolone
HPLC	High Pressure Liquid Chromatography
HSL	Homoserine lactone
IPTG	Isopropyl-β-D-Thiogalactopyranoside
K_2HPO_4	Dipotassium phosphate
Kan^R	Kanamycin resistant
Kb	Kilobase
KCl	Potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
KNO_3	Potassium nitrate
L	Litre
LB	Luria Bertani
LC-MS	Liquid chromatography-mass spectrometry
M	Molar
mA	mili ampere
MeOH	Methanol

$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MOPS	4-Morpholinepropanesulfonic acid
mRNA	Messenger Ribonucleic Acid
NA	Nutrient Agar
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
Na_2SO_4	Sodium sulphate
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NGM	Nematode growth media
Ni	Nickel
Ni-NTA	Nickel – nitrilo-triacetic acid
Nt	Nucleotide(s)
o/n	Overnight
OD	Optical density
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PQS	Pseudomonas quinolone signal; 2-heptyl-

	3-hydroxy-4-quinolone
QS	Quorum sensing
QSI	Quorum sensing inhibitor
RBS	Ribosome binding site
RLU	Relative light units
RNA	Ribonucleic Acid
Rsm	Regulator of secondary metabolites
S-D	Shine Dalgarno
SDS	Sodium Dodecyl Sulphate
Sm^R/Sp^R	Streptomycin/spectinomycin resistant
sRNA	Small RNA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet^R	Tetracycline resistant
TLC	Thin layer chromatography
UV	Ultraviolet
V	Volts
v/v	Volume per volume
v/w	Volume per weight
Wt	Wild type
x-gal	5-bromo-chloro-3 indoyl β-D-galactoside

LIST OF FIGURES

1 Introduction

1.1 BACTERIAL VIRULENCE

A bacterium that is capable of causing a disease is defined as a bacterial pathogen and its ability to cause a disease is called pathogenicity. The quantitative assessment of pathogenicity or its potential to cause disease is measured by virulence. Bacteria produce many virulence factors that enable them to survive and establish themselves within a host, enhancing their potential to cause a disease. The first step in bacterial pathogenicity is the adhesion to the host; this is usually accomplished by the adherence of the bacteria to a surface. To achieve this, bacteria have evolved attachment mechanisms for example pili and fimbriae. Some bacteria are invasive and have developed mechanisms that enable them to invade the eukaryotic cells; others have evolved virulence factors that act as protective mechanisms enabling their survival in the host (production of capsules, biofilm formation). Furthermore, in order to evade the host's immune responses, bacteria have evolved virulence factors that inhibit the host's immune system defenses. Finally, many virulence factors consist of toxins that cause tissue damage, thus facilitating host colonization [1].

In order to fine-tune the production of all these virulence determinants, bacteria have evolved complex regulatory networks, which enable them to respond to particular environmental conditions in a very efficient and specific manner.

1.2 *PSEUDOMONAS AERUGINOSA*

Pseudomonads are Gram-negative, motile, ubiquitous bacteria capable of colonizing a wide variety of environmental niches due to their remarkable metabolic and physiological versatility [2]. These microorganisms are studied for their role as mammalian and plant pathogens, plant growth-promoting bacteria, and for their huge biodegradative potential. In a disease context, the *Pseudomonas* pathogens of humans and animals are characterized as having a "low virulence", i.e., they are opportunistic and do not produce disease unless the animal's constitutive or immune defenses are compromised [3]. This does not mean that they lack any determinants of virulence. *P. aeruginosa* indeed contains a number of structural, biochemical and genetic properties that contribute to its virulence, which may be compared to those of leading pathogens such as *Staphylococcus* or *Streptococcus* [4].

In this genus, most attention from scientists has been focused on the opportunistic human pathogen *P. aeruginosa*. This microorganism is a highly adaptable bacterium that can colonize various environmental niches, including soil and marine habitats, plants, animals and humans [5]. Its versatility is reflected by its ability to tolerate low oxygen conditions and to grow at a wide range of temperatures [6]. The ability of *P. aeruginosa* to colonize and thrive in diverse environments is reflected by its relatively large genome (6.3 Mbp) and genetic complexity (at least 5570 open reading frames, ORFs) [5]. Compared to the majority of known sequenced bacterial genomes, the genome of *P. aeruginosa* possesses a larger overall number of genes coding for outer membrane proteins, efflux systems and multiple chemotaxis systems, which may contribute to its pathogenesis. Moreover, about 10% of the predicted ORF are predicted to be gene expression regulators, reflecting the ability of *P. aeruginosa* to respond and adapt to sudden and constant environmental fluctuations [5, 7].

P. aeruginosa may cause acute infections in hospitalized patients and is often associated with infections of immuno-compromised individuals, for example those affected by AIDS, cancer,

or severe burn wounds. It also causes many chronic infections, for example hospital-acquired pneumonia in patients receiving artificial ventilation and keratitis in users of soft contact lenses [5, 8]. Moreover, it has recently been recognized as one of the main causes of chronic wounds [9] and as the predominant cause of mortality in cystic fibrosis (CF) patients [10, 11]. CF is a genetic disorder affecting approximately 1 in 2,500 individuals, primarily Caucasians, and is a consequence of a mutation in the gene coding for the transmembrane conductance regulator, CFTR. The inability to regulate sodium and chloride transport due to an aberrant CFTR increases airway secretion viscosity. *P. aeruginosa* find a favorable niche in the lung within the resulting thick mucus [12]. Indeed, there is evidence of lung colonization by *P. aeruginosa* in 95% of CF affected children [13].

According to the National Nosocomial Surveillance System of the Centres for Disease Control and Prevention, *P. aeruginosa* infection in U.S. hospitals between 1985 and 1991 accounted for 10.1% of all hospital-acquired infections [14]. Another more recent survey indicated that, of the patients who developed nosocomial pneumonia, *P. aeruginosa* was isolated in at least 21% of cases [15].

P. aeruginosa possesses and produces a large variety of both cell-associated and extracellular virulence factors, a characteristic that reflects the various clinical diseases caused by this typical nosocomial pathogen [4]. Evaluations in various animal models of the roles of these different virulence factors led to the understanding that pathogenesis of *P. aeruginosa* is not related to a single virulence factor, but on the precise and delicate interplay between different factors, leading from efficient colonization to tissue necrosis, invasion and dissemination through the vascular system, as well as to activation of both local and systemic inflammatory response (Fig. 1) [4].

P. aeruginosa is capable of causing both chronic and acute infections by regulating the virulence determinants required for each of this type of infection [16]. In particular, chronic infections are defined as the one where the bacteria are mostly organized in biofilm structures, such as in the case of CF lung infections [17]. On the other hand, acute *P. aeruginosa* infections are characterized by bacterial penetration on the host epithelium and systemic spread, such as in severe burn wound infections.

1.2.1 Bacterial cell surface virulence factors

The first step in the pathogenic sequence of *P. aeruginosa* infections is the colonization of an epithelial surface [4].

Flagella are complex proteic structures forming a filamentous polar appendage at the surface of *P. aeruginosa.* As well as being involved in motility and chemotaxis, flagella have a critical role in pathogenesis by adhering to epithelial cells through the binding with a common membrane component, asialoganglioside gangliotetraosylceramide (asialoGM1) [18]. They also participate in virulence by eliciting a nuclear factor kappa B (NFkB)-dependent inflammatory response through interaction with the Toll-like receptors TLR5 and TLR2, leading to interleukin 8 (IL-8) production [19, 20]. However, flagella are also very immunogenic, making their presence an obstacle for *P. aeruginosa* once colonization is successful. This is why *P. aeruginosa* is capable of adapting by selecting aflagellar mutants to evade host response in chronic infections [21].

Pili, or fimbriae, are smaller filamentous surface appendages involved in *P. aeruginosa* motility. This flagella-independent motility, called twitching, is due to the retractile properties of *P. aeruginosa* pili and allows this bacterium to "spread" along hydrated surfaces rather

than "swim" (swimming requires flagella). This feature facilitates the rapid colonization of the airway [22]. Like flagella, pili are crucial for the adhesion phase of colonization through binding to asialoGM1 of the epithelial cell membrane [23, 24]. Studies found that both pili-mediated adherence and twitching motility are critical for *P. aeruginosa* virulence [25, 26].

Although the inner face of the outer membrane resembles a typical phospholipid bilayer, the outer face of the outer membrane is mainly composed of lipopolysaccharide (LPS). LPS is composed of a hydrophobic domain (Lipid A), inserted into the phospholipid bilayer, and a hydrophilic tail, composed by the core polysaccharide and the O-specific polysaccharide. The Lipid A component of *P. aeruginosa* LPS activates multiple pro-inflammatory pathways [27]. LPS is also critical to virulence, mainly due to its role in adhesion to various Toll-like receptors, through asialoGM1 binding [23].

Alginate is a mucoid exopolysaccharide produced by *P. aeruginosa*. It consists of repeating polymers of mannuronic and glucuronic acid. Alginate, like LPS, functions as an adhesin, anchoring *P. aeruginosa* to the colonized respiratory epithelium. Particular conditions in the CF patient's lungs and host inflammatory response increase alginate synthesis, leading to the conversion to an alginate overproducing mucoid phenotype [28]. This mucoid alginate-producing phenotype is commonly found in CF airways during *P. aeruginosa* chronic infections [29].

1.2.2 Secreted virulence factors

P. aeruginosa produces several extracellular products that, after the initial step of colonization, can cause extensive tissue damage, bloodstream invasion and dissemination. The relative contribution of a given factor may vary with the type of infection [30].

Pyocyanin is a blue pigment metabolite of *P. aeruginosa* that has numerous pathogenic effects such as increasing IL-8, depressing host-response, and inducing apoptosis in neutrophils [31-33]. In animal models of acute and chronic lung infection, pyocyanin was found to be essential for *P. aeruginosa* virulence [34]. Additionally, due to its oxidoreductive properties, pyocyanin oxidizes glutathione and inactivates catalase in respiratory epithelial cells, thus participating in oxidative-stress related damage [35].

Hydrogen cyanide (HCN) is a potent poison that blocks cytochrome oxidase, leading to the inhibition of mitochondrial respiration. This secondary metabolite of *P. aeruginosa* is responsible for rapid paralytic killing of the nematode *Caenorhabditis elegans* [36]. Its role during human infections is unclear, but it could participate in tissue destruction and might contribute to toxicity in *P. aeruginosa* infected burn wounds [37].

Pyoverdine and pyochelin are siderophores, small molecules chelating iron from the iron-poor environment encountered in the host, allowing its utilization in *P. aeruginosa* metabolism. Siderophore-dependent iron acquisition is key for the production of biofilm and for virulence in different animal models, possibly by regulating the secretion of other *P. aeruginosa* virulence factors [38].

Exotoxin A (ExoA) is an ADP-ribosyl transferase secreted into the extracellular space that inhibits the elongation factor-2 (EF-2), thereby inhibiting protein synthesis and leading to host cell death [39]. ExoA has also been found to depress the host response to infection [40].

Among a wide range of proteases, alkaline protease is one of the most important. This enzyme is a fibrin-lysing protease secreted by *P. aeruginosa.* Alkaline protease has been shown to degrade the complement components, as well as interferon Y [41].

The ability of *P. aeruginosa* to destroy elastin is considered to be a major virulence determinant during acute infection. The concerted activity of two enzymes, LasA and LasB

elastases, is responsible for elastolytic activity [42]. These enzymes, secreted into the extracellular space, have been discovered to have a role in the pathogenesis of *P. aeruginosa* respiratory infections by damaging the respiratory epithelium through tight-junction destruction, thus increasing epithelial permeability and facilitating neutrophil recruitment [43]. Elastases are also pro-inflammatory factors increasing IL-8 levels [44].

Among the hemolysins produced by *P. aeruginosa*, phospholipase C and rhamnolipids have been studied extensively. These virulence factors contribute to tissue invasion by cytotoxic effects [45].

1.2.3 Type III secretion system

Type III secretion systems are used by *Yersinia*, *Salmonella*, *Shigella* and *Pseudomonas* species as mechanisms to directly inject toxins into the host cells. The type III secretion system (TTSS) of *P. aeruginosa* is a complex pilus-like structure that allows the translocation of effectors proteins from the bacteria into the eukaryotic cytoplasm through a needle-like appendage that forms a pore in the eukaryotic membrane [46].

The TTSS system is involved in pathogenesis through the translocation of multiple toxins (ExoS, ExoT, ExoY and ExoU) into the host cell, however, it also appears that TTSS may participate in *P. aeruginosa* virulence by itself [47]. A complex regulon is involved in the biogenesis and regulation of TTSS. This reflects the importance of TTSS for *P. aeruginosa* pathogenicity. There appear to be five types of genes: those involved in the formation of the needle complex for the transport of the toxins from the bacteria cytosol into the extracellular environment; the proteins that are involved in the transport of the toxins into the host cells;

the proteins that regulate the secretion process; the chaperone proteins that assist the secretion of the TTSS machinery and finally the effector proteins which are injected into the host cells. The type III proteins are transported from the bacterial cytosol into the extracellular enviroment through a needle like complex, which facilitate their movement past the bacterial cytoplasmic membrane, the peptidoglycan layer and the outer membrane. This needle structure is organized in a multi-ring base and a needle filament. The filament, which consist of protein PscF subunits, is thought to act as a channel through which the secreted factor move into the external environment [47].

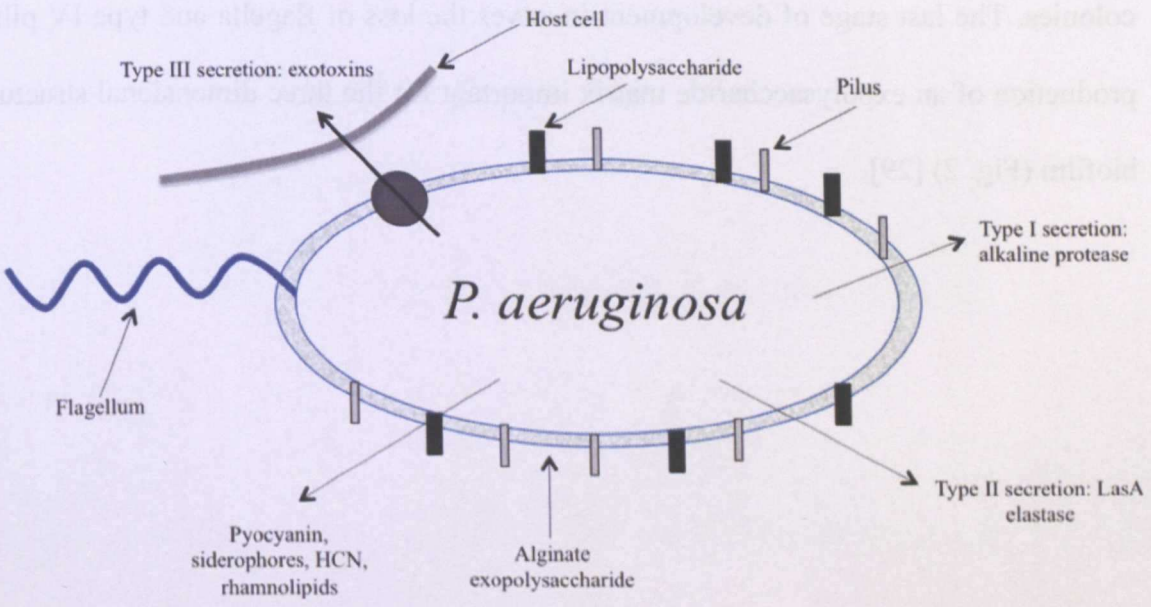


Figure 1-1. Overview of *P. aeruginosa* virulence determinants.

P. aeruginosa has both cell-associated (flagellum, pili, lipopolysaccharide, alginate) and extracellular virulence factors (elastases, exotoxin A, phopholipase, proteases, pyocyanin, siderophores HCN, and rhamonolipids). The type III secretion apparatus is used to inject exotoxins in the host cells (Figure extracted from van Delden, 2004).

1.2.4 Biofilm

When *P. aeruginosa* chronically infects patients, it adapts to the biofilm mode of growth [48]. The generally accepted definition of a biofilm is a "community of cells attached to a surface or to each other, imbedded in a self-made, protective matrix of extracellular polymeric substances (EPS)" (Fig. 2) [49]. The tendency of *P. aeruginosa* to colonize surfaces in these organized communities makes it less susceptible to antibiotics, anchors the cells to the surface they colonize and protects the bacteria from the host defenses [50, 51]. A number of components essential for biofilm formation and development have been identified (flagella, type IV pili and exopolysaccharides) [52]. Firstly, flagella enable the bacteria to swim to a specific surface and attach. Then, type IV pili allow the bacteria to move forming micro-colonies. The last stage of development involves the loss of flagella and type IV pili and the production of an expolysaccharide matrix important for the three dimensional structure of the biofilm (Fig. 2) [29].

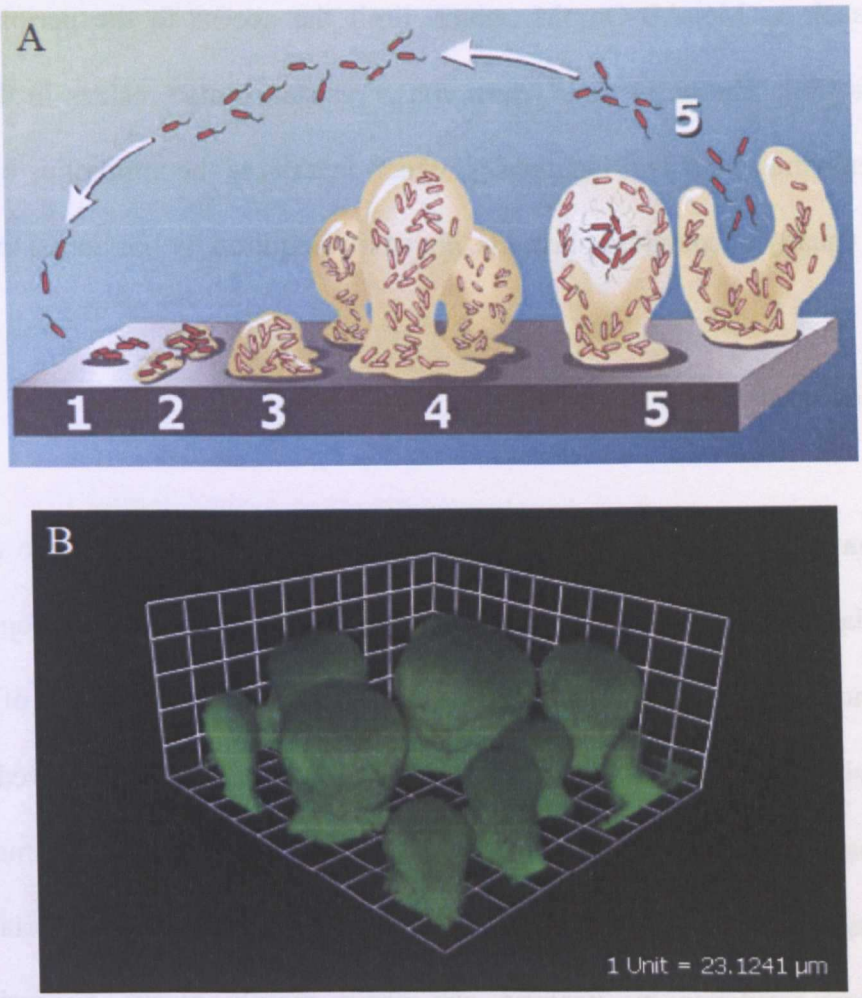


Figure 1-2. Representation of biofilm development in *P. aeruginosa*.

(A) Schematic representation of different stages of biofilm formation. In the colonization stage, planktonic cells adhere to the substrate (1) and start to proliferate forming microcolonies (2). During the maturation stage the microcolonies grow (3), forming three-dimensional structures known as "mushrooms" (4). Finally, bacterial cells leave the mushrooms leading to biofilm dispersion (5). Modified from Kiristis and Parsek, 2006. (B) Typical biofilm formed by *P. aeruginosa* PAO1.

Virulence factor production and the ability to form biofilms intertwine, making *P. aeruginosa* a particularly dangerous pathogen.

P. aeruginosa is also notorious for its intrinsic resistance to a wide range of antibiotics and for its ability to easily acquire new resistance phenotypes by genetic exchange, especially in the nosocomial environment [53]. The *P. aeruginosa* outer membrane is relatively impermeable to a wide range of antibiotics, as the outer membrane contains multidrug-efflux pumps

systems such as MexAB-OmpM, which limit the access to the periplasm of hydrophilic antibiotics [54]. The basis for *P. aeruginosa* resistance also resides in the expression of an array of enzymes such as ß-lactamases, which inactivate the antibiotics by hydrolysis. These enzymes, usually encoded on plasmids, are easily acquired by horizontal transfer [55].

1.3 REGULATION OF VIRULENCE

Bacteria have a long evolution history that allowed them to evolve into complex machines, able to adapt and respond to different conditions. In particular, pathogenic bacteria have evolved mechanisms to regulate the quick and effective production of virulence factors. Bacterial virulence has to be strictly coordinated in space and time to reduce the possibility that the host immune system will dismantle the bacterial attack, and to maximize the chance that the assault occurs in the most favorable conditions for the bacterial community. The regulation of virulence by bacteria can occur mainly at the transcriptional and post-transcriptional level.

1.3.1 Quorum sensing

Bacteria have evolved ways to quickly modulate their gene expression; this adaptability reflects their need to respond to the constantly changing environmental conditions. One way bacteria achieve this high flexibility is by regulating genes expression at the transcriptional level by using global regulators that are activated by specific stimuli, allowing them to coordinate a concerted response [56]. Among these global transcriptional regulators sigma factors (σ) represent an example (reviewed in Guber and Gross, 2003) [57, 58]; histone like

nucleotide structuring proteins (H-NS), which add stability to the DNA (reviewed by Atlung and Ingmer, 1997) [59]; signaling molecules, such as cyclic-AMP (c-AMP) or cylcic-di-GMP (c-di-GMP), which bind to a specific effector protein, activating a set of responses [60, 61].

In addition to these multiple levels of transcriptional regulation, a new system of regulation, called the quorum sensing (QS) system of communication, was described forty years ago [62]. Bacteria have been shown to posses a way of sensing and interacting with each other in a way similar to the one observed in highly evolved organisms. This interaction can be intra- or inter-species, and it probably evolved to allow co-existence of a bacterium into its host. However, in pathogenic bacteria this communication system is mainly used to coordinate a virulence response.

QS is a form of cell-to-cell communication via the use of small signaling molecules, called autoinducers. This form of regulation allows bacteria to monitor their population density by responding to the extra cellular concentration of autoinducer molecules they produce. Once a critical concentration (corresponding to a particular population density) of the signal has been reached, the whole population initiates a concerted action, activating or repressing target genes (Fig. 3). This cell-to-cell communication system has been shown to be important for biofilm formation, expression of virulence factors, synthesis of secondary metabolites, competence and colonization of superior organisms [63].

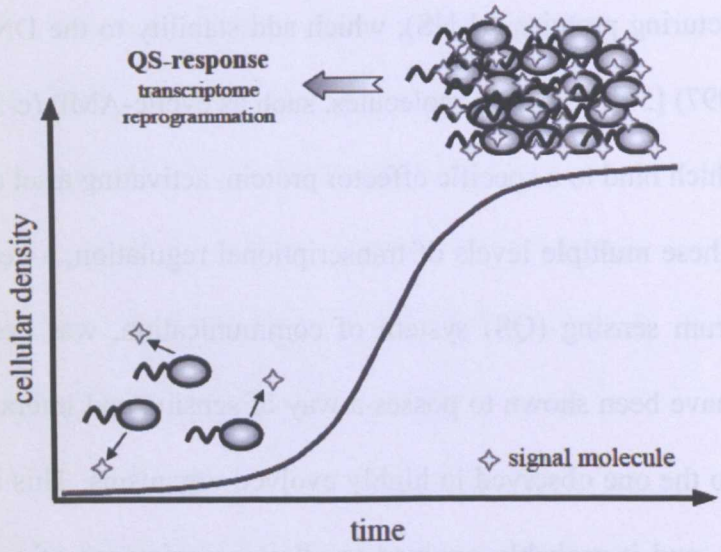


Figure 1-3. A schematic representation of QS.

The signal molecule is constitutively produced at a basal level by the bacterial cells. At low-cell densities, very little signal molecule is present. As cell density increases, the signal molecule accumulates in the media until a threshold level is reached. This signal molecule concentration is responsible for a coordinate transcriptome reprogramming in the whole bacterial population. Modified from Dunn and Stabb, 2006.

Several chemical classes of microbial derived signaling molecules have been now identified. These can be split into three main categories: 1) *N*-acyl-homoserine lactones (AHL), frequently utilized by Gram-negative bacteria, 2) aminoacids and short peptides, commonly utilized by Gram-positive bacteria, 3) the AI-2 signal, widespread in several Gram-positive and Gram-negative species [64-66].

In the AHL-dependent QS system, a single enzyme is required for the synthesis of the signal molecule. This enzyme is a synthetase, generally belonging to the LuxI family. The HSL ring is conserved, while the acyl side chain can vary in length, degree and type of substitutions, conferring strain-specificity to the signal [67]. When the cell density increases, so does the concentration of the signal molecule, until it interacts with a cytoplasmic DNA-binding receptor belonging to the LuxR family. The activated LuxR homologous protein modulates the expression of QS controlled genes [68, 69].

The first QS system that employs AHL as a signaling molecule was described in the marine bacteria *Vibrio fischeri* [62]. In this organism, QS regulates different phenotypes, among which is bioluminescence. *V. fischeri* is non-luminescent when free living in seawater (at low cell densities). When *V. fischeri* lives in symbiosis with the squid *Euprymna scolopes* (high cell densities), the bacteria activate its bioluminescence. The molecular basis of the bioluminescence induction involves an interaction between the freely diffusible signal molecule *N*-3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL) and the transcriptional regulator LuxR. *V. fischeri* cells express *luxI*, the AHL synthase gene, at a basal level when living in a low population density. In that way, the concentration of signal molecule remains low. However, as the population density increases within the confines of the hosts light organ, so does the concentration of 3-oxo-C6-HSL in the immediate environment. Once the critical concentration of the signal molecule has been achieved (corresponding to a critical population density), 3-oxo-C6-HSL binds to LuxR. The activated regulator binds to a 20 base pair (bp) DNA inverted repeat, known as the *lux*-box, located upstream from the *luxCDABE* operon responsible for the light production.

1.3.1.1 Quorum sensing in P. aeruginosa

Almost twenty years ago, Gambello and Iglewski provided evidence that the human pathogen *P. aeruginosa* possesses a QS dependent system of regulation controlling a battery of virulence factors, such as elastase [70]. Later, the AHL signal molecules at the basis of this regulatory system were identified [53, 71]. Subsequent studies revealed that *P. aeruginosa* mutants defective in the production of the QS signal molecules form an abnormal biofilm which, in contrast to the wild-type, is sensitive to low concentration of detergents and antibiotics [50, 72-74]. Several *in vivo* models showed attenuated virulence when infected

with mutated *P. aeruginosa* strains incapable of producing the QS signal molecule (reviewed by Smith and Iglewski, 2003) [75]. Furthermore, in the lungs of CF patients a clear correlation between biofilm production and QS has been established [11].

The current hypothesis on the relationship between QS and virulence in *P. aeruginosa* postulates that cell-to-cell signaling could enable this microorganism to overcome host defense mechanisms. Indeed, isolated production of extracellular virulence factors by a small number of bacteria would probably lead to an efficient host response, neutralizing these compounds and the microorganism itself. Conversely, the coordinated expression of virulence genes by an entire bacterial population, once grown to high cellular density, might allow *P. aeruginosa* to secrete high levels of extracellular factors, enough to overcome host defenses [76].

In *P. aeruginosa,* there are two QS systems homologous to the *lux* system of *V. fischeri.* The *las* system, which consists of the LasR transcriptional regulator, and the LasI synthase. LasI produces the signal molecule *N*-3-oxododecanoyl-homoserine lactone (3-oxo-C12-HSL). LasR requires 3-oxo-C12-HSL binding in order to become an active transcriptional factor. In the presence of 3-oxo-C12-HSL, LasR forms dimers. Only the dimeric form of this protein is able to bind to the *lux* box of the genes it regulates [77]. LasR is composed of two distinct domains: the amino-terminal domain responsible for AHL-binding, and the carboxy-terminal domain, containing the helix-turn-helix motif important for the DNA-binding activity of the protein. Studies proved that the interaction between the signal molecule 3-oxo-C12-HSL and LasR leeds to the dimerization of the regulator and promotes its binding to the promoter region of target genes [77]. The LasR/LasI system was initially shown to regulate the expression of various virulence factors such as elastase, exotoxin A and alkaline protease, as well as LasI itself, thereby creating a positive regulatory feedback loop (Fig. 4) [78, 79].

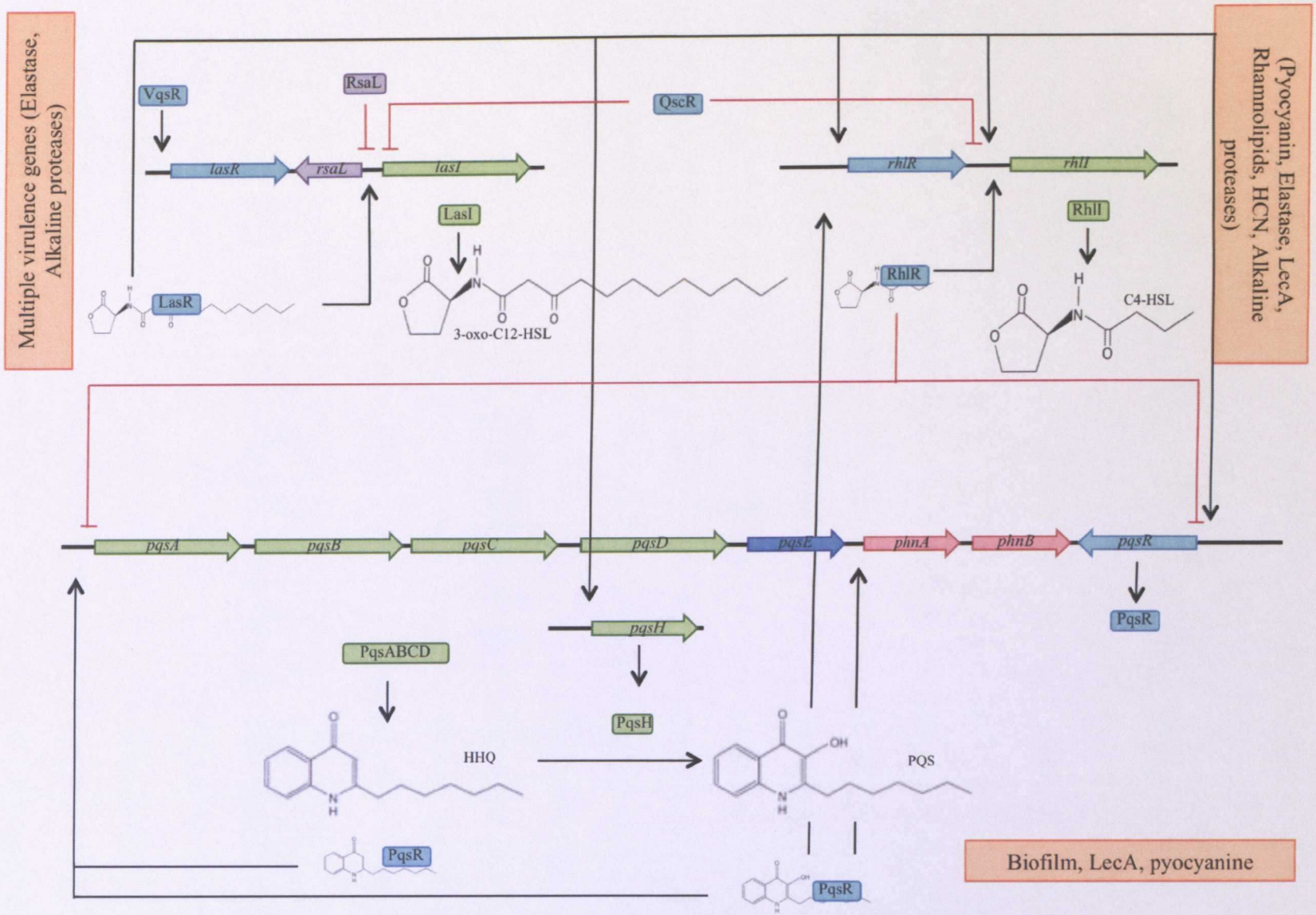


Figure 1-4. Diagrammatic representation of the AHL-dependent and AQ-dependent QS network in *P.aeruginosa*.

The LasR-autoinducer complex also activates the expression of the *rhl* QS system of *P. aeruginosa*. In particular, the expression of the RhlR transcriptional regulator is induced by the 3-oxo-C12-HSL-LasR complex [80]. RhlR binds to the signal molecule *N*-butyryl-homoserine lactone (C4-HSL) produced by the cognate synthase RhlI. Like the *las* system, a functional *rhl* system is necessary to regulate the expression of several virulence factors such as alkaline proteases, pyocyanin, hydrogen cyanide, lectins and elastase [81]. Moreover, the C4-HSL-RhlR complex promotes transcription of *rhlI*, establishing an autoregulatory loop (Fig. 4) [82]. The *las* system is hierarchically located above the *rhl* system. Indeed, 3-oxo-C12-HSL-LasR complex activates *rhlR* transcription, triggering the second signaling cascade [80, 83]. Furthermore, the 3-oxo-C12-HSL can compete with C4-HSL for the binding to RhlR, thus inhibiting this activator [84]. Therefore, the *las* system controls the *rhl* system at both transcriptional and post-translational level. Presumably, this second level of control exerted by the *las* system on the *rhl* system ensures that the two systems initiate their regulatory cascades sequentially and in the appropriate order (Fig. 4) [84].

In addition to LasR and RhlR, a third LuxR-like regulator was discovered in *P. aeruginosa* [85]. Unlike the homologues LasR and RhlR, this regulator is not associated with a cognate LuxI-like synthetase. Therefore, QscR is classified as an "orphan" QS signal receptor. A *P. aeruginosa qscR* mutant prematurely transcribes the *lasI* and *rhlI* AHLs generator genes, producing an increment in the expression of some QS controlled virulence genes, such as those involved in the expression of pyocyanin, elastase and HCN. Indeed, the *P. aeruginosa qscR* mutant is hypervirulent [85]. Several possible mechanisms have been proposed in order to explain the QscR mediated repression of *lasR* and *rhlR* expression. Filloux and co-workers demonstrated that, when expressed in the heterlogous host *E. coli*, QscR can form homomultimers, and also heterodimers with LasR and RhlR. Moreover, the same authors

showed that QscR is capable of binding both 3-oxo-C12-HSL and C4-HSL, thus competing with LasR and RhlR for these signal molecules [86]. On this basis, the role of QscR as inhibitor of QS through the formation of inactive heterodimers or through the competition for the signal molecules, is implicit [86]. Interestingly, it was recently demonstrated that the 3-oxo-C12-HSL-QscR complex can regulate the transcription of target genes (Fig. 4) [87]. Indeed, transcriptomic analysis provided evidence for the existence of a QscR regulon, which is not entirely overlapping with the LasR and RhlR ones [88].

A fourth LuxR-like protein, called VqsR (virulence and quorum sensing regulator), has been characterized in *P. aeruginosa*. Like QscR, VqsR is an "orphan" LuxI-like synthase. VqsR was shown to be involved in the positive regulation of QS, since *vqsR* inactivation abrogated the production of both 3-oxo-C12-HSL and C4-HSL signaling molecules. Indeed, the absence of *vqsR* leeds to a decrease of *lasI* transcription. Moreover, it was found that the promoter of *vqsR* contains a putative *las*-box, which places *vqsR* under the control of the *las* system [89]. Further studies confirmed this hypothesis, demonstrating the direct binding of LasR to the *vqsR* promoter (Fig. 4) [90].

P. aeruginosa QS response involves another chemical signal, distinct from the aforementioned AHLs. This signal, called 2-heptyl-3-hydroxy-4-quinolone (PQS), adds a further level of complexity to the QS network, providing another link between the *las* and *rhl* QS systems (reviewed by Williams and Cámara, 2009) [91]. The transcription of *pqsABCDE* operon, and therefore PQS production, is negatively regulated by the *rhl* system and positively regulated by the *las* system. On the other hand, PQS positively controls the expression of RhlR and RhlI (Fig. 4) [92-94].

Pesci and collaborators demonstrated that the effects of LasR and RhlR on the *pqsABCDE* operon occur indirectly through the control of the transcriptional regulator PqsR. PqsR in turn

has a positive effect on the transcription of *pqsABCDE* and *phnAB*, directly binding to their promoter [95]. Thus, the production of PQS is dependent on the ratio of 3-oxo-C12-HSL/C4-HSL, suggesting a delicate balance between the two QS systems [96]. PqsR binding to the *pqsABCDE* and *phnAB* promoters is augmented by the presence of PQS and HHQ, implying that these two molecules act as co-inducers of PqsR [97, 98].

PQS is also a multifunctional molecule, as it has an important role in iron transport, by acting as an iron trap and associating to the cell surface facilitating siderophore-mediated iron uptake [98]. PQS also has both oxidant and anti-oxidant properties acting on the population profile of the bacterial community [99]. PQS promotes the formation of membrane vesicles (MVs), which are thought to provide a mean of transporting PQS within bacteria [100]. This molecule has also been implicated in controlling biofilm maturation [101] and cell population densities. Under stressful conditions, PQS promotes cell lysis and the release of DNA (Fig. 5) [101, 102].

PQS is required for virulence in nematodes, plants and mice [34, 103-105]. This molecule was also found in the lungs of CF patients infected with *P. aeruginosa*, and it was shown to regulate diverse virulence genes including those coding for elastase, rhamnolipid and pyocyanin, as well as influencing biofilm development [92, 94, 98, 104]. Moreover, both *in vivo* and *in vitro* studies show how PQS interferes with the host during infection by deregulating the immune response [106-108].

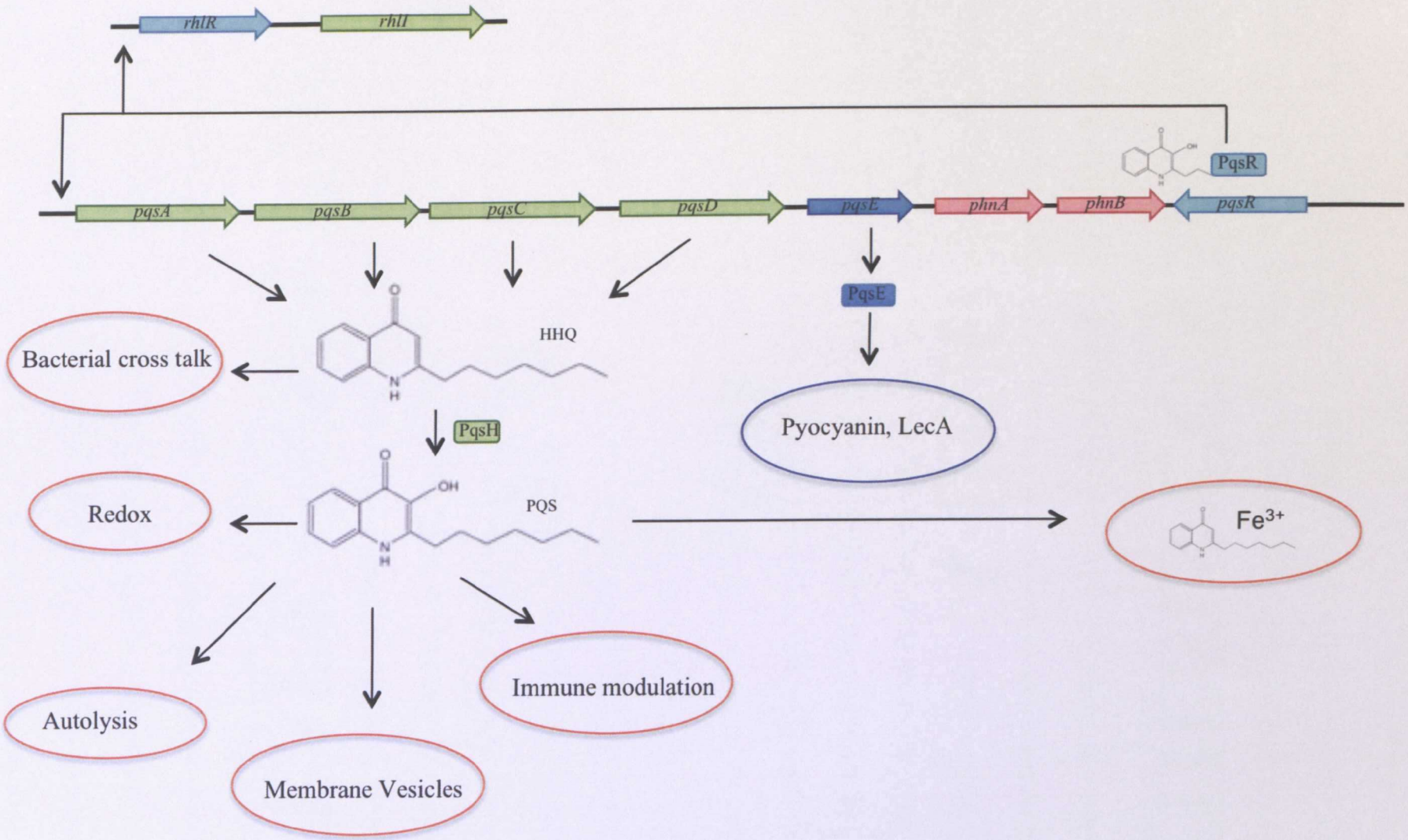


Figure 1-5. Diagrammatic representation of the biological roles of PQS and HHQ in *P. aeruginosa*.

The three different PQS pathways are also shown: the *pqsR* dependent (activation of genes does not require *pqsE*); the *pqsE* dependent (PQS induces the expression of genes through PqsE); and the iron sequestration pathway.

Two operons are implicated in the biosynthesis of 2-heptyl-4-quinolone (HHQ), the natural precursor of PQS: *phnAB* and *pqsABCDE* [109, 110]. More precisely, the *phnAB* operon is required for the conversion of 2-amino-2-deoxy-isochorismic acid to anthranilic acid, which is subsequently converted to HHQ by the enzymes encoded by the *pqsABCD* genes. HHQ is then converted to PQS by the product of the *pqsH* gene.

Besides PQS and HHQ, *P. aeruginosa* also produces a large number of related molecules which are part of the group of 2-alkyl-4(1*H*)-quinolones (AQs). The AQ biosynthetic enzymes present in this bacterium allow for the generation of over 50 different AQs. These can vary in the saturation of the alkyl chain and in the length (from one to thirteen carbons) or in the ring structure (containing N-oxides (HQNO)). The *pqsABCD* genes are involved in the biosynthesis of all AQs. Many of these molecules seem to lack a specific biological function [109-111].

In addition to the four biosynthetic genes encoded, the *pqsABCDE* operon also encodes for the PqsE. The sequence of PqsE resembles the one of the group of the metallo-ß-hydrolase superfamily. The proteins belonging to this group have different functions such as ß-lactamases or AHL-lactonases. However, the role of PqsE has not been unraveled, although its crystal structure is available [112]. A *pqsE* mutant is defective in the production of many virulence PQS-mediated exoproducts such as pyocianine, HCN and lectin. Moreover, the virulence in plant and animal infection models in the absence of AQs is dependent on the presence of this gene [113].

To summarize, PQS acts as a signal molecule controlling the expression of genes in a *pqsR* dependent way (such as *pqsA*), in a *pqsR/pqsE* dependent way (such as the expression of *lecA*) and in a signal independent way through the iron sequestration pathway (Fig. 5).

In silico analysis has revealed the presence of genes potentially involved in PQS synthesis in over 40 bacterial species. In a number of *Burkholderia* species such as *B. pseudomallei* and *B. thailandensis*, the complete putative *pqsABCDE* operon has been identified [114]. The main AQs produced by these species is HHQ, however, no PQS was detected, raising the possibility that related bacterial species which occupy similar ecological niches can cross-talk through the common molecule HHQ [115].

Since the discovery of cell-to-cell signaling in *P. aeruginosa*, the list of genes reported to be controlled by QS has increased steadily. Through the analysis of the QS-regulated transcriptome of *P. aeruginosa*, an overwhelmingly large number of genes were shown to be regulated by QS, with 3-7% of all *P. aeruginosa* open reading frames affected [51, 116, 117]. The majority of the QS regulated genes were classified as hypothetical or of unknown function, but a high proportion of them were shown to be secreted factors (toxins and extracellular enzymes), reinforcing the hypothesis that QS has an important role in virulence [118]. In addition, many genes involved in general metabolic functions, such as central intermediary metabolism, biosynthesis of cofactors and fatty acid metabolism, were affected by QS. This suggests that AHL signaling elicits major physiological changes in the cell, which reach far beyond virulence functions and may contribute to adaptation to a high cell-density environment [118].

Although QS is defined as a mechanism by which bacteria regulate target genes in a growth phase dependent way, in 1999 Greenberg and collaborators observed that, in *P. aeruginosa*, many QS controlled genes responded to the presence of signal molecules with different temporal patterns [119]. This observation was the first major indication that additional regulation was superimposed to the *P. aeruginosa* QS network. A number of "early" QS genes were found to respond immediately to exogenously added signals, while another group

of QS controlled genes (called "late" genes) were able to respond to the signals only during stationary growth phase. It was hypothesized that "late" genes might be under the control of some unknown regulatory mechanisms preventing their "early" expression [119]. The transcriptome analysis performed by Greenberg and collaborators at different points of the *P. aeruginosa* growth curve confirmed this pattern of delayed expression for most QS controlled genes [117].

In accordance with this hypothesis, recent studies have shown that in *P. aeruginosa* the QS regulatory circuit is integrated into specific aspects of cell physiology, and that it is modulated by environmental and metabolic signals that are independent from extracellular signal molecules concentrations. An increasing number of regulators which modulate the timing and level of QS-controlled gene expression has been described (reviewed by Venturi, 2006) [120]. These regulators provide additional control steps to the QS network in response to growth phase, environmental signals and yet unidentified stimuli.

1.3.2 Two component regulatory systems

Bacteria are exposed to different and constantly changing environmental conditions and stress. For this reason, they have evolved sensory systems that enable them to quickly and specifically respond to a particular input. Two component systems (TCSs) are the main mechanism by which bacteria and fungi react to their environment [121]. TCS are involved in the regulation of different responses such as drug resistance, motility, osmoregularity, host invasion, nitrogen fixation and virulence [122].

The TCSs are part of a transduction system, which recognizes an environmental signal and converts this signal into a transcriptional activation. The TCSs are generally composed of an

inner membrane-spaning sensor histidine kinase (HK), which detects the stimuli and activates the cytoplasmatic response regulator (RR) through a set of phosphorylation steps. Then, the RR converts the signal into a transcriptional regulation of target genes [123].

The TCSs can be divided into three categories. In the "classic" systems, the HK responds to a signal by undergoing trans-autophosphorylation at a conserved histidine residue in its transmitter domain. This phosphoryl group is then transferred to an aspartate-containing receiver domain of the cognate RR, which is generally a DNA binding protein [124]. In the phosphorelay-type system, three consequent phosphoryl transfer events (within the same sensor protein or through different proteins) result in the phsophorylation of the RR. In particular, the phosphorylation of the RR may travel through a Histidine phosphotransfer (Hpt) protein [125]. In the case the Hpt is a separate protein, the TCSs are termed "hybrid". When the Hpt domain is fused to the receiver domain of the HK protein, the TCSs are called "unorthodox" (Fig. 6).

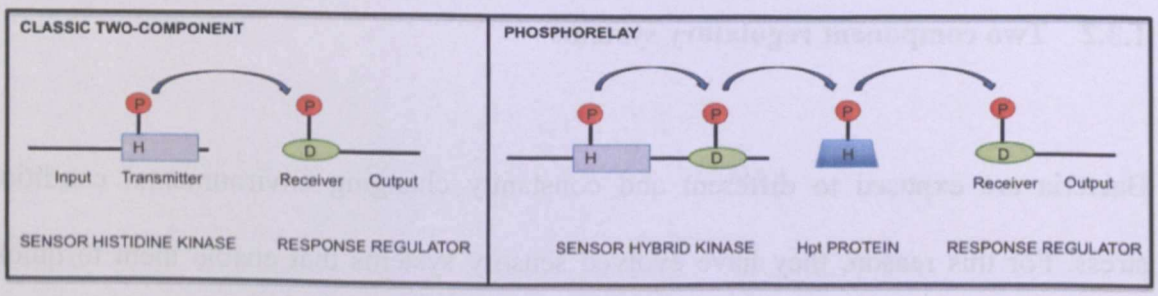


Figure 1-6. A Schematic representation of the different two-component systems.

The HK and RR coding sequences are often encoded adjacent to each other in the same operon, indicating that the system responds specifically to a signal (although most of the times the signal is unknown). However, in the case of "orphan" two-component system, the HK gene is not transcribed with the RR and *vice versa.*

One of the ways in which TCSs can act on the activation of a response is through the activation of small non-coding regulatory RNAs (sRNAs) [126-128]. Some response regulators promote the transcription of sRNAs, which in turn post-transcriptionally modulate target genes, thus providing a link between TCS and post-transcriptional regulation.

One way the sRNAs can act is by binding to their target mRNA, thus interfering with ribosome binding preventing their translation [129]. On the other hand, sRNAs can positively affect the rate of translation of a target mRNA by stabilizing their secondary structure [130]. The sRNAs can also affect post-transcriptionally gene expression in a more global way by binding and sequestrating global post-transcriptional regulators. The most studied sRNAs that act as post-transcriptional regulators are those that involve the CsrA global regulator in *E. coli*, homologues of which are also present in many bacteria [129, 131, 132].

The CsrA global regulator (carbon storage regulator A) was discovered by Romeo and collaborators [133]. In *E. coli*, the Csr system is composed of the CsrA protein and two small sRNAs, namely CsrB and CsrC [134]. CsrA binds to a site near the Shine-Dalgarno (SD) sequence of target mRNA, competing with the binding of the ribosome and increasing mRNA decay. Interestingly, CsrA can also act as a positive regulator by binding and stabilizing the target mRNAs or by releasing secondary structure, preventing their translation [135]. The activity of CsrA is in turn modulated by two sRNAs, CsrB and CsrC, which have a high affinity for CsrA. By binding to this global regulator, these sRNAs titrate CsrA, preventing its binding to target mRNAs [134, 136]. Finally, the transcription of these two sRNAs is controlled by the two-component signal transduction system BarA/UvrY, which is activated by yet unknown signal molecule(s) [137].

In *E. coli*, this global post-transcriptional regulatory system modulates multiple phenotypes such as biofilm formation and motility, carbon metabolism, virulence and QS. This discovery

links the transcriptional regulatory system of QS with CsrA global post-transcriptional regulation, providing new insights into the complex network of gene expression. Several homologues to CsrA have been identified in other bacterial species [138].

1.3.2.1 GacA/S and RsmA

In the *P. aeruginosa* genome, there is an astonishing number of putative two-component regulatory proteins that mediate the link between external stimuli, such as an extracellular signal, and a response. *P. aeruginosa* encodes 64 HK and 72 RR, 50 of which are organized in the same operon, while there are 13 HK and 15 RR orphan components [125, 139]. Additionally, there are 3 Hpt proteins (HptA, HptB and HptC) that act with the several hybrid kinases forming complex networks [140].

This section will focus on the Gac/Rsm transduction system in *P. aeruginosa*. This system is composed of the global post-transcriptional regulator RsmA, the two regulatory RNAs (RsmZ and RsmY), and a sensor kinase and the response regulator (GacS/GacA) [141].

The GacS/GacA (global activator of antibiotic and cyanide synthesis) TCS is one of the best-characterized virulence regulatory pathways. Both GacS and GacA are required for pathogenesis *in vivo* models such as plant, nematode, insect and mouse [105, 142-146]. GacA and GacS are required for the modulation of multiple virulence factors such as the ones involved in the regulation of biofilm formation and in the synthesis of the QS AHL signals [147, 148].

The GacS/GacA system exerts its regulatory function by modulating the activity of the global post-transcriptional regulator RsmA, which is the CsrA analogue in *E. coli* [149]. In detail, the GacS/GacA TCS acts exclusively through the modulation of the two sRNAs. In response to a still unknown stimuli, GacA positively regulates the expression of RsmZ and RsmY by

directly binding to the sequence upstream of these genes (called the GacA-box) [150]. These two sRNAs sequestrate RsmA protein to jointly antagonize the binding of RsmA to its target mRNA [151].

RsmA is a negative regulator involved in the formation of several extracellular products, such as pyocyanin, hydrogen cyanide, elastase and LecA (PA-IL) lectin. Moreover, it exerts its global regulatory effect by modulating QS. RsmA was shown to negatively regulate the production of QS signal molecules such as AHL, although the molecular mechanism is still unknown [152, 153]. Interestingly, RsmA is also able to positively regulate swarming, lipase and rhamnolipids production, although the mechanisms of its positive effect are not fully understood [151].

1.3.2.2 RetS and LadS

In addition, the presence of two additional sensors kinases, namely RetS (regulator of exopolysaccharide and type III secretion) and LadS (lost adherence sensor), which work in combination with GacS for the modulation of the phosphorylation of GacA, adds further complexity to the system [154, 155].

RetS is a hybrid sensor kinase which inhibits GacA phosphorylation by forming inactive heterodimers with GacS [155, 156]. The expression of the genes important for chronic infection, such as the ones involved in the early biofilm formation linked to the activation of the matrix exopolysaccharide *pls* operon, is enhanced in a *retS* mutant [7]. On the other hand, the expression of genes required for acute infection (such as type III secretion system, type IV pili and exotoxin A) is reduced [157, 158].

LadS is another hybrid sensor kinase, which was firstly identified by Ventre *et al.* [155]. LadS is important in the early and maturation steps of biofilm development by positively regulating

the exopolysaccharide operon *pel*. A *ladS* mutant is unable to form biofilm and shows an increase cytotoxicity relative to the wild type [155]. Thus, LadS and RetS have opposite effects on the Gac/Rsm system, although LadS was shown to be hierarchically located 'above' RetS [155].

The previous observations allowed for the establishment of a model that attempts to explain how this TCS (GacS/LadS/RetS/GacA) and its linked post-transcriptional regulator (RsmA) are involved in coordinating the gene expression associated with acute or chronic infection [154, 155, 158]. In this model, during chronic infection, environmental cues activate GacS and LadS, which in turn promote the transcription of *rsmZ* and *rsmY*. This decreases the levels of free RsmA causing the expression of genes involved in biofilm formation. On the other hand, RetS is activated in an acute infection. This sensor represses GacA activation, thus promoting RsmA activity by lowering the amount of sRNA. Once RetS is active, the genes responsible for type III secretion system and other genes involved in virulence and thus in acute infection, are upregulated (Fig. 7). Whilst RetS is known to regulate *rsmZ* and *rsmY* transcription through the inhibition of GacS phosphorylation [156]. The machanism by which LadS modulates *rsmZ* and *rsmY* transcription is still unknown. A possible explanation is that LadS could promote GacA phosphorylation [158].

1.3.2.3 BfiR/S

Recent studies conducted using a proteomic approach, led to the identification of BfiR/S, which is another TCS linked to the GacS/GacA regulatory system. In particular, the sensor kinase BfiS was shown to be essential for the early stages of the biofilm development regulating the irreversible attachment, by acting at the post-transcriptional level on *rsmZ* via RNase G (Fig. 7) [159]. In detail, the BfiSR TCS is activated in a biofilm-specific manner and

promotes the transcription of the gene *cafA*, encoding for RNase G. This RNase acts on the degradation of *rsmZ* and eventually on the progression of biofilm development. This discovery adds another layer of complexity to this complex regulatory network. The levels of *rsmZ* in the cell are regulated at the transcriptional level by the GacAS/RetS/LadS/Rsm system, and at the post-transcriptional level by BfiS/R. Moreover, BfiS/R TCS was shown to act also on the levels of *rsmY* transcripts, although this modulation does not appear to depend on RNase G activity.

1.3.2.4 HptB

As previously stated, *P. aeruginosa* codes for three Hpt proteins. Recent studies conducted on HptB showed how this protein is part of a phosphorelay TCS. HptB is phosphorylated by four sensor kinases, namely PA1611, PA1976, PA2824 and RetS. This finding suggested that HptB could be the phosphorylation relay of the RetS hybrid sensor kinase. This hypothesis was however discharged, as further studies showed that an overexpression of HptB in the *retS* defective strain was unable to restore the wild type phenotype [160]. Furthermore, it was shown that GacA regulation is located downstream of HptB. The *in vitro* ability of HptB to retro-transfer the phosphyl group to RetS, combined with the previous results, led to the hypothesis that HptB could control target genes through the GacS/GacA system [140]. HptB was also shown to inhibit the release of a putative sigma factor, responsible for promoting the transcription of *rsmY*, suggesting that RsmZ and RsmY are regulated through different pathways (Fig. 7) [140, 160].

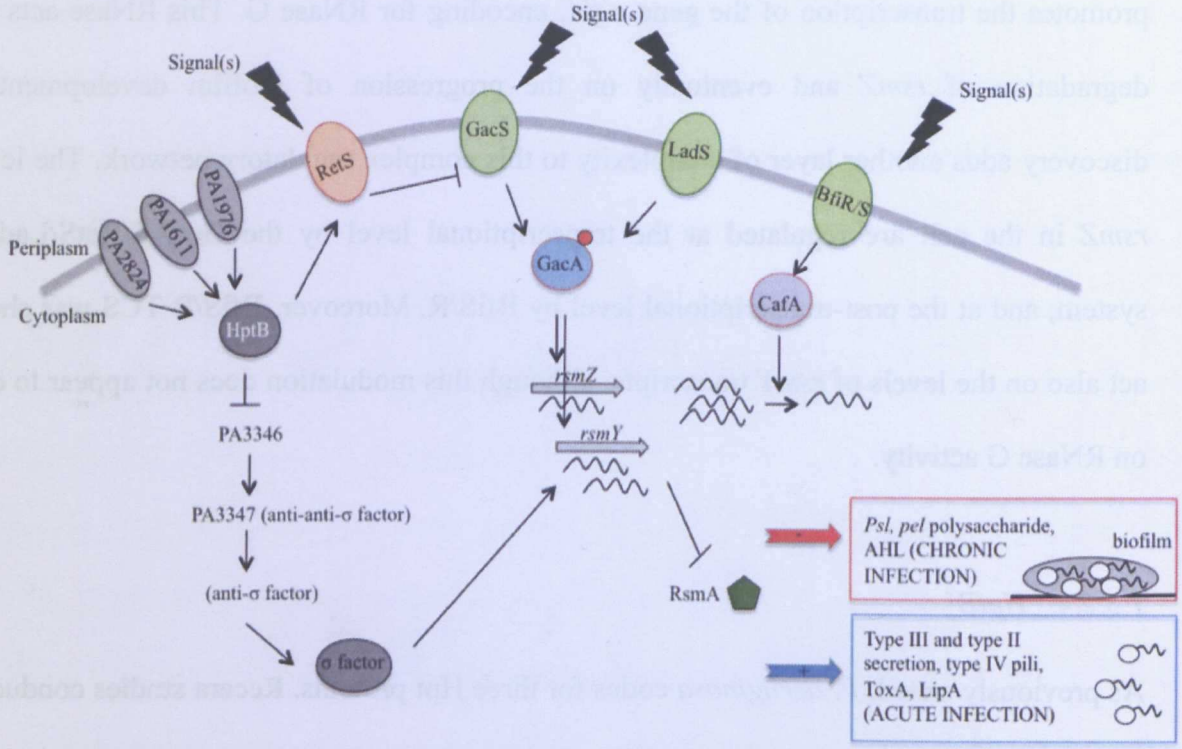


Figure 1-7. Model for the Rsm/Gac regulatory network in *P. aeruginosa*.

Three hybrid sensors (PA2824, PA1611, PA1976) activate HptB. This protein has a negative effect on the activity of PA3346, which activates PA3347 (an anti-anti-σ factor). PA3347 can bind a putative anti-σ factor, allowing the release of a yet uncharacterized σ factor. This σ factor may act specifically on *rsmY*, but not on *rsmZ* (Bordi et al., 2010). HptB has also a potential retro-transfer ability, from the three hybrid sensors to RetS (Hsu et al., 2008). RetS is forming dimers inhibiting GacS phosphorylation (Goodman et al., 2009), while GacS and LadS act positively on GacA phosphorylation and BfiRS act on the levels of *rsmZ* and *rsmY* transcripts (Petrova, Sauer, 2010).

1.4 INHIBITION OF VIRULENCE

Historically, the first approach to treat bacterial diseases was to inhibit their growth (bacteriostatic) or to directly eradicate them (bactericidal). Since the discovery of penicillin, the development of antibacterial drugs has been exponential. However, the production of novel antibiotics fails to compete with the fast development of anti-microbial resistance. For this reason, new approaches for the treatment of bacterial disease are needed. A better understanding of the capability of bacteria to coordinate the production of virulence factors in

a multicellular way could offer a novel way to interfere with this inter and intra-species communication. By inhibiting QS signaling, it could, therefore, be possible to interrupt the flow of information essential for the successful establishment of the infecting pathogen in the host.

1.4.1 Quorum sensing inhibition

Studies that have explored the role of QS in *P. aeruginosa* infection (in a burnt-mouse model, a murine model of acute pneumonia, and a rat model of chronic lung infection) have demonstrated that the inactivation of QS genes resulted in reduced *P. aeruginosa* virulence compared to the wild type [161]. In humans, QS was shown to be functional during *P. aeruginosa* infections. Thus, the signal molecules AHLs and AQs were directly measured in the sputum of CF patients chronically colonized with *P. aeruginosa* [11].

In addition to their role in modulating the QS regulatory system, these signal molecules were also shown to interfere with the host immune response [162]. In particular, low concentrations of 3-oxo-C12-HSL (<10 μM) were shown to inhibit the production of the cytokines interleukin (IL-) 12 and the tumor necrosis factor alpha (TNF-α) by the LPS-stimulated macrophages. The signal molecule 3-oxo-C12-HSL also inhibited the proliferation of T cells and the release of IL-2 in *vitro* [106]. Finally, 3-oxo-C12-HSL was proved to be a potential anti-inflammatory agent to treat autoimmune diseases such as diabetes in mice [163]. These observations led the authors to believe that 3-oxo-C12-HSL was involved in shifting the host immune response from the host protective T-helper response to the pathogen protective T-helper response. These data suggest a scenario in which after infection, the bacterial community starts producing AHLs, which on one side facilitate QS, and on the other

represses the host immune response. This eventually leads to an increased bacterial colonization.

In another series of investigations conducted using higher doses of 3-oxo-C12-HSL (100 μM), it was shown that 3-oxo-C12-HSL could also induce pro-inflammatory responses in fibroblasts [164]. These studies established that 3-oxo-C12-HSL induced the expression of cytokine and the chemokine interleukin-8 (IL-8). Interleukines are pro-inflammatory cytokines involved in attraction and activation of polymorpho-nuclear leukocytes (PMNs). Their production is important in keeping the immune response acute and morbid by constant recruitment of PMNs [165]. High levels of chemotactic factors such as IL-8 produced at the site of infection stimulate the migration of many different cell types to the site of infection. Neutrophils that enter the infection site secrete multiple mediators such as reactive oxygen species and elastases. Although the environment is filled with these caustic agents, *P. aeruginosa* tends to be resistant to their effect; this resistance is probably due to the growth of the bacteria on protective biofilms [17]. Moreover *P. aeruginosa* also produces catalases and superoxide dismutases that are able to neutralize the deleterious effects of hydrogen peroxide and oxidative stress [166]. Therefore, instead of clearing the bacterial infection, the release of neutrophils results in tissue destruction [162, 167]. 3-oxo-C12-HSL was also shown to activate T-cells production of interferon-gamma (IFN-Y) [165].

These observations led to the hypothesis that 3-oxo-C12-HSL was in fact acting as a promoter of the immune response. One of the possible explanations for this pro-inflammatory function of 3-oxo-C12-HSL is that, during *P. aeruginosa* acute infection, the 3-oxo-C12-HSL promotes inflammation and subsequent destruction of host tissue. This in turn leads to dissemination of the bacteria and sepsis.

Finally, AHLs have been shown to exert a pharmacological effect on the cardiovascular system, suggesting that host cardiovascular function may be modulated by bacterial QS molecules. In particular, it was observed that some AHLs and PQS exerted a vasodilatatory activity, thus favoring the dispersal and the nutrient supply of the microorganisms via the bloodstream [168].

Consequently, QS signal molecules production may not only have a role in activating the QS response, but also in modifying host immune response to maximize its own chances of survival and proliferation. This is achieved by increasing the supply of nutrients via the bloodstream and by regulating the host defenses.

Since QS is crucial for virulence, biofilm formation and the direct modulation of the immune host response, it is plausible that QS can be an ideal target for the design of novel drugs for the treatment of *P. aeruginosa* infections. This new approach, based on QS inhibitor (QSI) compounds that specifically block bacterial signaling systems, offers a novel opportunity to control infectious bacteria in a nonlethal way. By interfering with QS, a limited selective pressure for the survival of bacteria is exerted [169]. In contrast to traditional anti-microbial agents, QSI compounds work at concentrations well below the minimal inhibitory concentration. This concept is attractive, since such compounds will not create a selection pressure for the development of resistance. Moreover, since the selected QSIs are non-toxic for bacteria at the concentrations used, they are not expected to exhibit adverse effects on beneficial bacterial communities that live in symbiosis with the host (e.g., the gut flora) [169].

There are several strategies for disrupting *P. aeruginosa* AHL-dependent quorum sensing. One way is to reduce the concentration of the signal molecules by enhancing their degradation or by inhibiting their synthesis. Alternatively, one could block the transmission of the signal by antagonizing the formation of the LuxR-AHL complex [51].

1.4.2 Blockage of signal synthesis

One way of affecting QS system is by interfering with the signal production and in particular by blocking the AHL synthetase. Studies conducted on the LuxI and its homologues have revealed that the AHL synthesis is a conserved mechanism and that AHLs are synthesized from precursors involved in the amino acid and fatty acid metabolism: S-adenosyl methionine (SAM) and acyl-ACP [170]. Various analogues of SAM that compete with the natural precursors for the active site of LuxI have been developed. In particular, *in vitro* studies demonstrated that *S*-adenosyl-DL-homocysteine drastically reduces the activity of the RhlI protein [170]. However, due to the fact that SAM and acyl-ACP are common and necessary intermediates in many prokaryotic and eukaryotic pathways, this strategy has been disregarded as a possible approach for developing QSI molecules [51].

1.4.3 Signal degradation

The inactivation or degradation of the signal molecules could represent another strategy for the blockage of the QS systems. AHLs are very sensitive to elevated temperatures and to pH [171, 172]. In particular, high pH causes lactonolysis (ring opening) of the AHL ring. A number of higher organisms, such as the plant *Erwinia carotovora*, have adopted this strategy in defense against invading microorganisms, thus preventing the expression of QS controlled genes and virulence factors.

The AHLs can also undergo a spontaneous alkylation of the ß-ketoamide moiety, which leads to the formation of tetramic acids. Although these molecules have lost their signaling

properties, tetramic acids were shown to act as antibiotics against Gram-positive bacteria [173].

Lactonolysis of AHL can also be accomplished by enzymatic activity, which appears to occur in a very broad range of organisms. Numerous bacterial strains capable of either inactivating and/or metabolizing the signal molecule have been indentified. In particular, a *Bacillus* specie was found to produce an enzyme, namely AiiA, which hydrolyzes the ester bond of the lactone ring [174]. Other species are capable of utilizing the AHL as a sole energy source, such as *Variovorax paradoxus* [175]. AHL lactonases have also been reported in different eukaryotes and in mammalian serum [176]. These enzymes have been recently reported to interfere with QS regulation in *P. aeruginosa* biofilms [177].

1.4.4 Signal antagonists

Another way to interfere with bacterial QS is to prevent the signal from being perceived by the bacteria by blocking the receptor protein, the LuxR homologue. This can be achieved by using antagonists that compete or interfere with the cognate AHL for binding to the LuxR transcriptional regulator proteins.

There are two types of inhibitors, the competitive ones, which are structurally similar to the native AHL signal, and the non-competitive, which have no structural similarity to the AHL. In the former case, the inhibitor molecules bind and occupy the AHL binding pocket without activating the LuxR-type receptor. In the latter, they bind to allosteric sites on the regulator protein [169].

1.4.4.1 Natural QSI

Given the widespread occurrence of AHL-mediated cell-to-cell communication system, it is highly probable that higher organisms may have evolved specific means to interfere with bacterial communication and possibly escape colonization [178]. Eukaryotic interference with AHL-mediated signaling was observed in several plants which secrete substances that mimic bacterial AHL signal, thus affecting QS-regulated behaviors of the pathogenic bacteria [179]. Recently, a large amount of plants and fungal extracts have been screened for QSI activity. Extracts derived from carrot, soybean, water lily, tomato, pea seedlings, habanero and garlic have been found to produce compounds capable of inhibiting QS. However, the structure of the QSI molecules often remains unknown [180].

The first molecule discovered to have a QSI activity was the one produced by the Australian marine macroalga *Delisea pulchra.* This algae produces a number of halogenated furanones that inhibit bacterial biofilm formation [181].

More recently, furanones have been shown to inhibit AHL regulated processes, including biofilm formation and QS-regulated virulence genes in a number of bacteria [182]. Although it was originally thought that these compounds acted by competing with the natural AHL for a common binding site to the LuxR protein, recent evidence suggests that the inhibition of AHL gene expression is a consequence of an accelerated degradation of the LuxR protein [183].

1.4.4.2 Synthetic QSIs

The potential of the natural QSI compounds to control virulence, biofilm formation and pathogenicity, prompted researchers to focus on the development of synthetic molecules derived from the natural AHL or from the naturally occurring QSI molecules (i.e., furanones) capable of interfering with the microbial activity. The furanones produced by *D. pulchra* have

no effect on the QS system of *P. aeruginosa*. On the contrary, chemically modified furanones derived from *D. pulchra* were shown to repress QS by reducing the expression of virulence factors [169, 182]. Moreover, microarray analysis demonstrated that furanones and their derivatives specifically repress expression of QS controlled genes in *P. aeruginosa* [169]. However, it is important to note that furanones derived compounds are often too reactive (due to their halogenated group) and therefore too toxic for the treatment of bacterial infections in humans [169].

Another approach used for the development of QS inhibitors was based on the construction of synthetic chemical libraries composed of AHL derivatives. These compounds differentiated in the homoserine lactone ring or the acyl-side chain. It appears that the homoserine lactone ring is very important for biological activity, while the nature of the acyl chain is not as critical. For this reason, much effort to create effective AHL antagonists has been focused on the modification of the acyl side chain. A study of the *P. aeruginosa* LasR receptor revealed that the fully extended acyl chain geometry is necessary for its activation. This suggested that the minimum acyl side chain length determined by the cognate AHL signal is required for binding to LuxR-type proteins [184]. The flexibility of the acyl side chain also appears to be important for binding to the LuxR-type proteins. For example, it was shown that the reduction of the chain rotation by the introduction of an unsaturated bond close to the amide linkage nearly abolished the binding to the receptor [184]. The chirality of the homoserine lactone moiety is crucial for its biological activity. Natural AHL signals are L-isomers, whereas D-isomers are generally devoid of biological activity; D-isomers do not bind to the LuxR-type receptor [184]. The effects of changes in the composition and size of the homoserine lactone ring was studied. The main conclusion was that the ring size, the keto group adjacent to the amino group and the presence of saturated carbons in the ring strongly affected the inhibitory

activity of the molecule on the LasR/LasI system. In fact, only slight variations in these key positions, such as change from a saturated ring to an aromatic benzene ring, transformed the molecule from an agonist into an antagonist [184].

Screening of random compound libraries has also identified QSI. Using this method, several compounds whose structure was unrelated to the signal molecules were identified. Among these, the most effective was the 4-nitro-pyridine-N-oxide (4-NPO) compound. Microarray analysis showed how 4-NPO was lowering the expression of 37% of the QS-regulated genes in *P. aeruginosa* [180].

1.5 AIMS OF THE PRESENTED THESIS

This study aimed to unravel the molecular mechanisms linking QS with other signaling regulatory networks in *P. aeruginosa*, in order to obtain a deeper understanding of the global gene regulation networks that determine the virulence properties of this opportunistic pathogenic bacterium. In particular, the work presented here aimed to achieve a deeper understanding of the modulation of the two regulatory RNAs (RsmZ and RsmY) by internal and external signaling, and to uncover novel pathways regulating this post-transcriptional regulatory network. Moreover, it was interesting to exploit QS as an antibacterial target by conducting *in vitro* studies and a clinical study on CF patients infected with *P. aeruginosa*.

In order to achieve this, the following approaches were considered:

- Construction of reporter strains, based on QS regulated genes, for the analysis of the effect of synthetic and natural compounds on QS inhibition.
- Construction of reporter strains for the study of the Gac/Rsm regulation by environmental signal molecules and by known QS and intracellular signal molecules.

- Construction of post-transcriptional based fusions for the study of the regulation of virulence genes by PQS.
- Creation of a genetic library and a transposon mutagenesis bank of mutants for the identification of new regulators acting on the Gac/Rsm post-transcriptional regulation.
- Exploitation of the promoter-pull down approach to identify regulators directly acting on the transcription of the two regulatory sRNAs.

2 Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

The strains of *E. coli* and *Pseudomonas* used in this study were cultivated at 37 °C and 30°C depending on the strain in solid and liquid media, with or without antibiotics. The stocks were stored at -80 °C in Luria Bertani broth (LB) plus glycerol 15% [v/v]. The strains used in this study are reported in Table 1.

Unless otherwise stated the *P. aeruginosa* strain used was from Nottingham collection strain *P. aeruginosa* PAO1-N.

Rich media: Luria Bertani (LB) (tryptone 1% [w/v], yeast extract 0.5% [w/v], NaCl 1% [wv], pH 7.5, sterilized by autoclaving).

M9 Minimal Medium: Basal salt solution 2x (Na_2HPO_4 13.6 g/l, KH2PO4 6 g/l NaCl 2 g/l), 100x supplements solutions: NH_4Cl 100 mg/ml, CaCl2 2.22 mg/ml, $MgSO_4$ 24.2 mg/ml, glucose 360 mg/ml .

Glycerol-alanine minimal medium: DL-alanine 1% (w/v), glycerol 2% (v/v), K_2HPO_4 0.014% (w/v), $MgCl_2 6H_2O$ 0.4% (w/v), Na_2SO_4 1.42% (w/v), ferric citrate 0.01% (w/v), pH 7.0 to 7.2.

Casamino Acid media, CAA consists of 5 g Difco casamino acids, 20 g glycerol, 0.573 g of anhydrate K_2HPO_4 and 0.75 g of $MgSO_4 \cdot 7 H_2O$ in a total of 1 liter H_2O.

Swarming motility agar was prepared according to a previous published method [185].

This consisted of 0.5% (w/v) Bacto agar (Difco) and 0.8% (w/v) Nutrient broth No. 2 (Oxoid) in distilled water [186]. After autoclaving, filter sterilized D-glucose (Sigma) in distilled water was added to a final concentration of 0.5 % (w/v). When adding signal molecules to the

swarming media, the molecules were added in a clean Falcon tube and the solvent was then evaporated (under the fume hood). The warm swarming agar was then added to the Falcon tube and shaken well to allow the compound to go into solution.

For plasmid selection and maintenance, antibiotics were added to growth media at the following concentrations: gentamicin (Gm) 10 mg/l; ampicillin (Amp) 100 mg/l; tetracycline 10 mg/l for *E. coli* and gentamicin (Gm) 25 mg/l; streptomycin (Sm) 1000 mg/l; tetracycline 200 mg/l for *P. aeruginosa*. Isopropyl β-D-1-thiogalactopyranoside (IPTG) 0.5 mM and 5'-bromo4'-chloro-3'- indolyl β-D-galactopyranoside (X-gal) 40 mg/l were included in the media, respectively for gene induction and for detection of β-galactosidase activity.

The liquid cultures were incubated in shaking conditions (200 rpm). Agar plates contained 15 g/l no.1 agar (Oxoid). All the media were sterilised by autoclaving or filtration.

Table 1. Bacterial strains used in this study

Strain	Genotype/Characteristics	Reference/Source
Escherichia coli:		
DH5α	F⁻ *endA1 hsdR17*(r_K- m_K⁺) *supE44 thi-1* Δ⁻ *recA1 gyrA96 relA1 deoR* Δ (*lacZYA-argF*)-U169 φ80d*lacZ*ΔM15	[187]
S17-1 λpir	*rec*A, *thi*, *pro*, *hsd*R17 (r_K-, m_K⁺), RP4-2-Tc::Mu-Km::Tn7, λ*pir*	[188]
BL21(DE3)[pLysS]	F–, *omp*T, *hsd*S_B (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm^R	[189]
RVH-E1	*sdiA* mutant derived from MG1655 (F⁻ λ⁻ *rph-1 sdiA*::Km^R)	[190]
Pseduomonas fluorescens		
CHA0	Wild type	[191]
Pseudomonas aeruginosa:		
PAO1-N	Wild type, Nottingham strain	Laboratory collection
PAO1-L	Wild type, Lausanne strain	Laboratory collection
PAO1-DSM	Wild type, DSM strain	Laboratory collection
Clinical isolates 1,4,7,9,14,23,35,73	Strain isolated from CF pediatric patients infected *P. aeruginosa*	S. Cruz PhD thesis
PAKR02	Δ*lasI*::Gm^R; mini-CTX::*lasB-luxCDABE* obtained with pKR6	This study
PAKR04	Wild type mini-CTX::*rsmY-luxABCDE* obtained with pKR77	This study
PAKR08	Δ*pqsA* mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PAKR09	Δ*pqsA*; mini-CTX::*rsmY-luxCDABE* obtained with pKR77	
PAKR21	PASK10 mini-CTX::*rsmZ-luxABCDE* obtained with	This study

	pSH50	
PAKR26	Δ*wspF* obtained by transposon insertion of Tn5, GmR	This study
PAKR29	Δ *gacA* mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PAKR38	Δ*lasI::Gm*^{R}; mini-CTX::*lasI1-luxCDABE* obtained with pKR7	This study
PAKR39	Δ*lasI::Gm*^{R}; mini-CTX::*lasI3-luxCDABE* obtained with pKR9	This study
PAKR40	Δ*gacS* mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PAKR41	Δ*pqsE* in frame mutant obtained with pKR3	This study
PAKR42	Δ*pqsE* mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PAKR43	Δ*gacS* in frame mutant obtained with pKR1	This study
PAKR44	Δ*ladS* mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PAKR45	Δ*ladS* in frame mutant obtained with pKR2	This study
PAKR46	Δ*lasI::Gm*^{R}; mini-CTX::*lasI2-luxCDABE* obtained with pKR8	This study
PAKR47	Δ*lasI::Gm*^{R}; mini-CTX::*lasI4-luxCDABE* obtained with pKR10	This study
PAKR48	Δ*pqsR* mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PAKR50	Δ*wspF* mini-CTX::*rsmZ-luxCDABE* fusion, obtained with pSH50 and PAKR26	This study
PAKR51	Δ*aguR* in frame mutant obtained with pKR51	This study
PAKR52	Δ*retS* in frame mutant obtained with pKR4	This study
PAKR53	Δ*retS* mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PAKR54	Wild type mini-CTX::*rsmZ-luxABCDE* altered *lux*-box obtained with pKR5	This study
PAKR55	Δ*pqsR* in frame mutant obtained with pCP1	This study
PAKR56	Δ*aguR* mini-CTX::*rsmZ-luxCDABE* fusion, obtained with pSH50 and PAKR51	This study
PAKR57	Δ*wspF* in frame mutant obtained with pKR57	This study
PAKR59	Δ*gacA* in frame mutant obtained with pME6111,SpR SmR	This study
PAKR60	Δ*pqsA* mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAKR61	Δ*pqsE* mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAKR62	Δ*pqsR* mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAKR63	Δ*pqsA*/Δ*pqsR* mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAKR64	Δ*pqsA*/Δ*pqsE* mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAKR65	Δ*rhlR* mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAKR66	Δ*lasI::Gm*^{R}; mini-CTX::*lasI5-lux*CDABE obtained with pKR66	This study
PAKR67	Δ*lasI::Gm*^{R}; mini-CTX::*lasI6-lux*CDABE obtained with pKR67	This study
PAKR68	Δp*qsE* mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study
PAKR74	Δ*lasR::Gm*^{R}; mini-CTX::*rsmZ-luxCDABE* obtained with pSH50	This study
PAKR75	Δ*rhlR*; mini-CTX::*rsmZ-luxCDABE* obtained with pSH50	This study
PAKR76	Δ*phz1/phz2* mini-CTX::*rsmZ-luxCDABE* fusion, obtained with pSH50 and PAKR82	This study
PAKR82	Double Δ*phz1/phz2* mutant unable to produce phenazines, obtained with pKR23 and pKR24	This study
PAKR83	Δ*rhlR* mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study

PAKR84	Δ*pqsA*/Δ*pqsR* mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study
PAKR85	Δ*pqsA*/Δ*pqsE* mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study
PAKR86	Δ*pqsA*/Δ*rhlR* mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study
PAKR87	Δ*pqsA*/Δ*rhlR* mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAKR88	Δ*pqsA* mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study
PAKR89	Δ*pqsR* mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study
PAKR94	*lasI::Gm*^{R} mutant, obtained with pSB219.8	This study
PAKR101	Δ*pqsA* Δ*rsmY* double deletion mutant obtained with Δ*pqsA* and pME3087 Δ*rsmY*	This study
PAKR102	Δ*pqsA* Δ*rsmZ* double deletion mutant obtained with Δ*pqsA* and pME3332 Δ*rsmZ*	This study
PACP10	Δ*rhlR* in frame mutant	C. Pustelny PhD thesis
ΔrhlI	*rhlI::*Tc^R mutant (*P. aeruginosa* strain from Beatson laboratory strain collection)	[192]
PASDP233	*lasR::*Gm^R mutant	S. Diggle
PASDP123	Δ*pqsA* in frame mutant	[193]
PASK09	P_{tac} -*rsmA*; *rsmA* constitutively expressed from the chromosome	S. Kehüne PhD thesis
PASK10	*lacI*^{q}, P_{tac}-*rsmA*; inducible *rsmA*, (Sm^R/Sp^R)	S. Kehüne PhD thesis
PASH50	Wild type mini-CTX::*rsmZ-luxABCDE* obtained with pSH50	This study
PASH51	*lacI*^{q}, P_{tac}-*PA2567*; inducible *PA2567*, (Sm^R/Sp^R) obtained with the pSH10 suicide vector	M. Messina PhD thesis
PAO1 *lecA-lux*	*lecA::luxCDABE* fusion at the 73th codon	[194]
PACP2	Wild type mini-CTX::*lecA-luxCDABE* fusion at the 18th codon obtained with pCP2	This study
PACP3D	Triple Δ*lasI::*Gm^R/*rhlI::*Tc^R/*pqsA* mutant obtained from the double Δ*pqsA lasI::Gm*^{R} and with pSB224.12B	C. Pustelny PhD thesis
PACP04	Double Δ*pqsA* Δ*lasI* mutant, obtained from Δ*pqsA* and pSB219.8	C. Pustelny PhD thesis
PACP05	Δ*rhlR* in frame mutant	C. Pustelny PhD thesis
PAMM84	Wild type mini-CTX::*lecA-luxCDABE* fusion at the +1 transcriptional starting site, obtained with pMM42	This study
PAMM94	Δ*fleQ* mini-CTX::*rsmZ-luxCDABE* obtained with pSH50 and pMM58	M. Messina PhD thesis

Unless otherwise stated all the *P. aeruginosa* strains that were used and generated in this study derive from *P. aeruginosa* PAO1 Nottingham strain.

Table 2. Plasmids used in this study

Plasmids	**Characteristics**	**Reference/Source**
pBLS	pBluescript-II KS (+) cloning vector; ColE1 replicon	(Stratagene)
pET28(b)+	Plasmid for the overexpression of proteins	(Novagen)
pUC6S	Clonig vector; ColE1 replicon	[195]
pLM1	Mini-Tn5 delivery vector, Gm^R, *ori*VR6K, *ori*TRP4	[196]
pETAguR	Vector derived from pET28 (b)+ for the overexpression of AguR 6-His (*NdeI*/*HindIII*)	This study
pDM4	Suicide vector, *sacBR*, oriR6K, Cm^R	[197]
pHKBS1	Mini-CTX::*lux* delivery vector, Tc^R	[198]
pFLP2	Mobilizable broad-host-range plasmid providing Flp	[199]

	recombinase, ApR	
pKR1	pDM4::Δ*gacS*, for *gacS* in-frame deletion, made with GACS primers Cm^R	This study
pKR2	pDM4::Δ*ladS*, for *ladS* in-frame deletion, made with LADS primers Cm^R	This study
pKR3	pDM4::Δ*pqsE*, for *pqsE* in-frame deletion, made with PQSE primers Cm^R	This study
pKR4	pDM4::Δ*retS*, for *retS* in-frame deletion, made with RETS primers Cm^R	This study
pKR5A	Derived from pBLS containing the promoter of *rsmZ** (mutated in the *lux*-box) obtained using the primers PRSMZ2 and pSH50A	This study
pKR5	pHKBS1::$P_{rsmZ'}$(mini-CTX::rsmZ'-*lux*CDABE), replacement of the 66-bp *HindIII-PstI* fragment of pSH50 with annealed PRSMZ2 oligonucleotides. Substituted *lux* box	This study
pKR6	mini::CTX-*lux* P*lasB-lux* derived from pHKBS1 and oligos PlasB	This study
pKR7	mini::CTX-*lux* P*lasI*1-*lux* derived from pHKBS1 and oligos PlasI	This study
pKR8	mini::CTX-*lux* P*lasI*2-*lux* derived from pHKBS1 and oligos PlasI2	This study
pKR9	mini::CTX-*lux* P*lasI*3-*lux* derived from pHKBS1 and oligos PlasI3	This study
pKR10	mini::CTX-*lux* P*lasI*4-*lux* derived from pHKBS1 and oligos PlasI4	This study
pKR14	pME6032::*aguR* obtained using AGUR primers	This study
pKR17	pBLS containing *rhlR* (*Eco*RI/*Cla*I) obtained with RHLR primers	This Study
pKR18	pME6032 containing *rhlR* (*Eco*RI/*Cla*I) derived from pKR17 and *rsmZ*::*luxCDABE* from pSH50	This Study
pKR19	pME6032 containing *rhlR* (*Eco*RI/*Cla*I) derived from pKR17 and *rsmZ'*::*luxCDABE* (*Xba*I) derived from pKR5	This Study
pKR20	pME6032 containing *rhlR* (*Eco*RI/*Cla*I)	This Study
pKR23	pDM4::Δ*phz1*, for *phz1AG* in-frame deletion Cm^R, made with PHZ1 primers	This Study
pKR24	pDM4::Δ*phz2*, for *phz2AG* in-frame deletion Cm^R, made with PHZ2 primers	This Study
pKR51	pDM4::Δ*aguR*, for *aguR* in-frame deletion Cm^R, made with AGUR primers	This study
pKR57	pDM4::Δ*wspF*, for *wspF* in-frame deletion Cm^R, made with WSPF primers	This study
pKR66	mini-CTX-*lux* P*lasI5-lux* derived from pHKBS1 and oligos PlasI1FW and PlasI2RV	This study
pKR67	mini-CTX-*lux* P*lasI6*::*lux* derived from pHKBS1and oligos PlasI4FW and PlasI1RV	This study
pKR69	Chromosomal *Sau*3AI fragment containing *lasR* cloned in pME6000	This study
pKR70	Chromosomal *Sau*3AI fragment containing *rhlR* cloned in pME6000	This Study
pKR71	Chromosomal *Sau*3AI fragment containing *upf* cloned in pME6000	This study
pKR72	pME6032 containing the third ORF of the *upf* 3.1 kb fragment, and oligos UPF3	This study
pKR73	pME6032 containing the second ORF of the *upf* 3.1 kb fragment, and oligos UPF2	This study
pKR77	mini-CTX-*lux* P*rsmY-lux* derived from pHKBS1 and	This study

	oligos PrsmY	
pKR78	pME6000, containing the first ORF of *upf* 3.1 kb fragment derived from pKR71	This study
pKR79	pME6000, containing the second and third ORFs of *upf* 3.1 kb fragment derived from pKR71	This study
pKR80	pME6000, containing the first and second ORFs of *upf* 3.1 kb fragment derived from pKR71	This study
pKR81	pME6000, containing the first and third ORFs of *upf* 3.1 kb fragment derived from pKR71	This study
pCP1	pDM4::Δ*pqsR*, for *pqsR* in frame deletion, Cm^R, made with oligos PQSR	[113]
pCP2	pHKBS1::P_{lecA} (mini-CTX::lecA$_{(-18\ codons)}$-*lux*CDABE), obtained with oligos PLECA18	This study
pSB219.8	pRIC380 suicide vector carrying *lasI*::Tc^R for construction of *lasI* mutants	[192]
pSB224.12B	pRIC380 suicide vector carrying *rhI*::Tc^R for construction of *rhlI* mutants	[192]
pSH50A	pBLS containing the promoter region of *rsmZ* using oligos PRSMZ1	This study
pSH5	pME6032 containing the coding region for PA2567 using oligos PA2567START and PA2567H6	This study
pSH8	pUC6S containing the PA2567 coding region under the control of Ptac and the *lacIQ*. Derived from pSH5	This study
pSH9	pSH8 in which 100 bp up-stream from PA2567 coding region was cloned	This study
pSH10	pDM4 and pSH9 to construct the inducible PA2567 strain	This study
pSH50	mini-CTX::*lux* PrsmZ-*lux* obtained with oligos PrsmZ	This study
pMW02	pMM45 containing the promoter of $rsmZ_{CHA0}$ and the leader sequence of $aprA_{CHA0}$	Q. Wu MSc dissertation
pSK61	Vector for the overexpression of RsmA derived from pME6031	S. Kehune PhD thesis
pMM0	*Hin*dIII/*Xho*I fragment of pME6013 containing *'lacZ* cloned in pME6031 digested with *Hin*dIII/*Sal*I, Tc^S	M. Messina PhD thesis
pMM1	Synthetic constitutive 44-bp P_{km}promoter made with PKMFW and PKMRV inserted with *Xho*I and *Eco*RI in pMM0. Reporter vector for translation control analysis Tc^R	M. Messina PhD thesis
pMM-B10	pME6000 carrying the PA2567 gene	M. Messina PhD thesis
pMM11	*Eco*RI/*Cla*I fragment of pME6016 containing *'lacZ* (including RBS) cloned in pMM1 digested with the same enzymes, Tc^R	M. Messina PhD thesis
pMM42	pHKBS1::PlecA (*mini-CTX::lecA*$(_{-1})$-*luxCDABE*) made with PLECA1primers, Tc^R	M. Messina PhD thesis
pMM43	P_{Km}-*lecA'-'*cherry reporter plasmid. Synthetic 80-bp *lecA* RBS inserted in pMM45. Reports translation from *lecA* RBS, Tc^R	M. Messina PhD thesis
pMM44	P_{Km}-*lecA'-'lacZ* reporter plasmid. Synthetic 80-bp *lecA* RBS made with PLECAFWII and PLECARVII inserted in pMM1. Reports translation from *lecA* RBS, Tc^R	M. Messina PhD thesis
pMM45	pME6032 containing the *DsRedE2* red cherry gene	M. Messina PhD thesis
pMM58	pDM4::Δ*fleQ*, for the *fleQ* in frame deletion, made with WSPF primers Cm^R	M. Messina PhD thesis
pME6013	pVS1-p15A shuttle vector for translational *lacZ* fusions, Tc^R	[200]
pME6016	pVS1-p15A shuttle vector for transcriptional *lacZ* fusions, Tc^R	[201]
pME6031	pVS1-p15A shuttle vector, Tc^R	[200]
pME6032	pVS1-p15A shuttle expression vector, Tc^R	[202]

pME6111	ColE1 suicide plasmid for *gacA*::ΩSm/Sp mutation, Tc^R Sm^R/Sp^R	[147]

Table 3. Oligonucleotides primer sequence

Oligonucleotides	Sequence 5′-3′	Description
PlasBFW	TGGCCCAAGCTTACCGTGCG	Forward *lasB*,250bpUS+1*Hin*dIII
PlasBRV	ACCCAGGATCCCAACGAACAAC	Reverse P*lasB*,250bpUS+1*Bam*HI
P*lasI*1FW	ATAAAGCTTTCTGTGTGAAGCCATTGCTCTGA	Forward P*lasI*1 & P*lasI*5, *Hin*dIII
P*lasI*1RV	ATAGGATCCCTCCAAATAGGAAGCTGAAGAATT	Reverse P*lasI*1 and P*lasI*6, *Hin*dIII
P*lasI*2FW	ATAAAGCTTGCCCGGAAGGCCATGTTTT	Forward P*lasI*2, *Hin*dIII
P*lasI*2RV	ATAGGATCCAACTCTTCGCGCCGACCAA	Reverse P*lasI*2,P*lasI*3, P*lasI*4&P*lasI*5, *Hin*dIII
P*lasI*3FW	ATAAAGCTTCAGAAAGTTTCCTGGCTTTCCCG	Forward P*lasI*3, *Hin*dIII
P*lasI*4FW	ATAAAGCTTGTGCGGGTGGCCTTTGCC	Forward P*lasI*4 & P*lasI*6, *Hin*dIII
PHZ1FW1	TATAAGCTTCCTGTTCCAGAGCCTTTTCCT G	Forward 0.5 kbUS*phz1AG*, *Hin*dIII
PHZ1RV1	TAT GAATTC CTG ACC GTT CAT GCG CCG	Reverse US *phz1AG*, *EcoRI*
PHZ1FW2	TAT GAATTC CTG CAA CCG TGA CGA CAC	Forward 0.5 kb DS *phz1AG*,*Eco*RI
PHZ1RV2	TATTCTAGAGACCATACCGTGGCGCC	Reverse US *phz1AG*, *Xba*I
PHZ2FW1	TATTCTAGACATCGGCCTGCTCAACTG	Forward 0.5 kb US *phz2AG*, *Xba*I
PHZ2RV1	TATCTGCAGGTACTCTCGCATGGTGCG	Reverse US *phz2AG*, *Pst*I
PHZ2FW2	TATCTGCAGCAACCGTGACGACACCGC	Forward 0.5 kb DS *phz2AG*, *Pst*I
PHZ2RV2	TATGATATCGTTGCCGGAACAAGCCCT	Reverse US *phz2AG*, *Eco*RV
UPF2FW	TATGAATTCATGAATGTGAACTTAGAGAACG	Forward *upf* ORF2 *Eco*RI
UPF2RV	TATCTCGAGTCAGTCATCCTTGATGGGGAAA	Reverse *upf* ORF2 *Xho*I
UPF3FW	TATGAATTCATGAGCACAATACCAGACCG	Forward *upf* ORF3 *Eco*RI
UPF3RV	TATCTCGAGTCATAAGTTTATCTCCGGATG	Reverse *upf* ORF3 *Xho*I
WSPFFW1	ATATCTAGACTGGACGACGGTTCGCCG	Forward 0.5 kb US *wspF*, *Xba*I
WSPFRV1	ATAGGATCCTCCGATCCTCAACCCTGTG	Reverse US *wspF*, *Bam*HI
WSPFFW2	ATAGGATCCTTCGATTAGCCGGGTTCGAC	Forward 0.5 kb DS *wspF*, *Bam*HI
WSPFRV2	ATAAAGCTTCGCCACCAGCTCGATGGC	Reverse US *wspF*, *Hin*dIII
GACSFW1	ATAAAGCTTATCGTCGGCGCCCTGGC	Forward 0.5 kb US *gacS*, *Hin*dIII
GACSFW2	ATAGGATCCGAACTCTGACCATGCGCATC	Forward DS *gacS*, *Bam*HI
GACSRV1	ATAGGATCCTCCTTGAACACACGTCTCTC	Reverse US *gacS*, *Bam*HI
GACSRV2	ATATCTAGAGAAGCCGGCCATGATGCG	Forward 0.5 kb US *ladS*, *Hin*dIII
LADSFW1	TATAAGCTTTCGGCCACCGTGTCGACC	Forward 0.5 kb US *ladS*, *Hin*dIII
LADSFW2	TATGAATTCGCCTGAAGCCGTTCCGCG	Forward DS *ladS*, *Eco*RI
LADSRV1	TATGAATTCCCAGTGCCGCATGATGCC	Reverse US *ladS*, *Eco*RI
LADSRV2	TATTCTAGAAAGATTTCCGCCGCCAGCT	Reverse 0.5 kb DS *ladS*, *Xba*I
LUXC-RV	TCGGGAAAGATTTCAACCTGGC	*luxC* reverse sequencing primer
PQSEFW1	ATAAAGCTTGCCGGCGAGAGTCTCGAA	Forward 0.5 kb US *pqsE*; *Hin*dIII
PQSEFW2	ATAGGATCCCTGGACTGAGACGGGACAT	Forward DS *pqsE*, *Bam*HI
PQSERV1	ATAGGATCCAAGCCTCAACATGGCCGGT	Reverse US *pqsE*, *Bam*HI
PQSERV2	ATATCTAGAAGGCTGGACAGGCCATGC	Reverse 0.5 kb DS *pqs*E, *Xba*I
PQSRFW1	ATAAAGCTTTCTTAGAACCGTTCCTGG	Forward 0.5 kb US *pqsR*; *Hin*dIII
PQSRFW2	ATAGGATCCAGAGTAGAGCGTTCTCCA	Forward DS *pqsR*, *Bam*HI
PQSRRV1	ATAGGATCCAGGTTATGAATAGGCATC	Reverse US *pqsR*, *Bam*HI
PQSRRV2	ATATCTAGAAGTTCTGCCTGCTCGGCG	Reverse 0.5 kb DS *pqsR*, *Xba*I
PRSMY1FW	TATCTCGAGGGCGCCCTTGTCGAGGTT	Forward *rsmY* promoter, *Xho*I site at -327 from the +1 starting site
PRSMY1RV	TATCTGCAGCGGTTTGAAGATTACGCATCT	Reverse *rsmY* promoter, *Pst*I site at +6 from the +1 starting site
PRSMZ1FW	TATCTCGAGGGAAAACCTTAGACCCACTGA	Forward *rsmZ* promoter, *Xho*I site at -250 from the +1 starting site
PRSMZ1RV	TATCTGCAGGCAGGAGTGATATTAGCGATTC	Reverse *rsmZ* promoter, *Pst*I site at +2

		from the +1 starting site
PRSMZ2FW	AGCTTCGTAGGGTTTTCTGGCGTGTTGCGGGAGC ACTGGGAATCGCTAATATCACTCCTGCCTGCA	Forward *rsmZ* promoter, anneals PRSMZ2RV forming *Hin*dIII - (natural) and *Pst*I-compatible ends.
PRSMZ2RV	GGCAGGAGTGATATTAGCGATTCCCAGTGCTCCC GCAACACGCCAGAAAACCCTACGA	Reverse *rsmZ* promoter, anneals PRSMZ2FW forming *Pst*I - and *Hin*dIII - compatible ends
PKMFW	TCGAGCCGGAATTGCCAGCTGGGGCGCCCTCTGG TAAGGTTGGG	Forward Km promoter, anneals PKMRV forming *Xho*I- and *Eco*RI-compatible ends
PKMRV	AATTCCCAACCTTACCAGAGGGCGCCCCAGCTGG CAATTCCGGC	Reverse Km promoter, anneals PKMFW forming *Eco*RI- and *Xho*I-compatible ends
PLECA18FW	ATACTCGAGCCGGTTCGACCCCGGCTC	Forward *lecA*, *Xho*I, -373 from +1 transcriptional starting site
PLECA18RV	TATGGATCCCGACGTTACCTGCCCTGC	Reverse *lecA*, *Bam*HI, +54 from +1 transcriptional starting site
PLECAFW1	ATACTCGAGTTGTGTTTCCTGGCGTTCAG	Forward *lecA* promoter, *Xho*I site at position -345 from +1 transcriptional starting site
PLECARV1	ATAGAATTCCCTGTTAGCAAAGCACAG	Reverse *lecA* promoter, *Eco*RI site at position +2 from +1 transcriptional starting site
PLECAFWII	AATTCGAAGGATCGCGAATCAGGGTTTTTCGCCT CTTTCGTTTATGAACAGGATTCATATATCGGAGA TCAATCATGGC	Forward *lecA* RBS, anneals PLECARV forming *Eco*RI - and *Hin*dIII -compatible ends
PLECARVII	AGCTGCCATGATTGATCTCCGATATATGAATTCC TGTTCATAAACGAAAGAGGCGAAAAACCCTGATT CGCGATCCTTCG	Reverse *lecA* RBS, anneals PLECAFW forming *Hin*dIII - and *EcoRI*-compatible ends
RHLRFW	ATAGAATTCATGAGGAATGACGGAGGCTT	Forward *rhlR*
RHLRRV	ATAATCGATTCAGATGAGACCCAGCGC	Reverse *rhlR*
PRSMZCHAOF	AGCAGCACCCTTGAAGATGT	Forward for amplification of *rsmZ* promoter from CHAO
PRSMZCHAOR	TATGAATTCACAGGGCTGATATTAGAGAGTTC	Reverse for amplification of *rsmZ* promoter from CHAO, *Eco*RI
APRAF	AATTCCTGTTCCATTGATTACAAGGAAGTGTGTT TATGGA	Oligo $aprA_{CHAO}$ leader sequence anneals APRACHAO2 forming *Eco*RI - and *Hin*dIII -compatible ends
APRAR	GGACAAGGTAACTAATGTTCCTTCACACAAATAC CTTCGA	Oligo $aprA_{CHAO}$ leader sequence anneals APRACHAO1 forming *Eco*RI- and *Hin*dIII -compatible ends
AGURFW	ATAGAATTCATGCAGCCCACCTCGCC	Forward for the overexpression of AguR, *Eco*RI
AGURRV	ATACTCGAGCTATCGATTTAAATCGGATATAT	Reverse for the overexpression of AguR, *Xho*I
AGURFW1	ATAAAGCTTCTGCGCTACGTCGACAGC	Forward 0.5 kb US *aguR*; *Hin*dIII
AGURRV1	ATAGGATCCGGGCTGCATGTGGCGGG	Reverse US *aguR*; *Bam*HI
AGURFW2	ATAGGATCCAATCGATAGAAAATCCATAACAC	Forward 0.5 kb DS *aguR*; *Bam*HI
AGURRV2	ATATCTAGATGCCGCCATCGGCGTCG	Reverse DS *aguR*; *Xba*I
FLEQFW1	TATTCTAGAATCGGTGAGCTGGATCAGG	Forward 0.5 kb US *fleQ*, *Xba*I
FLEQRV1	TATGAATTCCCACATTTTGATCAGCTGCC	Reverse 0.5 kb US *fleQ*, *Eco*RI
FLEQFW2	TATGAATTCGATTGACAGGTCGTTTCGCA	Forward 0.5 kb DS *fleQ*, *Eco*RI
FLEQRV2	TATACTAGTTCGCGCGGAGCGAAGCAG	Reverse 0.5 kb DS *fleQ*, *Spe*I
PLECARBSFW	AATTCGAAGGATCGCGAATCAGGGTTTTTCGCCT CTTTCGTTTATGAACAGGATTCATATATCGGAGA TCAATCATGGC	Forward *lecA* RBS, anneals PLECARV forming *Eco*RI/ *Hin*dIII compatible ends
PLECARBSRV	AGCTGCCATGATTGATCTCCGATATATGAATTCC	Reverse *lecA* RBS, anneals PLECAFW

	TGTTCATAAACGAAAGAGGCGAAAAACCCTGATT CGCGATCCTTCG	forming *Hin*dIII / *Eco*RI compatible ends
PA2567START	TATGAATTCATGGCAACCCCACCCTATCC	Forward for the cloning of PA2567; *Eco*RI
PA2567H6	TATATCGATTCAGTGAGGTGATGGTGATGCGCCG CAGCCAGTCCTCC	Reverse for the cloning of PA2567, six histidine tag; *Xho*I

Sequences in 5′ to 3′ order; artificial restriction sites are underlined; US and DS primers indicate the oligos used to amplify the upstream and downstream region of the genes for in-frame mutation. All the primers were synthesised and purchased by Sigma-Aldrich. AUS: Analysis Unit Services (University of Nottingham, UK).

2.2 DNA MANIPULATIONS

2.2.1 Rapid extraction of plasmid DNA

Rapid extraction of plasmid DNA was performed as described previously [203]. Strains were grown in LB with the addition of appropriate selective antibiotics for 10-12 hrs at 37°C in shaking conditions. Afterwards 1.5 ml of bacterial cultures were centrifuged at 12,000 rpm for 3 min at room temperature (RT), and the bacterial pellet was resuspended in 200 µl of STET buffer (sucrose 8% w/v, Triton X-100 0.1% v/v, EDTA 50 mM, Tris-HCl 50 mM [pH 8.0]). After addition of 4 µl of lysozyme (50 mg/ml) the samples were incubated for 5 min at RT. The samples were then boiled for 45 secs and centrifuged at RT for 10 min at 13,000 rpm. The pellet was removed and 8 µl of CTAB (5% [w/v]) (Sigma) was added to the supernatant. The samples were then centrifuged 10 min at 14,000 rmp and the resulted precipitate was resuspended in 300 µl of NaCl 1.2 M. The DNA was precipitated using 600 µl of ethanol 95%, and then washed with 1 ml of ethanol 70%. Finally, the DNA was resuspended in 20 µl of H_2O containing RNAse A at a final concentration of 0.5 mg/ml [204].

2.2.2 Total DNA extraction

Total DNA extraction was performed as described before [205]. Strains were grown in LB with the addition of appropriate selective antibiotics for 10-12 hrs at 37°C in shaking conditions. Afterwards, 1.5 ml of culture was centrifuged at 12,000 rpm for 3 min, and washed with 1 ml of TE buffer (10 ml Tris [pH7.5], 2 ml Na$_2$EDTA 0.5 M in 800 ml H$_2$O). The bacterial cells were finally resuspended in 400 µl of TE buffer. Then 50 µl of SDS 10% [v/v], 50 µl of proteinase K (2.5 mg/ml) and 10 µl of RNAse A (10 mg/ml) were added and the sample that was incubated at 37°C for 3 hrs. The dense cell lysate was passed through a syringe five times with a needle, then extracted at least three times with phenol:chloroform:isoamylic alcohol (25:24:1). This extraction procedure was followed by a further extraction with water-cloramfenicol. DNA in the aqueous phase was precipitated using 1 ml of ethanol 95%, and then washed with 1 ml of ethanol 70%. Finally, DNA was resuspended in 50 µl of H$_2$O [204].

2.2.3 Cloning procedures

Large-scale plasmid DNA extractions were performed using the Plasmid Midi kit (Qiagen), in accordance with the manufacturer's specifications.

In order to identify and quantify specific DNA fragments, DNA derived from plasmids was digested with appropriate restriction enzymes (Promega) and subsequently separated by electrophoresis on agarose gel 1% (w/v) [204]. The DNA ladder 1 Kb (Promega) was used as molecular weight marker. Restriction fragments were purified from agarose gels using the QIAquick Gel Extraction kit (Qiagen), in accordance with the manufacturer's specifications.

PCRs (Polymerase Chain Reactions) were performed with Taq polymerase (Promega), following manufacturer's specifications. Nucleotides (Promega) and synthetic oligonucleotides (Sigma) were utilized at the final concentration of 0.5 mM and 0.5 μM, respectively. PCR products were purified using the QIAquick PCR DNA Purification kit (Qiagen), in accordance with the manufacturer's specifications.

Ligation reactions were performed with T4 DNA ligase (Promega) in accordance with manufacturer's specifications.

Automated sequencing was performed by Biopolymer Synthesis for Analysis Unit Services (University of Nottingham, UK).

2.3 PLASMID DNA TRANSFER: TRANSFORMATION AND CONJUGATION

In order to create electro-competent *E. coli* and *P. aeruginosa* PAO1 or *P. fluorescens* CHA0 cells, the strains were grown in LB for 10-12 hrs at 37°C in shaking conditions. Once reached OD_{600} of 2.5, the cultures were diluted 1:200 in 200 ml of LB and then incubated at 37°C in shaking conditions for 6 hrs. After a centrifugation at 10,000 rpm for 10 min at 4°C the bacterial pellets were washed twice with 40 ml of Buffer 1 (MOPS 1mM [pH7.2], glycerol 10% v/v) and the resuspended in 1 ml of Buffer 1. Aliquots of 50 μl were stored at -80°C. All the solutions were pre-cooled before using and the cells were maintained on ice during the whole procedure.

For transformation, 80-150 ng of DNA was added to the competent cells. These were then electoporated and incubated at 37°C in shaking conditions for 1 hrs. Finally, the transformed cells were grown on LB plates with the addition of the appropriate antibiotics.

When required, plasmid DNA was transferred in *P. aeruginosa* PAO1 or *P. fluorescens*

CHA0 strains by conjugation. The donor and the recipient strains were grown in LB with the addition of appropriate selective antibiotics for 10-12 hrs at 37°C in shaking conditions. 1.5 ml of each culture was centrifuged at 4000 rpm for 5 min. After two washes with LB the strains were mixed together in a final volume equivalent to the volume of the bacterial pellet. The mixed cells were transferred as a drop to the centre of a LB plate and incubated at 37°C for 6 hrs. Finally the conjugation drop was resuspended in 1 ml of NaCl 0.9% [w/v] solution and 200 µl of the bacterial suspension was spread into the selective plates.

2.4 Plasmid and Strain Construction

2.4.1 Construction of suicide plasmid using the pDM4 vector

All the *P. aeruginosa* mutants constructed are derived from *P. aeruginosa* PAO-N laboratory strain. For the in-frame deletions of *gacS, lads, retS, aguR, wspF, phz1, phz2, pqsE* and *pqsR* a common strategy was used. Upstream and downstream flanking regions were amplified by PCR from PAO1 chromosomal DNA using the oligonucleotides described in Table 3. The 500 bp products for the up and downstream sequence were cloned separately into pBLS and verified by sequencencing. Then, the up and downstream fragments were ligated into the pDM4 suicide vector (*XbaI/XhoI*).

2.4.2 Construction of inducible PA2567 strain

The suicide plasmid pSH10 to introduce the inducible $lacI^{Q}$ P_{tac} promoter locus immediately upstream of PA2567 in the chromosome was constructed in several steps. Firstly, a PCR

product corresponding to a 0.5 kb region upstream of PA2567 was amplified from PAO1 chromosomal DNA using the primers PA2567UF and PA2567D1 and cloned in pBLS as a 0.54 kb *Spe*I-*Sal*I fragment to produce pSH1. This construct was verified by sequencing. Secondly, the PA2567 open reading frame was amplified from PAO1 chromosomal DNA by PCR using the primers PA2567START and PA2567H6. Due to the presence of two natural *Eco*RI sites downstream of a central *Bam*HI site in PA2567, the 1.78-kb PCR fragment was digested separately with *Eco*RI and *Bam*HI and with *Bam*HI and *Cla*I to produce two moieties of 0.83 and 0.95 kb respectively. The two moieties were then inserted in a triple ligation into pME6032 digested with *Eco*RI and *Cla*I, to produce pSH5. The resulting 2.4 kb *Bam*HI fragment of pSH5 was then subcloned in pUC6S digested with *Bgl*II in the *Not*I-*Kpn*I-[*Bgl*II/*Bam*HI]-lac^{q}-P_{tac}-PA2567- [*Bam*HI/*Bgl*II] orientation to produce pSH8. The 0.54 kb *Not*I-*Kpn*I fragment of pSH1 was then subcloned in pSH8 digested with these same enzymes to produce pHS9. Finally, the 2.9 kb *Spe*I fragment of pHS9 carrying PA2567-'lac^{q}-P_{tac}-'PA2567 was subcloned into pDM4 digested with the same enzymes to produce pSH10. Allelic replacement in the chromosome using pSH10 allowed the construction of *P. aeruginosa* strains in which the expression of PA2567 could be induced by IPTG.

2.4.3 Construction of chromosomal *lux*-based transcriptional fusions

Transcriptional chromosomal *rsmY-lux*, *rsmZ-lux*, *lasB-lux*, *lasI1-lux*, *lasI2-lux*, *lasI3-lux*, *lasI4-lux*, *lasI5-lux*, *lasI6-lux*, $lecA_{18codon}$-*lux* and $lecA_{+1}$-*lux* fusions were constructed as follows: the promoter regions were amplified from *P. aeruginosa* PAO1 genomic DNA by PCR using primers listed in Table 3. Each amplicon was digested with appropriate restriction enzymes and cloned into pHKBS1 digested with the same enzymes, in *E. coli* S17-1λpir.

After verifying the inserts by sequencing with primer LUXCRV, the resulting plasmids were mobilized by conjugation into the recipient wild type *P. aeruginosa* PAO1-N or its mutant derivatives (Table 1). The integration of the mini CTX::*lux* promoter fusion-*lux* in the chromosomes of the recipient strains, was revealed by Tc resistance and bioluminescence. The luminescence phenotype was detected with a Luminograph LB980 photon video camera (EG&G Berthold). When required, the Tc resistance marker was excised from the chromosomes of the reporter strains by mobilizing pFLP2 followed by plasmid curing as previously described [199].

2.4.4 Construction of P*rsmZ*-lux* transcriptional fusion

The promoter of *rsmZ** mutated in the *lux*-box region was constructed by self-annealing of two oligos PRSMZ2FW and PRSMZ2RV which anneal forming a *Hin*dIII (natural) and *Pst*I compatible ends. The vector containing the natural P*rsmZ* fragment (pSH50A) was digested with *Pst*I/*Hin*dIII (thus eliminating the 62 bp region) and ligated with the annealed oligonucleotides containing the mutated *lux*-box from the *rsmZ* promoter. The new construct pKR5A was then digested with *Xho*I/*Pst*I and cloned into the mini CTX::lux vector (pHKS1) giving rise to pKR5.

2.4.5 Construction of *upf* fragments derived from pKR71

For the generation of pKR78, pKR71 was digested with *Hin*dIII/*Acl*I and the resulting 618 bp fragment (containing ORF1) was subcloned into pBlueScript (pBLS) plasmid previously digested by *Hin*dIII/*Cla*I (*Cla*I and *Acl*I generate compatible sticky ends). The resulting

vector was then digested with *Sac*I/*Xho*I and the fragment was inserted into pME6000 digested with the same restriction enzymes.

For the generation of pKR80, pKR71 was digested by *Hind*III/*Sca*I/*Not*I and the resulting 2.4 Kb fragment (containing ORF1 and ORF2) was cloned into pME6000 previously digested with *Hin*dIII/*Eco*RV.

For the generation of pKR79, pKR71 was digested with *Acl*I/*Not*I and the resulting 2.6 Kb fragment (containing ORF2 and ORF3) was inserted into pBLS previously digested with *Not*I/*Cla*I. The resulting vector was then digested with *Sac*I/*Xho*I and the fragment inserted into pME6000 digested with the same restriction enzymes.

For the generation of pKR81, pKR71 was digested with *Msc*I/*Pvu*II (blunt ends were generated) and the plasmid (containing ORF1 and ORF3) was re-ligated.

2.4.6 Construction of double transcriptional-translational *P. fluorescens* CHA0 reporter

The double transcriptional-translational DsRedE2 reporter was constructed using the *rsmZ* promoter amplified from *P. fluorescens* CHAO and cloned (*Xho*I/*Eco*RI) into the pMM45 vector already containing the DsRedE2 fluorescent protein gene. The annealing of the oligonucleotides of the leader sequence of the $aprA_{CHAO}$, was carried out by a semi-cycle PCR reaction. The oligos were diluted to a concentration of 100 nM and mixed, the starting temperature was of 95 °C for 30 seconds, and then lowered at 0.05 °C per second until reaching 4 °C. This resulted in the annealing of the two oligos and the formation of double-stranded DNA having compatible extremities with *Eco*RI and *Hind*III. The leader sequence was then inserted in pMM45 digested with *Eco*RI and *Hind*III by ligation, resulting in the

plasmid pMW02 that was then transformed into *P. fluorescens*.

2.4.7 Construction of pKR18 and pKR19

The plasmids pKR18 and pKR19 were constructed for the overexpression of *rhlR* in the heterologous *E. coli* system. These plasmids are derived from pME6032. The *rhlR* gene is under the regulation of the inducible P_{tac} promoter. The *luxABCDE* has also been inserted into the plasmid and it is under the regulation of the *rsmZ* ptomoter (pKR18) or the *rsmZ** promoter (pKR19). The coding region of *rhlR* was amplified with the RHLR primers and subcloned into pBS giving rise to the pKR17 vector. Once the sequences was checked by sequencing analysis, *rhlR* was cloned into pME6032 (pKR20) with *Eco*RI/*Cla*I.

To construct pKR18, the *rsmZ-lux* fusion (comprising the promoter of *rsmZ* fused with the *luxCDABE* operon) was excided with *XbaI* from pSH50 and inserted into pKR20.

In the case of pKR19, the P*rsmZ*-lux* fusion (comprising the promoter of *rsmZ* mutated in the *lux*-box fused with the *luxCDABE* operon) was excided with *Xba*I from pKR5 and cloned into pKR20.

2.4.8 Construction of translational fusions

Translational fusions based on *'lacZ* under the control of a constitutive promoter P_{Km} were constructed. In brief the *'lacZ* gene truncated at the 8th codon was inserted into the vector pME6031, generating plasmid pMM0 (Tab. 2). A synthetic Km promoter was inserted upstream of in pMM0, generating the vector pMM1. Finally the synthetic RBS (Ribosome Binding Sites) was inserted generating the post-transcirptional fusion P_{Km}-*lecA'*$_{RBS}$-*'lacZ*,

generating pMM44 (M. Messina PhD thesis for details).

The P_{Km}-*lacZ*$_{RBS}$-'*lacZ* was constructed by exchanging the *lecA*'$_{RBS}$-'*lacZ* sequence with the *lacZ*'$_{RBS}$-'*lacZ* sequence, generating pMM11 (M. Messina PhD thesis for details).

2.5 GENERATION AND ANALYSIS OF THE GENETIC BANK AND THE TRANSPOSON LIBRARY

The genetic bank was generated using the PAO1-L strain chromosomal DNA. The reason is that the PAO1-N strain was found to have a 50 Kb deletion in its chromosome (S. Heeb personal communication). Random 2-4 Kb DNA fragments of the PAO1-L chromosome partially digested by *sau3*A-1 were cloned in pME6000, previously linearised by *Bam*HI and treated with alkaline phosphatase to avoid the plasmid re-ligating into itself. The product of the ligation was used to transform *P. aeruginosa* PAO1-N *rsmZ-lux* (PASH50).

The genetic bank strains were screened by using autoclaved toothpicks to transfer colonies from the selective plates onto the LB agar plates at a density of 50 colonies per plate. The plates were incubated for 10-12 hrs at 37°C. As control the strain PASH50 with the empty pME6000 vector, was used. The clones were then analysed under the light camera, clones which produced a stronger or weaker light signal, were retested individually in new plates. Once identified a clone of interest, the plasmid was extracted and sequenced using the oligos Ptac/P6032 (Tab. 3).

The transposon mutagenesis library was obtained by introducing the transposon Mini-Tn5 delivery vector pLM1 into the recipient strain *P. aeruginosa* PASH50 (*rsmZ-lux*).

The transposon mutants were screened as for the genetic bank derived clones as a negative control the PASH50 strain was used. Once identified a particular clone, to identify which gene was the one interrupted by the transposon the following strategy was carried out. The

chromosome of the interesting clones was extracted and digested (5 µl of the extracted chromosome in 50 digestion mix) with *Bam*HI. The chromosomal DNA was then relegated using the T4 Ligase enzyme (Promega) generating plasmids able to replicate in *E. coli* (*ori* contained in the transposon fragment). The plasmids, containing part of the interrupted gene of interest were then transformed in *E. coli* S17.1 *λpir*. The plasmid was then extracted and sequenced using the oligos 13.2 and 17-1 (Tab. 3).

2.6 PURIFICATION OF AGUR SDS-PAGE ANALYSIS AND EMSA

To purify AguR, *aguR* gene was amplified by PCR with primers FWaguR and RVaguR from genomic DNA of the *P. aeruginosa* PAO1-N strain. The resulting PCR product was digested with *Nde*I/*Hind*III and cloned into similarly digested pET28b(+) (Novagen), resulting in the plasmid pET*aguR*, which was verified by sequence analysis. In this plasmid the *aguR* gene was fused to a 6xHis tag at its N-terminus. The pET*aguR* plasmid was introduced into *E. coli* BL21(DE3)pLysS strain by electroporation.

E. coli BL21(DE3)[pLysS] strain carrying pET*aguR* was grown over-night at 37° C in shaking conditions in LB supplemented with Km 25 mg/l, Cm 30mg/l and glucose 1% (w/v). The overnight culture was diluted 1:1000 in 600 ml of LB supplemented Cm 30 mg/ml, Km 25 mg/ml and glucose 0.2% (w/v). Cultures were then grown at 37° C in shaking conditions to OD_{600} = 0.6. At this point, 2 ml aliquots of the non induced cultures were centrifuged and stored at -20° C, as a control. At this stage AguR expression was induced with 1 mM IPTG and 1mM agmatine (the natural ligand of AguR). After additional incubating for an overnight at 37°C two aliquots of 2 and 6 ml were collected to perform analyses of the soluble and insoluble fraction and the remaining culture was centrifuged at 4000 rpm for 45 min at 4° C.

The resulting pellet was stored at -20° C.

The eluate containing the purified AguR, was desalted using Zeba Spin Desalting Columns (Pierce) according to the manufacturer's instructions. Aliquots of the protein were stored at 4° C, at -20° C or at -80° C.

To estimate the protein concentration of a sample the Bradford assay was used [206].

Analysis of *E.coli* overexpressing AguR total proteins was performed by resuspending bacterial pellets deriving from 2 ml of culture collected before and after the induction in loading dye 3x (Tris-HCl 175mM [pH 6.8], SDS 5% [w/v], glycerol 15% [v/v], dithiothreitol 50 mM, Bromophenole Blue 0.125 %[w/v]). Moreover it was interesting to analyse also the soluble/insoluble fractions of the induced coltures [204]

The electrophoretic protein separation was accomplished following the discontinue Laemmli method. Acrylamide was used at 12% (v/v), the ratio acrylamide/bisacrylamide used was of 13:1. The electrophoretic run was performed at 140 V for approximatively 60 min. Gels were subsequently fixed in a 10% (v/v) isopropanol solution, stained with Comassie Brilliant Blue G-250, and then destained with acetic acid 10% (v/v) [207].

The electrophoretic mobility shift assays (EMSAs) were performed on a probe encompassing the promoter of *rsmZ*, generated by PCR using the primers FWrsmZ RVrsmZ. The resulting PCR fragment was purified and run in a bandshift assay. Increasing concentrations of AguR (from 0.1 ng/ml to 1 mg/ml) were incubated RT for 30 min with the digoxygenine-labeled probe generated with primers FWrsmZ and RVrsmZ and labeled with the DIG High Prime Labeling Kit (Roche) according to manufacturer's instructionat [204]. The detection was performed with Anti-Digoxygenine-AP Fab fragment (Roche) and CDP-Star Detection Reagent (Amersham) according to manufacturer's instruction.

2.7 PROMOTER PULL-DOWN

The promoters of *rsmZ* and *rsmY*, were amplified with the PRSMZ and PRSMY primers respectively, the reverse primers used where biotynilated (bio-RVPrsmZ and bio-RVPrsmY) at the 5'. To bind the DNA the Dynabeads M-280 Streptavidin (Invitrogen) were used.

Firstly the PCR product was optimised and purified so that the final concentration of the product was higher than 20 ng/µl in a final volume of 200 µl. The protein extract was prepared from a 400 ml of LB colture taken at a specific time point depending on the maximal activity of the promoter, usually at OD_{600} = 1. The pellet was resuspended in 4 ml lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.05% Triton X100) and lysed using a French press. After the lysate was centrifugate 14.000 rpm 4 °C and the supernatant was quantified using a Bradford curve, 0.1 mg/ml of protein extract was usually obtained. To the extract 1 mM PMSF (Phenylmethyl sulfonyl fluoride) a strong proteinase inhibitor, and 50 µg/ml of salmon DNA sperm was added before storing at -80 °C. Salmon DNA sperm was necessary to avoid unspecific reaction between the biotynilated probe and the protein extracts. The Dynabeads were washed three times, using the magnetic rack, with Wash Buffer 2X (WB2X) (2 M NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA). Then resuspended in 200 µl of WB2X and 200 µl of biotynilated DNA and incubated at RT for 30 min with gentle agitation. The Dynabeads were washed in 1 ml Wash Buffer 1X three times, then resuspended in the protein extract and incubated for 2 hrs at RT with gentle agitation. Finally the beads were washed in six times in 1 ml of lysis buffer and the final pellet was resuspended in 20 µl Elution buffer (1.2 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.05 % Triton X100) and incubated for 30 min at RT with gentle agitation. The eluted fraction was separated from the beads using the magnetic rack and run on an SDS-PAGE. The visible bands were then

analysed by Maldi Tof for protein identification. As control the protein extracts were incubated with the Dynabeads without the PCR product, in this case no bands are expected.

2.8 GENE REPLACEMENT IN *P. AERUGINOSA*

Plasmid transfer from *E. coli* S17-1λ*pir* donor strains to *P. aeruginosa* recipient cells was carried out by conjugation. The first step of recombination was selected by antibiotic resistance. By sucrose counter-selection the second event of recombination was selected [208]. The suicide plasmids used to perform gene replacements during this study derive from pDM4 carrying the *sacBR* locus that allows its counter-selection. Single colonies from the first cross over were re-streaked and grown o/n in LB broth. Then they were diluted 1000 times in LB broth containing 10% (w/v) sucrose and allowed to grow over night to counterselect for cells having achieved the second cross-over. After repeating this step, dilutions were plated onto sucrose plates to obtain single colonies. Colonies that grew were checked for loss of the suicide plasmid by screening for antibiotic sensitivity. All the mutants obtained in this work were confirmed by PCR.

Deletion of *rsmY* or *rsmZ* in a Δ*pqsA* in-frame deletion mutant was obtained as described elsewhere [151]. Construction of *lasI*, *rhlI*, *rhlR* of *lasR* mutants were obtained using the suicide vectors derived from pRIC380, which produces mutant by insertion of a gentamicin resistance marker within the gene, as described elsewere [192].

Mutants with a disrupted *gacA* gene were constructed by allelic exchange using the suicide plasmid pME6111 as described elsewhere [147].

2.9 SOUTHERN BLOT ANALYSIS

The genomic DNA was digested with restriction enzymes, run on agarose gel and then denatured. The DNA was blotted onto a nylon membrane (Hybond N+, Amersham Life Sciences, UK) by dry capillary transfer at room temperature as described elsewhere [204]. Blotted membranes were hybridized with probe labelled with a DIG DNA Labelling Kit (Roche Inc). After the hybridisation, the membrane was washed, saturated in blocking solution (1% (w/v) Blocking Reagent (Roche), and then incubated with anti-digoxigenin-AP antibody (diluted 1:10,000 in blocking solution) for 30 min. After a series of washing, the membrane were treated with CDP Star ready to use chemiluminescence substrate. After incubation at 37°C for 20 min, membranes were then exposed to autoradiograph films for 5-20 min and the films were developed [204]. The blocking reagent, antidigoxigenin-AP antibody, and CDP Star substrate were supplied by Roche Applied Science, UK.

2.10 ß-GALACTOSIDASE ACTIVITY

This biochemical assay was used to evaluate *lacZ* expression of strains of *P. aeruginosa* which contained a post-transcriptional *'lacZ* based fusion. For each analysed strain, the bacterial growth rate was followed measuring the optical density at 600 nm in different time intervals. At different growth points 1 ml of culture was collected and the β-Galactosidase activity was measuring as described elsewhere [209].

2.11 BIOLUMINESCENCE

Bioluminescence of reporter strains carrying transcriptional *luxCDABE* fusion was measured during growth as previously described [210]. To simplify the comparison between diverse conditions the peak values were extracted from the curves. The maximal values were typically reached at optical densities at 495 nm (the wavelength at which the Anthos Labtech LUCY 1 automated luminometer-spectometer reads turbidity) of between 0.85 and 1.15. The reporter strains were grown overnight in LB broth medium without antibiotics at 37°C. Overnight culture of the reporters were centrifuged, supernatant discarded and the residue pellet was washed in 1 ml of fresh LB medium. The cultures were diluted to a starting OD_{600} 0.001 in LB broth in a total volume of 200 µl for each well used in the LUCY or TECAN assay, in triplicate.

To measure the production of bioluminescence throughout growth, light levels and OD were monitored in 96 well microtitre plates using the Anthos LUCY1 combined photometer/luminometer controlled by the Stingray software (Dazdaq). The program measures OD_{492} and luminescence from the wells every 30 min for 24 hrs. Readings were analysed using Microsoft Excel. Alternatively the Infinite 200 (TECAN) multimode micro-plate reader controlled by XFluor software was used with the same procedure.

2.11.1 QS-agonist assay

Screening of individual molecules for their ability to activate P*lasB-lux* fusion was conducted using 5 µM of 3-oxo-C12-HSL as a positive control. In the negative control the solvent used for dissolving the compound tested was added (5% v/v of methanol). Test compounds were added to the tubes containing the biosensor (OD_{600} of 0.001) to a final concentration of 100 µM. Values were expressed as a percentage of the maximal bioluminescence measured

after exposure of the bacterial reporter cells to the analogues (approximately after 8 hrs from inoculation at 37 °C) relative to positive control (100%). Measurements were conducted in triplicates.

2.11.2 QS-antagonist assay

The assays for QS antagonists were based on testing the ability of individual molecules to compete with 3-oxo-C12-HSL for the activation of the P*lasB-lux* or the P*lasI-lux* based reporter strains. To each sample tube containing the diluted reporter (final OD_{600} of 0.001), 3-oxo-C12-HSL (the concentration used was specific for each reporter and corresponds to the concentration at which the promoter activity is at its half maximal: K_{ind}) and individual analogues were added at a concentration range of 1 μM, 10 μM and 100 μM. As a negative control the solvent used to dissolve the compounds tested was added. In the positive control only the K_{ind} concentrations of 3-oxo-C12-HSL were added, at 0.5 μM and 5 μM respectively for P*lasB-lux* and P*lasI-lux* fusions. Values were expressed as a percentage of the maximal bioluminescence measured after exposure of the bacterial reporter cells to the analogues (approximatively after 8 hrs from inoculation at 37 °C) relative to positive control (100%). Measurements were conducted in triplicates.

2.12 FLUORESCENCE

To measure the production of fluorescence throughout growth, the strains were monitored using 96 well micro-titre plates and the detection was conducted with the Infinite 200 (TECAN) multimode micro-plate reader controlled by XFluor software. The wavelength

settings for the detection of DsRedE2 were of 535 nm for the excitation and 595 nm for the emission. The measurements of the optical density were conducted at OD_{600} every 30 min for 24 h. Readings were analysed using Microsoft Excel. The strains were treated as described before for the luminescence assays.

2.13 Thin Layer Chromatography (TLC)

An appropriate volume of the extract was spotted onto the aluminum backed reverse phase 2 (RP-2) plate (Merck) used for separating long chain AHLs, as an alternative the reverse phase 18 (RP-18) plate (Merck), for separating the short chain AHLs, or the normal phase (NP) plate (Merck), for separating the quinolones molecules, were used. The sample was loaded 2 cm above the base of the plate and dried in a stream of warm air. The extracts were separated using methanol-water 45:55 v/v (for long chain AHLs), 60:40 v/v (for short chain AHLs) or dichloromethane-methanol 95:5 v/v (for quinolones) as the mobile phase. Once the solvent front reached the top of the plate, the TLC plate was removed and dried.

The reporter strains used for the TLC overlaying detection PAKR08 (*ΔpqsA rsmZ-luz*) and *P. fluorescens* CHA0 containing the pMW02 plasmid, were grown overnight in liquid LB at 37° C and 30° C respectively. The next day 5 ml of the reporters were mixed with 100 ml of soft top agar and the mixture was poured onto the TLC plate as already described [210]. In the case of the TLC performed with the PAKR08 a light camera was used. On the other hand, in the case of the double transcriptional-translational reporter strain, a visual method for the detection of fluorescence was used. In particular, the TLC plate was placed into a black-box and the red fluorescence was excited using a green light. To detect fluorescence a filter was used, this was placed on the top of the box, above the TLC plate.

2.14 HIGH-PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

The extracts from *P. aeruginosa* PAO were analysed by HPLC using a Waters 625 LC System linked to a Waters 996 Photo Diode Array detector (Waters, Manchester, UK). The 250 x 2.1 mm Kromasil C8 (Hichrom, Reading, Berkshire, UK) column was equilibrated with 50% (v/v) acetonitrile/water. In detail, 600 ml LB culture were grown at 37 °C to an OD_{600} corresponding to the end of the exponential phase. The colture supernatants were extracted and concentrated to a final volume of 50 µl. The whole volume was injected onto the column and run on a linear gradient 20-100 % (v/v) acetonitrile/water; the samples were scanned within the UV range, 190 to 400 nm, to determine spectral profiles. The HPLC column was connected to a fraction collector (Gilson, UK) and seven fractions were collected each 5 min for 35 min, the fractions were dried by rotary evaporation.

2.15 MASS SPECTROMETRY (MS)

The HPLC column was also connected to a mass spectrometer (MS). The apparatus used was a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectometer (Applied BIosystems, USA) equipped with TurboIon source used in positive ion electrospray mode [211].

2.16 *P. AERUGINOSA* SIGNAL MOLECULES

2.16.1 Extraction from culture supernatant

P. aeruginosa wild type and mutant strains were grown in 200 ml of Casamino Acid medium in 1-liter flask for 16 hrs with shaking to an OD_{600} of 2.0 to 2.5. Cells were then removed by centrifugation. The supernatant was passed though a 0.45-m-pore-size filter, adjusted to pH 5.0, and extracted twice with 200 ml of dichloromethane. The extracts were pooled and evaporated to dryness using a Rotavapor R-210 machine and the dried extracts stored at 20°C. The extracts were dissolved in methanol so that the extracted molecules were 200 times more concentrated than in the culture media. For the luminescence and fluorescence experiments, the ratio of reporter culture to signal extracts was 1:2, 1:4 1:6, 1:8 and 1:10. For the negative control methanol was added.

2.16.2 Pyocyanine extraction

Pyocyanine extraction was performed following the protocol already described by Frank and Demoss (1959) [185] . The strains were grown in 600 ml of Gylcerol-Alanine minimal medium for 36 hrs at 37 °C. The culture was then centrifuged and the supernatant was filtered. The supernatant was then extracted three times with 100 ml of chloroform, the organic phase was collected and re-extracted twice with HCl 0.1 M. The aqueous phase was collected each time and 1 M borate-NaOH buffer pH 10.0 was added until the solution become blue. The aqueous phase was re-extracted with chloroform and the organic phase was collected. The chloroform phase was then extracted again with HCl 0.1 M into a reduced volume. Addition of NaOH 1 M until the color turns blue.

2.16.3 Synthetic quorum sensing signal molecules

Synthetic 3-oxo-C12-HSL, C4-HSL and PQS were made by S.R. Chhabra and A. Turman at the School of Molecular Medical Sciences, University of Nottingham and kept as 10 mM stocks in methanol (PQS) or acetonitrile (3-oxo-C12-HSL, C4-HSL) as described by Chhabra *et al.*, (2003) [184], and Pesci *et al.*, (1999) [92]. Compounds were stored at -20°C.
Synthetic AHL analogues for competition studies were synthesized by S.R. Chhabra and A. Turman at the School of Molecular Medical Sciences, University of Nottingham and kept as dry form at -20°C.

2.16.4 Calculation of IC50

The half maximal inhibitory (IC_{50}) concentration of the tested compounds was determined by constructing a dose response curve and examining the effect of different concentrations of the molecule on the promoter activity of the reporter (P*rsmZ* or P*rsmY*). The IC_{50} was calculated by determining the concentration of the compound needed to inhibit half of the maximum activity of the reporter. In this study the IC_{50} values were calculated with the sigmoidal dose-response curve obtained using the program Prism2 (Graphpad, San Diego, USA).

2.17 CLINICAL STUDY

2.17.1 Patients

Patients over eight years, with CF and chronic pulmonary infection with *P. aeruginosa* were enrolled when they were free of an exacerbation of pulmonary symptoms. Participant were

only enrolled if they could expectorate sputum, perform pulmonary function tests reliably and swallow capsules. As a result of this selection, twenty six CF patients, 14 male and 12 women, mean age of 18 years (range 11-54 years) with chronic bronchopulmonary *P. aeruginosa* infection entered the study.

2.17.2 Study design

The study was a double-blind clinical trial in which patients were randomly assigned to receive either active treatment or the placebo. Patients were divided into adult (over 16) and paediatric (under 16).

2.17.3 Treatment

Active treatment consisted of intake of one capsule daily with their evening meal (656 mg of garlic oil macerate and 10 mg of cardamom oil), or placebo (656 mg of olive oil and 10 mg cardamom oil). The cardamom oil was present as an odour control agent so that it was difficult for the patients to understand in which group they were assigned. Treatment went on for 8 weeks. The patient therapy (enzymes, vitamins and antibiotics) was maintained unchanged throughout the study.

2.17.4 Investigation parameters

Investigational parameters included a clinical score, the Jensen score, pulmonary function tests, sputum samples for microbiological examination, haematological samples for clotting,

and liver funtion (alanine transaminase and gamma glutaryl transferase), sputum and blood samples for chemical analysis. Data were collected every two weeks, at the beginning and at the end of the therapy.

2.17.5 Clinical score

The Jensen clinical score included evaluation of general conditions as appetite and evaluation of lungs conditions. The parameters used in this score range from 0 to 6. A low score indicates a good clinical condition, while a high score indicates pulmonary exacerbation. The baseline score from the beginning of the treatment was subtracted from the final score obtained at the end of 8 weeks. A negative value for the change in clinical score represents an improvement in symptoms.

2.17.6 Lung function test

The pulmonary function was measured using a MicroLab 3300 spirometer (Micro Medical Ltd, Rochester, Kent, UK) according to joint ERS/ATS criteria. Forced expiratory volume in the first second (FEV_1) was recorded and expressed in percentage of change from the baseline (from the beginning of the treatment) to the end of the treatment.

2.17.7 Hematological tests

Liver function and blood parameters were samples were examined by routine laboratory methods and include clotting, platelet count and alanine transaminase and gamma glutaryl

transferase levels.

2.17.8 Sputum and plasma signal molecules extraction

Both sputum and plasma were frozen at -70° C and later analysed for 3-oxo-C12-HSL and C4-HSL levels. Sputum samples were resuspended in 5 ml of NaCl 0.9% (w/v) and vortexed until a homogeneous mixture was obtained. Plasma samples were resuspended in 5 ml of NaCl 0.9% (w/v) and vortexed. In either case equal amounts of acidified ethyl acetate was added to the mixture, vortexed for 2 min and left for the two phases to separate. The solvent phase, containing the QS molecules was collected and solvent allowed to evaporate to dryness under reduced pressure. This procedure was repeated twice to maximise the extraction. The resulting extracts were dissolved in 50 µl of methanol ready for analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2.17.9 Sputum and plasma signal molecules analysis

The samples derived from sputum and plasma extraction were first run trough an HPLC column, Phenomenex Gemini C18, 150 x 2 mm (5 µm particle size) used at 50° C. Mobile phase A was 0.1% formic acid and 200 µM EDTA in water. Mobile phase B was 0.1% formic acid in acetonitrile. To the HPLC it was coupled a hybrid triple-quadrupole linear ion trap mass spectometer (4000 QTRAP, Applied Biosystems, Foster City, CA, USA) equipped with Turbolon source was used in positive ion electrospray mode based on a previously published method [13]. Quantification was performed using a specific software (Analyst 1.4.1, Applied Biosystems) in Quantitate mode and raw peak areas were used. The analytical lower limit of

detection (LLOD) for 3-oxo-C12-HSL and C4-HSL was determined as a signal to noise ratio of 10:1 and values below this were considered to be not measurable. Synthetic signal molecules were run as standards.

2.17.10 Statistical analysis

Statistical analysis was conducted using SPSS version 15.0. The two treatments were compared using the t-test for normally distributed data (percentage change in FEV_1 and weight from the baseline) and the Mann-Whitney test for non-parametric variables (change in symptoms score from baseline). The relationship between 3-oxo-C12-HSL in sputum and plasma was compared using a Pearson correlation coefficient.

Chapter 3

Study of *rsmZ* regulation by signal molecules

3 Study of *rsmZ* regulation by signal molecules

3.1 INTRODUCTION

The ability of bacteria to colonise and thrive in a wide variety of environments is partly facilitated by their quick response to different stimuli. In order to do this, bacteria possess multiple two-component-systems (TCS). These regulatory systems allow them to respond with great sensitivity to specific environmental signals such as pH, oxygen, nutrient levels, osmotic pressure and the presence of antibiotics [212]. The importance of TCS for the survival of the bacteria is leading to the development of anti-TCS drugs. By targeting TCS, it would be possible to prevent them from activating growth-essential genes, or from activating virulence genes. Several TCS are also responsible for promoting antibiotic tolerance [213]. Thus, inhibitors of the TCS could be used in synergy with antibiotics to improve their efficacy and to prevent or slow down processes that lead to the development of resistance [214].

One way to identify TCS inhibitor molecules is to screen among natural products and synthetic compounds libraries using a reporter fusion regulated by this system. By using this approach, many inhibitor molecules were successfully identified [215]. TCS can be inhibited at different steps (reviewed by Gotoh *et al.* 2010) [216]. The autophosphorylation step can be targeted, by acting on the sensor kinase. This is the case of the molecule walkmycin B, that targets the WalK sensor kinase of the WalK/WalR TCS of *Bacillus subtilis* [215]. TCS can be also inhibited at the phosphotransfer stage, as in the case of walrycin B. This molecule targets the WalR phosphorylation by WalK of the WalK/WalR TCS of *B. subtilis* [217]. Finally,

molecules that are able to inhibit TCS at the sensing step have also been identified. These molecules interfere with the binding between the signal molecule and the sensor kinase. An example is the LED209 molecule that inhibits the binding of AI-3, of epinephrine and of norepinephrine to QseC of the QseC/QseB TCS of enterohemorrhagic *E. coli* [218].

P. aeruginosa contains a variety of TCS that are involved in virulence or antibiotic resistance [219]. However, until now no synthetic or natural compounds have been shown to be capable of inhibiting a TCS. Only recently, in the species *Pseudomonas putida* synthetic antagonists of TolS have been identified. TolS is a sensor kinase part of the TolS/R TCS involved in the metabolism of toluene [220].

This chapter will focus on the identification of the signal(s) modulating the Gac/Rsm signal transduction pathway. Three sensor kinases (GacS, LadS, and RetS) are 'at the top' of this pathway, as they sense putative signals and modulate the phosphorylation of the GacA response regulator. When GacA is phosphorylated, it promotes the transcription of the two regulatory sRNAs (RsmZ and RsmY), which in turn modulate the activity of RsmA. This global regulator post-transcriptionally controls the expression of a vast range of virulence factors such as biofilm formation, pyocyanin, lipase, elastase, motility and quorum sensing (QS) [151]. Due to its central role in *P. aeruginosa* virulence, the search for the signal(s) activating/repressing this TCS transduction pathway is crucial for the development of new drugs.

The first studies on the identification of the signal molecules acting on Gac/Rsm were conducted on *P. fluorescens* CHA0 [221]. These studies demonstrate that the signal molecules are produced at high cell population densities, suggesting that the Gac/Rsm pathway was the manifestation of social behaviour such as QS. However, the signal(s) appear to be unrelated to known QS signals such as AHL, since *P. fluorescens* CHA0 does not possess an AHL driven

QS system [221]. Furthermore, the signals are produced in a GacA-dependent way, thus linking the signal transducers to the signal generators in a positive feedback loop [221].

Although the chemical structure of the signal(s) molecules remains unknown, the molecule(s) can be extracted from culture supernatants with dichloromethane, yielding crude signal preparations [221-223]. Moreover, studies have shown that the signals that activate *P. fluorescens* Gac/Rsm cascade are also able to activate the expression of Gac/Rsm regulated genes in *P. aeruginosa* and vice-versa, suggesting the existence of a possible cross-talk between different species of *Pseudomonads* [221].

One of the approaches used to identify the signal molecules was to target by random mutagenesis the genes involved in the biosynthesis of the signal. Using this approach, the thiamine biosynthetic *thiC* gene was identified as being important for signal production in *P. fluorescens* CHA0. However, the mechanism by which thiamine causes a reduction in the signal concentration is not known [222]. Another way to identify the signals was to fractionate crude signal preparations on silica gel column, and to look for the fraction where the signal was present. This strategy was not effective because the signal appeared to be lost during fractionation (S. Heeb, personal communication). Interestingly, studies conducted on the sensor kinase GacS of *P. fluorescens* CHA0 revealed that the periplasmic loop of this sensor is not essential for the detection of the signal and thus can activate GacS through its cytoplasmic membrane domain [224], suggesting a lypophilic nature of the signal(s).

3.2 AIM OF THE STUDY

With the aim of identifying the GacA/S signal(s) in *P. aeruginosa* two approaches were used. On one hand, it was interesting to study the regulation of the synthesis of these extra-cellular

signal(s) molecules, as this would provide a starting point for their purification and characterisation. On the other hand, by using specifically constructed reporters, the *P. aeruginosa* extra-cellular extracts were analysed for the direct identification of the signal molecules responsible for the activation of the Gac/Rsm system. The involvement of the cis-2-decenoic acid (CDA) molecule in the modulation of the Gac/Rsm system was also investigated. This molecule has been previously found to be important for biofilm dispersal in *P. aeruginosa* [225].

3.3 RESULTS

3.3.1 Construction and analysis of the *rsmZ-lux* and *rsmY-lux* reporter fusions

From recent studies, it appears that GacA exerts its function on the Gac/Rsm transduction cascade exclusively through the activation of *rsmZ* and *rsmY* transcription [226]. Thus, the study of *rsmZ* and *rsmY* transcriptional modulation is crucial for the understanding of the signal(s) involved in this regulatory cascade. For this reason, *rsmZ-lux* and *rsmY-lux* fusions were constructed and inserted into the unique *attP* site in *P. aeruginosa* chromosome using a mini-CTX delivery system (Becher and Schweizer 2000). The plasmids produced were called pSH50 (*rsmZ-lux*) and pKR77 (*rsmY-lux*) respectively. These vectors were then used to insert the transcriptional fusions into *P. aeruginosa* PAO1-N strain to generate strains PASH50 and PAKR04, respectively. The promoter regions cloned contained 326 bp for *rsmY-lux* and 249 bp for *rsmZ-lux* upstream from the +1 transcriptional starting site of each sRNA. Both of these fusions contained the Gac-box responsible for the GacA-dependent transcriptional activation (Fig. 1) [226]. The expression of the sRNAs was monitored by growing PASH50

and PAKR04 at 37 °C in static conditions in LB medium. In accordance with what has already been found in *P. fluorescens* and in *P. aeruginosa* [202, 227, 228], the basal activity of the P*rsmZ* is significantly lower than the one of P*rsmY* (Fig. 2). The maximal P*rsmY* activity was calculated as approximately nine times higher than what was observed for P*rsmZ* (Fig. 2). Moreover, both *rsmZ* and *rsmY* promoters are activated in a growth-phase dependent manner (Fig. 2).

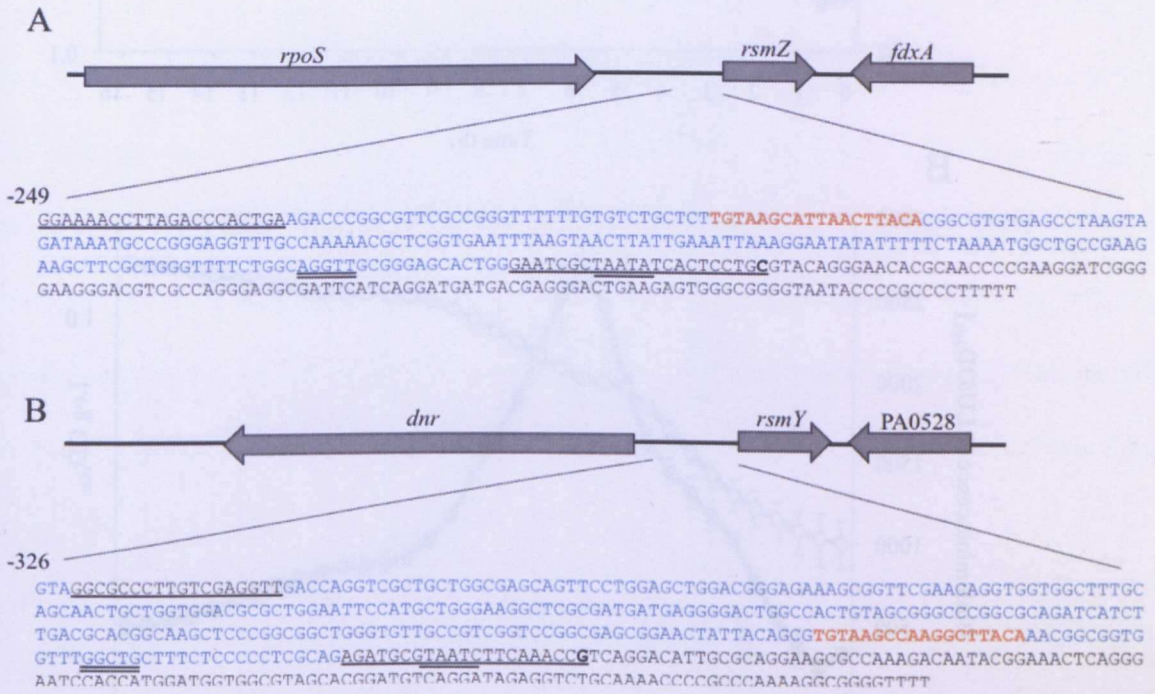


Figure 3-1. Representation of *rsmZ* and *rsmY* promoter region.

The 250 bp region of *rsmZ* promoter used to construct the PASH50 (*rsmZ-lux*) (A) and PAKR04 (*rsmY-lux*) (B) reporter fusions (in blue). The regions comprise a Gac-box (in orange). The transcriptional starting site of *rsmZ* and *rsmY* (in bold) and the -10 and -35 regions (double underlined). The annealing sites are also indicated (back single underlined).

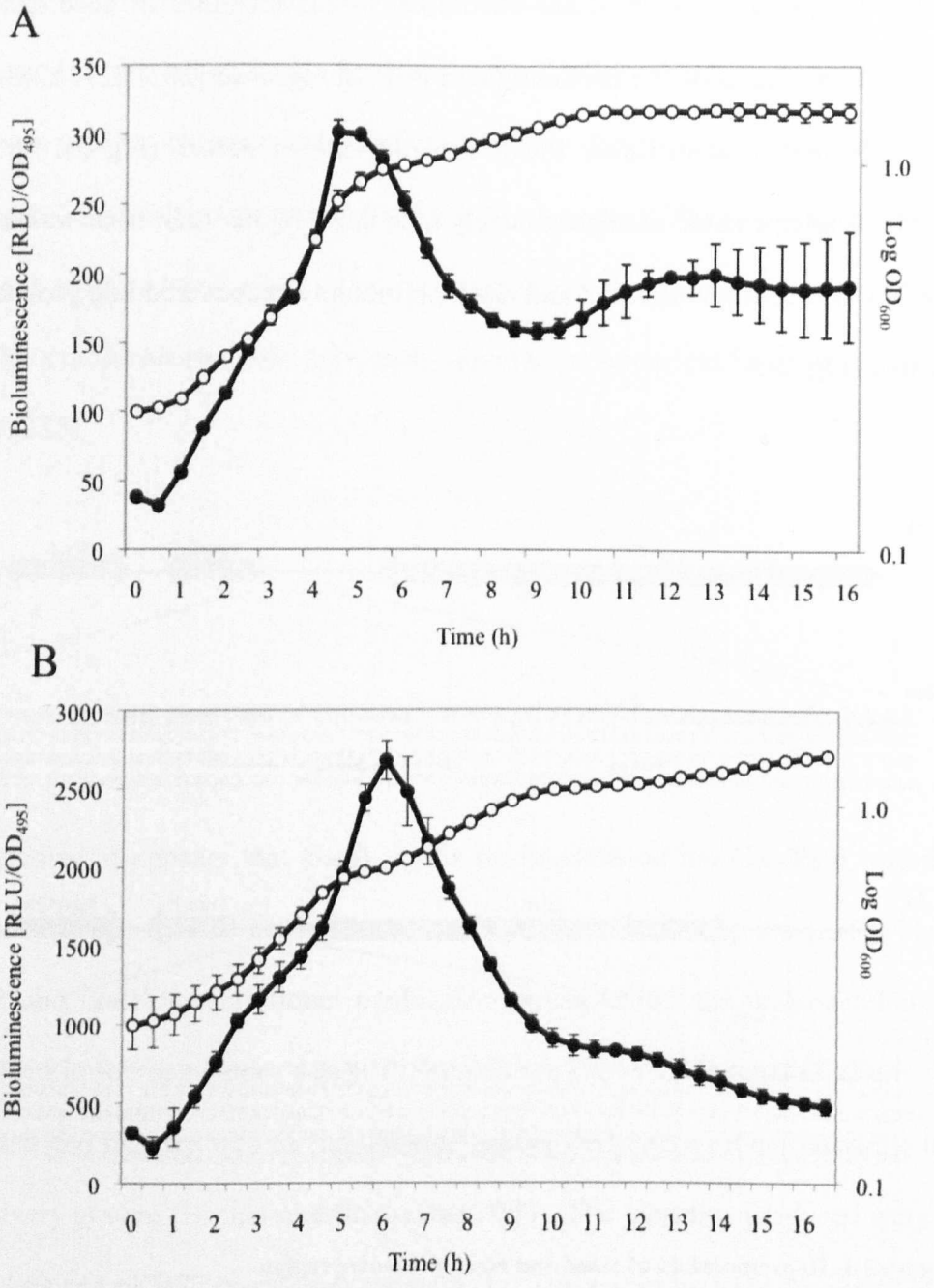


Figure 3-2. Regulation of *rsmZ* and *rsmY* expression in relation to growth

The transcription from *rsmZ* (A) and *rsmY* (B) promoters was monitored by measuring the light production RLU (filled circles) of the transcriptional fusions carried by *P. aeruginosa* strains PASH50 (*rsmZ-lux*) and PAKR04 (*rsmY-lux*) over growth (empty circles).

3.3.2 Effect of culture supernatant from *P. aeruginosa* WT on the activity of the *rsmZ* and *rsmY* fusions

Previously, the regulation of *rsmZ* and *rsmY* transcription was analysed using *–lacZ* based fusions. It was interesteing to analyse of the new *–lux* based reporter systems constructed. As expected, the addition of extracellular extract from *P. aeruginosa* PAO1 activated the *rsmZ* promoter fusion in PASH50 [221]. On the other hand, *rsmY* transcription could not be enhanced by the addition of extra-cellular extracts in PAKR04 (Fig. 3). The *rsmY* promoter has been reported to be one of the strongest one of *P. aeruginosa* (Kay *et al.*, 2005), and bioluminescence is a highly energy-demanding process. Thus, it is not surprising that when the *lux* operon is fused to the *rsmY* promoter, great quantities of energy must be consumed to produce bioluminescence, affecting growth (Fig 2B). For this reason, the growth limiting conditions may be affecting the response of the *rsmY-lux* reporter to the addition of extra cellular extracts. The light production reaches plateau levels and cannot be further enhanced (Fig. 3A).

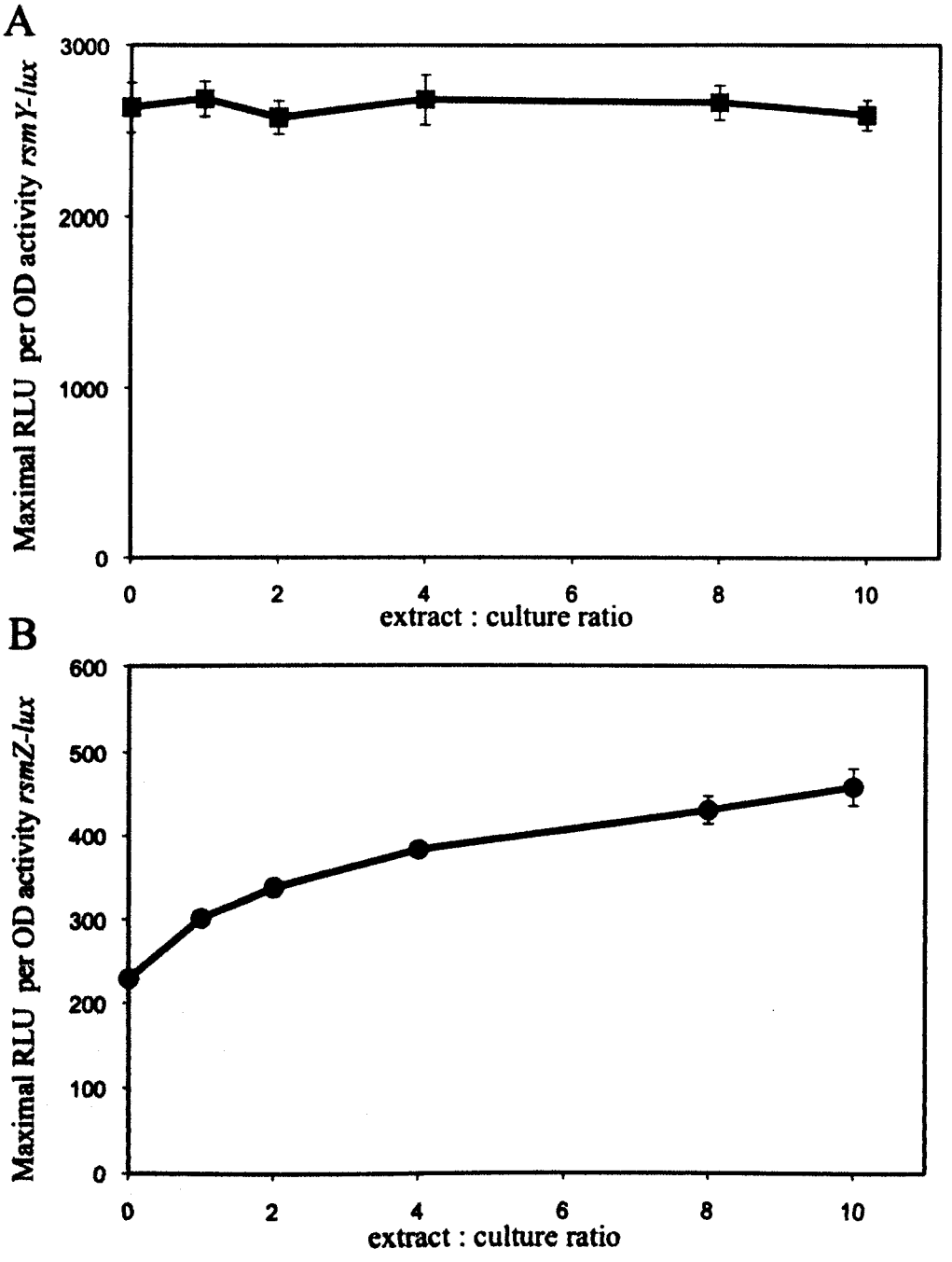


Figure 3-3. Regulation of *rsmZ* and *rsmY* expression in *P. aeruginosa* by extra-cellular extracts.

Maximum expression of PAKR08 (*rsmY-lux*) (A) and PASH50 (*rsmZ-lux*) (B) reporters in strains growing in the presence of increasing amounts of extracellular extract from *P. aeruginosa* PAO1-N.

3.3.3 Effect of the quorum sensing systems on the production of the signals inducing *rsmZ* transcription

The inability to enhance the *rsmY* promoter activity by adding extra-cellular *P. aeruginosa* extracts prompted us to use exclusively the PASH50 reporter strain (*rsmZ-lux*) in the further studies aimed to the identification of the signal(s) modulating GacA/S. The role of the Gac/Rsm system in the control of the QS system has been reported previously. The production of AHLs is positively regulated by GacA and negatively regulated by RsmA, which post-transcriptionally represses *rhlI* and *lasI* expression [147, 152]. Previous studies have also shown that the regulation of the Gac/Rsm system in *P. aeruginosa* and *P. fluorescens* is not modulated by 3-oxo-C12-HSL, C4-HSL or 2-alkyl-4(1*H*) quinolones QS molecules [151, 221].

Due to the important role of the QS networks in the modulation of different phenotypes in *P. aeruginosa*, it was interesting to investigate whether the three QS systems (*las*, *rhl*, and *pqs*) were involved in the production of the Gac/Rsm system signal molecules. For this purpose, it was necessary to construct mutants defective in each of the QS systems. In each mutant, the synthase gene responsible for the production of the signal molecule was deleted. The *lasI* mutant (PAKR94) was constructed, while the mutant *rhlI* (PAO1ΔrhlI) was already available in our laboratory [229]. These strains have an insertion of a gentamycin (Gm) and a tetracycline (Tc) gene cassette in the coding sequence of *lasI* and *rhlI*, respectively. The in-frame mutant *pqsA* (PAOpqsA) was already available in our laboratory [193], while the triple mutant PACP3D, defective in the production of the three QS signal molecules, was constructed.

The extracts from PAKR94 (Δ*lasI*::Gm^R), PAO1ΔrhlI (Δ*rhlI*::Tc^R), PAOpqsA (Δ*pqsA*) and PACP3D (Δ*lasI*::Gm^R-Δ*rhlI*::Tc^R-Δ*pqsA*) supernatants were analysed for their ability to activate the expression of the *rmsZ-lux* reporter fusion. The strains were grown at 37 °C in aerobic conditions in CAA media until they reached the stationary phase of growth. The OD_{600} was normalised to 1.5 before extracting the molecules from the supernatant (Materials and Methods, 2.16.1). All the extracts were able to induce *rsmZ* transcription. However, the extracts derived from the triple mutant, activated *rsmZ* expression later in growth (Fig. 4).

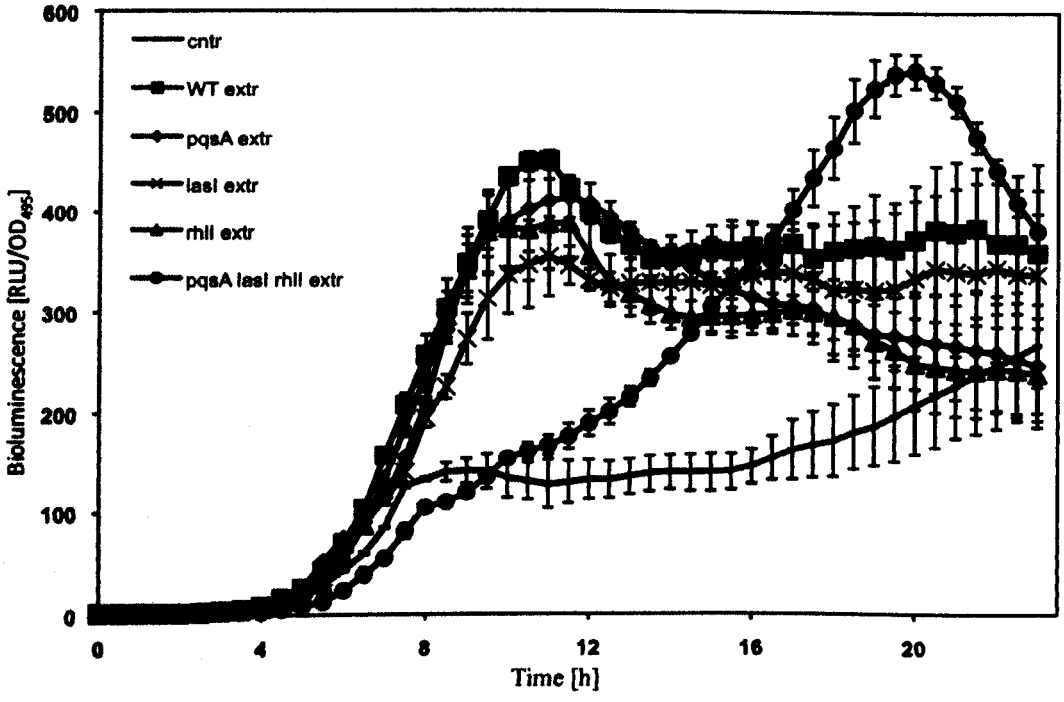


Figure 3-4. Impact of QS systems on the production of signal molecules acting on *rsmZ* transcription.
Extracts from WT, PAKR94 (Δ*lasI*::Gm^R), Δ*rhlI* (Δ*rhlI*::Tc^R), PAOpqsA (Δ*pqsA*) and PACP3D (Δ*lasI*::Gm^R-Δ*rhlI*::Tc^R-Δ*pqsA*), were tested on the *rsmZ-lux* reporter PASH50 (*rsmZ-lux*). The culture:extract ratio used was 1:4. As a control (cntr) only methanol was added to the culture 5% (v/v) ratio.

3.3.4 Impact of GacA/S, LadS and RetS on the production of *rsmZ* inducing molecules

Several studies conducted on *P. aeruginosa* using a transcriptional *rsmZ-lacZ* fusion suggest that, similar to *P. fluorescens*, *P. aeruginosa* produces a GacA-dependent signal [221]. The impact of the three sensor kinases (GacS, LadS and RetS) was investigated and of GacA on the production of the signals inducing *rsmZ* transcription, using our *–lux* based transcriptional fusion. Each strain was grown overnight and the OD_{600} was normalised to 1.5. The extracts were prepared as described in the previous section and added in a ratio of 1:4 (culture: corresponding volume extracted). Surprisingly, the results previously obtained by Dubuis and Haas (2007) were not reproducible when using the *rsmZ-lux* fusion [221]. Extracts from PAKR29 (Δ*gacS*), PAKR43 (Δ*gacA*), PAKR45 (Δ*ladS*) or PAKR52 (Δ*retS*) strains, all retained a wild-type inducing activity (Fig. 5).

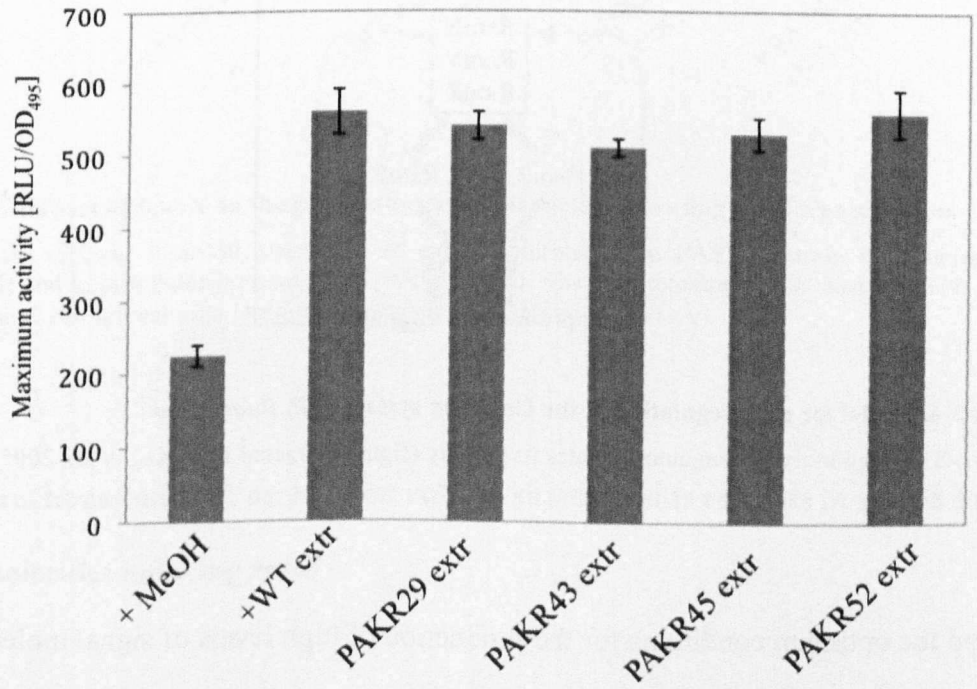


Figure 3-5. Impact of GacA/S, LadS and RetS mutation on the production of *rsmZ* activating signal molecule.

Extracts from WT, PAKR29 (Δ*gacA*), PAKR43 (Δ*gacS*), PAKR45 (Δ*ladS*) and PAKR52 (Δ*retS*) tested on the reporter strain PASH50 (*rsmZ-lux*). The culture:extract ratio used was 1:4. As a control methanol, the solvent used to dilute the extracts, was added 5% (v/v).

3.3.5 Effect of RsmA on the production of *rsmZ*-inducing molecule

To continue the analysis of *rsmZ-lux* modulation in *P. aeruginosa* PAO1-N strain, the effect of the global post-transcriptional regulator RsmA on the production of signal molecules activating *rsmZ* was investigated. Previous studies suggested that the two CsrA homologues RsmA and RsmE, found in *P. fluorescens*, are indirectly involved in modulating the Gac/Rsm system by repressing the signal production (Fig. 6) [223].

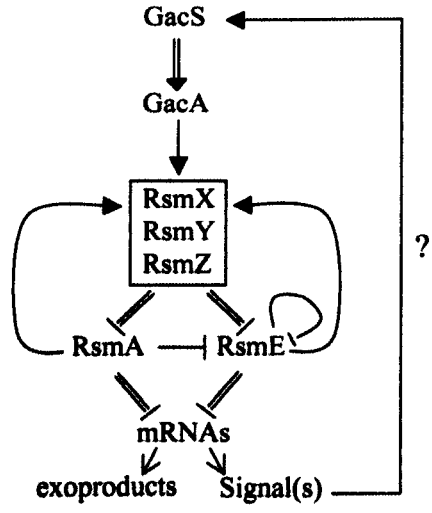


Figure 3-6. Model for gene regulation in the Gac/Rsm system of *P. fluorescens*
The Gac/Rsm regulatory system autoregulates its activity (figure extracted from Kay *et al.*, 2005).

To find the optimum conditions for the production of high levels of signal molecules, extracts derived from an *rsmA*-inducible strain PASK10 were used. This strain is an RsmA negative mutant in the absence of IPTG and RsmA overproducer in the presence of IPTG (1 mM). Interestingly, the extracts resulting from the RsmA over-expressed strain (grown with IPTG addition) or from the *rsmA* mutant (no IPTG added) were equally effective at inducing *rsmZ-*

lux fusion, with respect to WT extracts, indicating that in our *P. aeruginosa* PAO1-N strain, the signal production is not regulated by RsmA (Fig. 7).

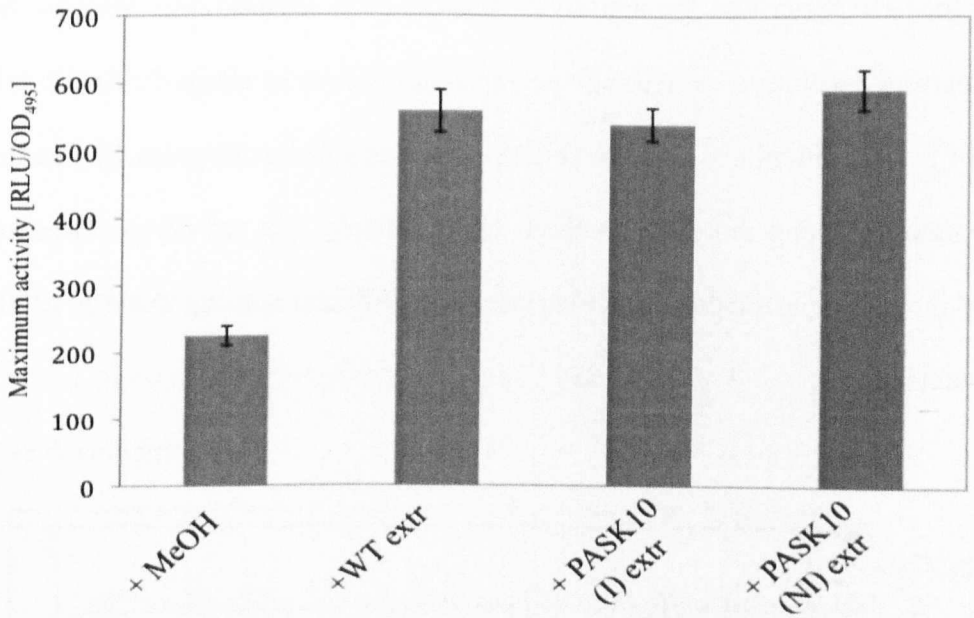


Figure 3-7. Impact of RsmA on the production signal molecule activating *rsmZ* transcription.

Effect on the reporter PASH50 (*rsmZ-lux*) of extracts derived from PASK10 strain in inducing conditions (PASK10 I) and in non inducing conditions (PASK10 NI). The culture:extract ratio used was 1:4. As a control only methanol, the solvent used to dilute the extracts, was added at 5% (v/v).

3.3.6 Fractionation of *P. aeruginosa* culture supernatants extracts in search for the molecules inducing *rsmZ*

As no strain enhanced in the signal molecules production were found, further analysis were conducted using the extracts derived from *P. aeruginosa* PAO1-N. Fractions derived from this strain were tested for their activating potential on the PASH50 (*rsmZ-lux*) reporter strain. *P. aeruginosa* was grown in the aforementioned conditions and the extracts were concentrated

200 fold in methanol. The extracts (final volume of 50 µl) were then injected into the HPLC column and were analysed in order to determine the spectra profile. The eluate was fractionated and collected at different time points (Materials and Methods, 2.14). Each of the seven fractions collected was dried and diluted in 10 µl of methanol. Each fraction was then added to the reporter and the activity of the *rsmZ-lux* reporter was assayed. More than one fraction was capable of activating the *rsmZ-lux* fusion in strain PASH50, while only one showed a weak inhibitory activity (Fig. 8). Further sub-fractionation of fractions 1, 4 and 7 was conducted using the same method. Unfortunately, the activity of the extracts resulting from this second purification was lost, due to insufficient starting material (200 ml of culture supernatant).

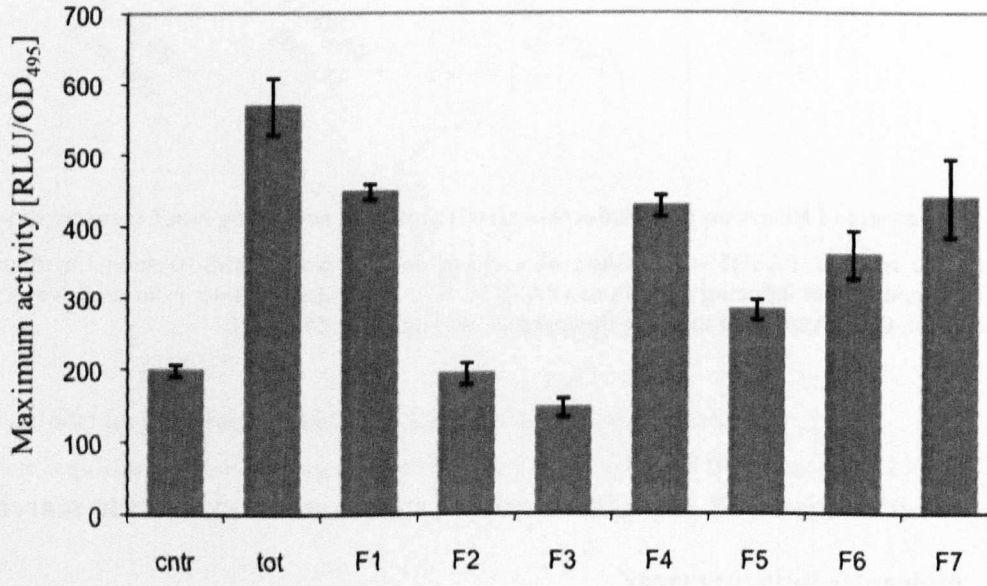


Figure 3-8. Activation of the rsmZ-lux reporter by different fractions of the *P. aeruginosa* extracellular extracts.

Maximum activity of the PASH50 (*rsmZ-lux*) reporter when incubated with extracts derived from HPLC fractionation. The fractions obtained were added to the biosensor in a 1:4 ratio. Also shown is the activity obtained when adding all the fractions together (tot). As a control (cntr), only methanol was added to the reporter strain at 5% (v/v).

3.3.7 Identification of PQS in one of the fractions activating *rsmZ* expression

In order to investigate whether any of the active compounds corresponded to known molecules for which synthetic standards were available, the HPLC fractions were analysed using mass spectrometry (MS). This technique allowed to compare fractions according to retention times and fragmentation patterns. Interestingly, PQS was identified in one of the fractions (F6) activating the *rsmZ-lux* reporter PASH50 (Fig. 9). This led to the hypothesis that PQS was acting on the promoter of *rsmZ*. Although previous experiments conducted on *P. fluorescens* CHA0 showed that PQS does not activate P*rsmZ*$_{CHAO}$ [221], the results obtained here suggest that PQS can activate *rsmZ* expression in *P. aeruginosa* (see Chapter 4 for a further development).

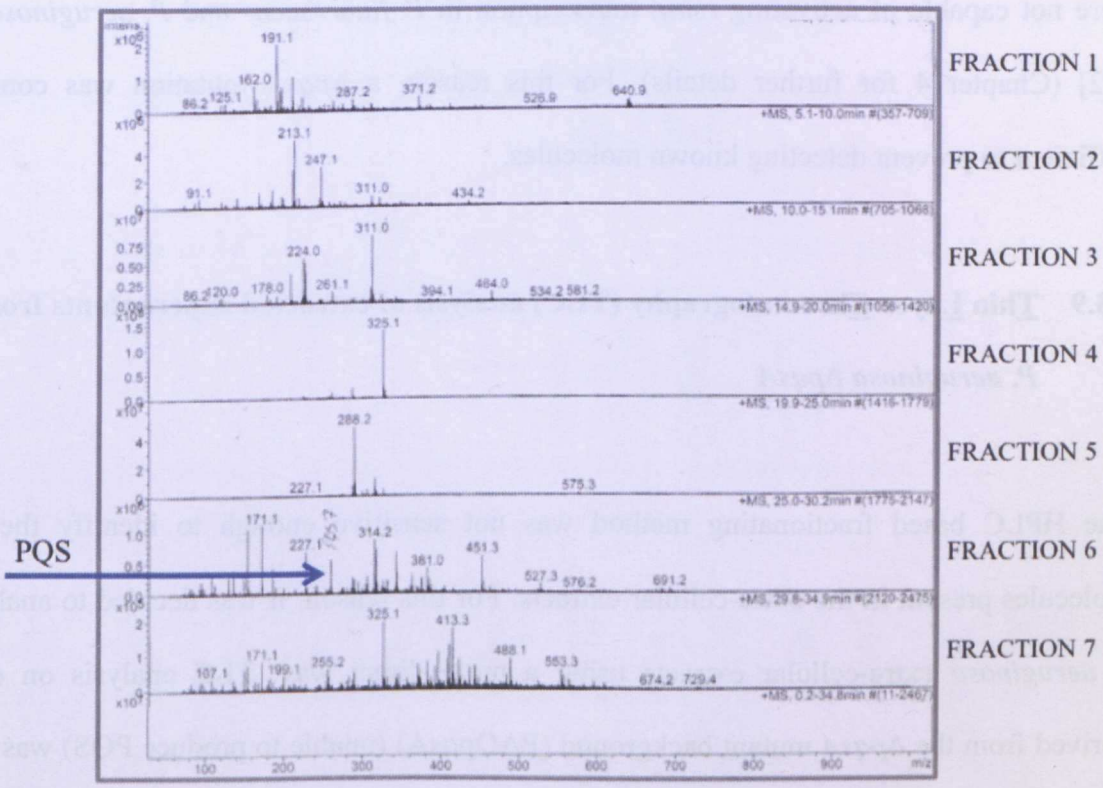


Figure 3-9. LC-MS analysis of different supernatant fractions from *P. aeruginosa* cultures.
The m/z peak of 260 corresponding to PQS, was identified in fraction 6.

3.3.8 Analysis of *P. aeruginosa* Δ*pqsA* supernatants for the presence of *rsmZ* inducing molecules

The aim of this study was to search for unknown molecules, which can activate the Gac/Rsm cascade. The discovery that PQS was partially responsible for the activation of *rsmZ* transcription, prompted us to use *P. aeruginosa* extracts derived from a Δ*pqsA* mutant (unable to produce PQS). Moreover, the reporter *rsmZ-lux* fusion integrated into the Δ*pqsA* background (PAKR08) was used. In order to identify additional signalling molecules, it was important to rule out the involvement of PQS on the observed effect on *rsmZ* induction. In accordance to previous observations, it was shown that 3OC12-HSL and C4-HSL molecules were not capable of activating *rsmZ* transcription in *P. fluorescens* and *P. aeruginosa* [151, 202] (Chapter 4 for further details). For this reason, a Δ*pqsA* mutation was considered sufficient to prevent detecting known molecules.

3.3.9 Thin Layer Chromatography (TLC) analysis of extracted supernatants from *P. aeruginosa* Δ*pqsA*

The HPLC based fractionating method was not sensitive enough to identify the signal molecules present in the extra-cellular extracts. For this reason, it was decided to analyse the *P. aeruginosa* extra-cellular extracts using a more direct way. TLC analysis on extracts derived from the Δ*pqsA* mutant background (PAOpqsA) (unable to produce PQS) was carried out and coupled with an overlay of the reporter PAKR08 (Δ*pqsA rsmZ-lux*). Using this method, the molecules, which are present in the extra cellular extracts, can be separated according to their polarity (Materials and Methods section 2.13). Once the molecules have

migrated, the TLC plate is overlaid with the reporter strain and incubated at 37 °C. From this experiments the detection of specific active spots on the plate was expected, corresponding to the molecules inducing *rsmZ* transcription. Because the chemical identity of the signal molecules is unknown, different TCL plates and liquid phases were used.

The extraction procedure was identical to the one described previously, and the extracts derived from the PAOpqsA (Δ*pqsA*) strain were concentrated 200 times in methanol to a final volume of 10 µl. The whole volume was then loaded into the TLC plate (Materials and Methods, 2.14). After the migration of the liquid phase, the plate was dried and an overnight culture of the reporter strain PAKR08 (Δ*pqsA rsmZ-lux*) was used to overlay the plate. The light production was checked each 30 min for an 8 hrs period using the light camera. Unfortunately, the background light emission of the reporter was too high to visualise a light spot corresponding to the signal molecule. Hence, it was carried out the construction of an alternative biosensor.

3.3.10 Construction of a double transcriptional-translational reporter in *P. fluorescens* CHA0 for the detection of *rsmZ* inducing molecules

In order to minimise the background effect observed when conducting the TLC analysis of the extracellular *P. aeruginosa* extracts using the strain PAKR08 (Δ*pqsA rsmZ-lux*), an alternative biosensor was constructed. The aim was to generate a biosensor whose activity would be repressed in the absence of the signal molecules, while strongly activated in the presence of the signal molecules. This biosensor was constructed in the *P. fluorescens* CHA0 background, which is unable to produce AHL or 2-alkyl-4(1*H*)-quinolones signals [202]. Extracts from *P. aeruginosa* PAO1 were previously found to be capable of inducing

P. fluorescens CHA0 *rsmZ* transcription [221]. Moreover, this strain is capable of growing at room temperature, thus facilitating the detection process using fluorescence imaging equipment. The reporter was constructed using the promoter $rsmZ_{CHA0}$ fused to the ribosome-binding site (RBS) of $aprA_{CHA0}$, a gene that is strongly regulated at the post-transcriptional level by RsmA [230]. The promoter and RBS were linked to the open reading frame (ORF) encoding the red fluorescent protein gene *DsRedE2* (Material and Methods, 2.4.5).

The final double transcriptional-translational report was carried on the plasmid pMW02 and transformed into *P. fluorescens* CHA0 strain. This bioreporter was regulated both transcriptionally and post-transcriptionally by the *P. fluorescens* Gac/Rsm system. In the absence of the signal molecules, RsmA binds to the RBS of $aprA_{CHA0}$ inhibiting the translation of DsRedE2. In this way, the background noise is minimised. On the other hand, the addition of extra-cellular extracts activates both the intrinsic $rsmZ_{CHA0}$ promoter, activating the transcription of *rsmZ* and the promoter controlling the transcription of *DsRedE2*. The enhanced production of the small regulatory RNAs, *rsmZ, rsmY* and *rsmX* [223] would titrate out RsmA, which is then incapable of binding to the RBS of $aprA_{CHA0}$. The translation of DsRedE2 protein is thus activated (Fig. 10).

The result is a reporter system activated both transcriptionally and post-transcriptionally by the signal molecules. A reduced background and an amplified sensitivity to the molecules are expected.

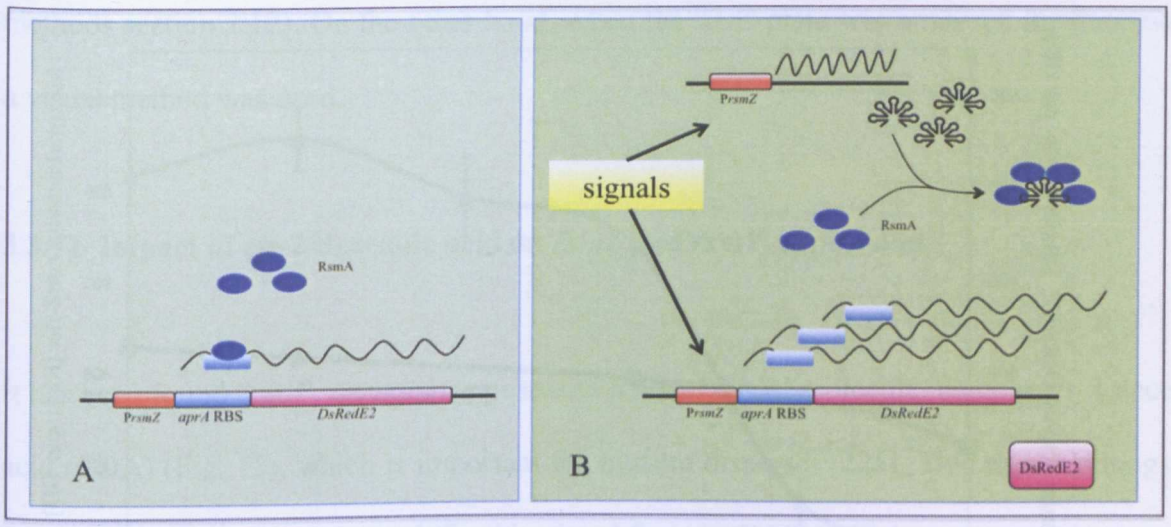


Figure 3-10. Model for the double trasnscriptional-translational Gac/Rsm-dependent *DsRed2* reporter fusion.

At low concentrations of signal molecules, RsmA exerts its post-transcriptional negative regulation on $aprA_{CHA0}$ RBS thus inhibiting the DsRedE2 translation (A). When adding signal molecules activating GacA, the transcription of the reporter fusion is induced ($rsmZ_{CHA0}$ promoter) and the effect of RsmA on the post-transcriptional repression is simultaneously alleviated of by the small RNAs (B).

The response in liquid media of the *P. fluorescens*/pMW02 strain to the addition of signal molecules was investigated. The extracts derived from PAOpqsA (Δ*pqsA*) strain were added at a range of different culture to extract ratio. The results indicated a stronger activation of the new biosensor and a significant decrease of the background activation level, compared to the activation of the biosensor *P. aeruginosa* PAKR08 (Δ*pqsA rsmZ-lux*) (Fig. 11).

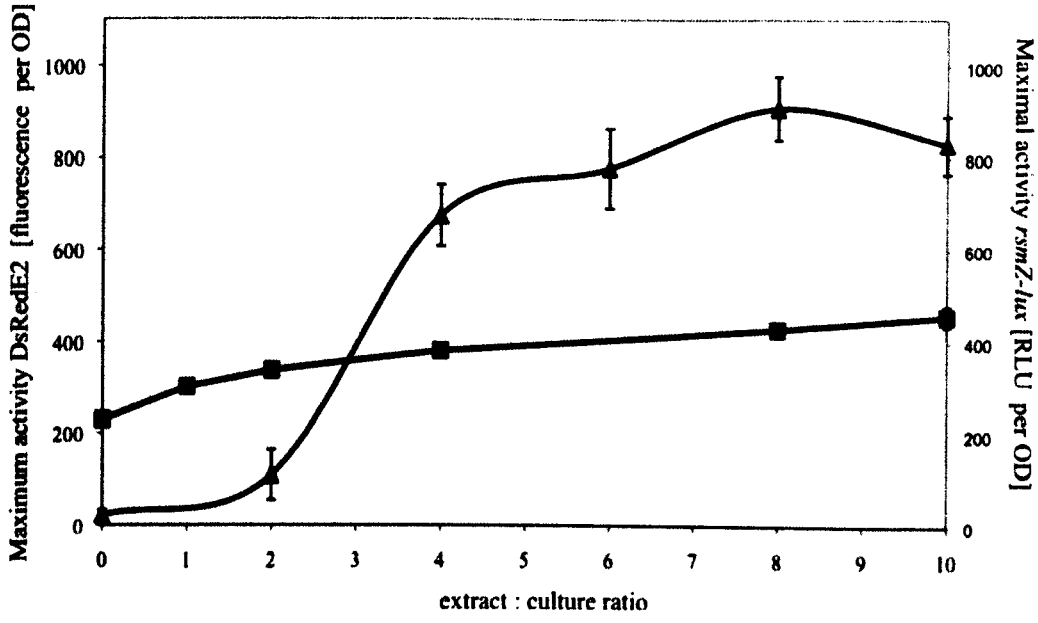


Figure 3-11. *P. fluorescens* CHA0 DsRedE2 reporter compared to *P. aeruginosa* PAKR08.

Culture supernatant extracts derived from *P. aeruginosa* PAOpqsA (Δ*pqsA*) were added to the reporters PAKR08 (Δ*pqsA rsmZ-lux*) squares, and to the double transcriptional-translational DsRedE2, triangles. The maximal fluorescence was measured over an incubation time of nine hrs (OD_{600} = 0.8).

The promising results obtained with the new reporter strain in liquid media, compared to the *lux* based reporter strain PAKR08 (Δ*pqsA rsmZ-lux*), prompted us to further analyse *P. aeruginosa* PAO1 extra-cellular. A TLC analysis was carried out using DsRedE2 reporter as the overlaying strain. Extracts from PAOpqsA (Δ*pqsA*) strain were loaded into a TLC plate as previously described. The DsRedE2 biosensor (*P. fluorescens* CHA0/pMW02) was then overlaid onto the dried plate and the appearance of red fluorescence was analysed every half an hour. Although the background noise was drastically reduced, it was not possible after 48 hrs of incubation to visualise any defined red spot on the TLC corresponding to activating signal molecules. The results obtained in liquid and in TLC conditions indicate a different sensitivity of the detection method used. When fluorescence was analysed in liquid, the detection was performed in micro titre plates using the sensitive TECAN reader (material and

methods section 2.12). On the other hand, when the TLC plate was analysed for fluorescence a visual method was used.

3.3.11 Impact of cis-2-decenoic acid on *rsmZ* and *rsmY* expression.

It has been found that *P. aeruginosa* produces a novel signal molecule, namely *cis*-2-decenoic acid (CDA) (Fig. 12), which is important for biofilm dispersal [225]. This signal belongs to a family of molecules known as diffusible signal factors (DSF), which are present in a number of human and plant related pathogens [231-233].

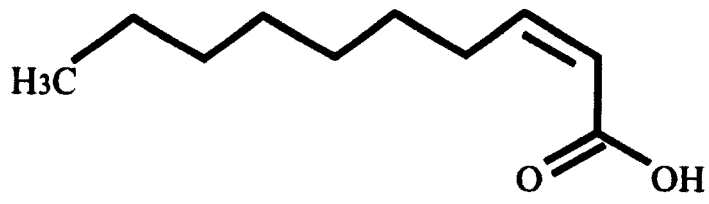


Figure 3-12. *Cis*-2-decenoic acid (CDA).

Since the swarming (thus biofilm dispersal) phenotype has already been linked to the activity of the RsmA in *P. aeruginosa* [151], the interaction between CDA and the Rsm post-transcriptional system was investigated. The hypothesis was that CDA could act on biofilm dispersal by repressing the expression of the sRNAs RsmZ and RsmY. The concentration of free RsmA in the cell would increase, promoting swarming. The reporters PASH50 (*rsmZ-lux*) and PAKR04 (*rsmY-lux*) were assessed for their activity after the addition of CDA. As expected, CDA was shown to inhibit the expression of both sRNAs (Fig. 13).

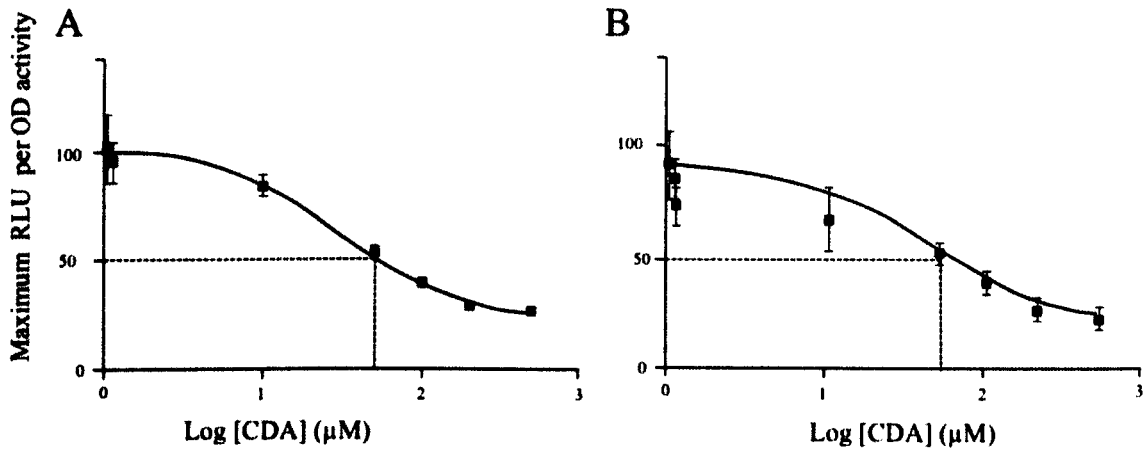


Figure 3-13. Effect of *cis*-2-decenoic acid on *rsmZ-lux* and *rsmY-lux* reporter fusions.

The maximum activity of PAKR04 (*rsmY-lux*) (A) and PASH50 (*rsmZ-lux*) (B) biosensors after the addition of increasing concentrations of CDA. The IC_{50} (half maximal inhibitory concentration) was calculated as 33 μM ($R^2 = 0.99$) and 45 μM ($R^2 = 0.94$), respectively.

It was previously reported that the Rsm/Gac system is negatively regulated by the action of the sensor RetS on GacS [154, 156]. It was investigated whether the action of CDA exerted on these two small regulatory RNAs was channelled through this sensor kinase, hoping to uncover the identity of a signal molecule acting on RetS. To test this hypothesis, we analysed the effect of CDA in the PAKR53 (Δ*retS rsmZ-lux*) background. The results indicated that the activity of *rsmZ* promoter was significantly higher in the PAKR53 background compared to the PASH50 (*rsmZ-lux*), in agreement with previous results [155]. However, CDA was still able to repress *rsmZ* transcription in both strains (Fig. 14). This rules out a possible involvement of this signal molecule in the RetS-mediated regulation of RsmZ.

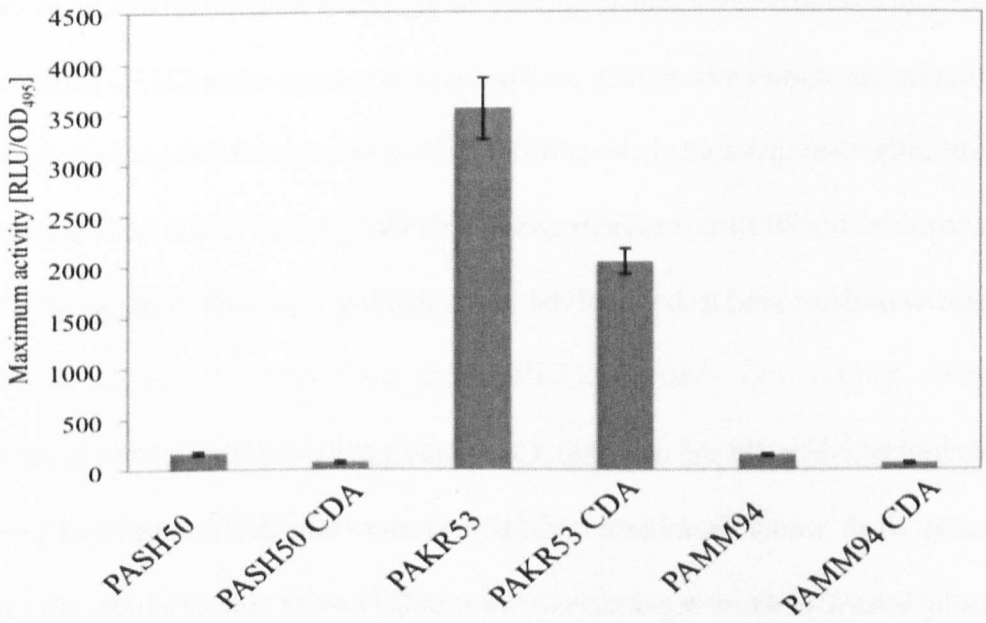


Figure 3-14. Modulation of *rsmZ* transcription by CDA in the WT, *retS* and *fleQ* mutant backgrounds.

Effect of the addition of 50 μM CDA on *rsmZ* transcription in the PASH50 (*rsmZ-lux*), PAKR53 (Δ*retS rsmZ-lux*) and PAMM94 (Δ*fleQ rsmZ-lux*) strains. As negative control methanol (used to dissolve CDA) was added to a corresponding concentration of 5% (v/v).

3.3.12 Cis-2-decenoic action on biofilm dispersal is not dependent on FleQ

Previous studies indicate that bis-(3'-5')-cyclic di-guanosine monophosphate (c-di-GMP) is a key intracellular signal controlling the equilibrium between biofilm formation and biofilm dispersion phenotypes. An increase in c-di-GMP levels enhances biofilm formation while decreasing swarming motility [234-237]. A possible way in which c-di-GMP might impact on biofilm formation could be by activating the Rsm/Gac system. This hypothesis was supported by the observation that high intracellular concentrations of c-di-GMP induce the *rsmZ-lux*-based fusions (M. Messina, PhD thesis). Since CDA is involved in biofilm dispersion, the hypothesis that the CDA molecule could act by promoting the turnover of c-di-GMP seemed

plausible. Intracellular levels of c-di-GMP have been found to be linked to the DSF signal transduction in studies conducted on *Xanthomonas campestris* [232]. Moreover, FleQ has been recently demonstrated to be a c-di-GMP-responsive transcriptional factor, repressing genes required for biofilm formation when c-di-GMP levels in the cells are low [238]. This regulator was also found to be involved in modulating c-di-GMP turnover (M. Messina, PhD thesis).

The formulated hypothesis was that CDA was acting on biofilm dispersal through FleQ activation. FleQ would promote c-di-GMP turnover, thus decreasing *rsmZ* transcription and increasing RsmA positive regulation on swarming. Lower concentration of c-di-GMP would in turn promote FleQ repression activity on biofilm formation genes. In order to test this hypothesis, the ability of CDA to inhibit *rsmZ* transcription was compared in the wild type PASH50 (*rsmZ-lux*) and PAMM94 (Δ*fleQ rsmZ-lux*). These experiments showed that CDA was still able of reducing *rsmZ* activity in both strains (Fig. 14). Hence, FleQ does not mediate the effect of CDA on *rsmZ* expression. However, this data does not exclude the possible direct effect of CDA on intracellular c-di-GMP concentrations. Further experiments are needed to prove this hypothesis.

3.4 Discussion

The Gac/Rsm global post-transcriptional regulation system regulates the expression of many virulence associated genes in *P. aeruginosa* [154, 155, 157]. The signals acting on the sensors GacS, LadS and RetS remain unknown. In this study, it was examined the modulation of the signal synthesis, in an attempt to identify these molecules and to understand the mode of action of the biofilm dispersal molecule CDA *via* the Gac/Rsm system.

The behaviour of the *rsmZ* and *rsmY* transcriptional fusions was analysed for the first time using the *lux* operon as the reporter gene instead of the commonly used *lacZ*. A higher promoter activity of *rsmY* compared to *rsmZ* was observed (Fig. 2), confirming previous data [228]. Recently, the lower activity of the *rsmZ* promoter was shown to be caused by the presence of a region containing the binding site of MvaT or MvaU, which act as repressors [226]. While adding *P. aeruginosa* extra-cellular extracts can further enhance *rsmZ* transcription, the same extracts failed to activate *rsmY* (Fig. 3). This is in discordance to what was previously observed [221], and can be explained by the difference in the reporter genes used to create the fusion (*-lacZ* based transcriptional fusion used in the past, compared to the *–lux* based fusion used in this study).

Since the identification of signal molecules activating the Rsm/Gac system was the aim of this study, the investigation continued using the *rsmZ-lux* reporter exclusively.

The regulatory pattern of the *rsmZ* expression, in particular its growth-phase dependance, could be linked to a cell density-dependent type of regulation such as QS. For this reason, it was investigated whether the production of signal molecules activating the Rsm/Gac system was somehow modulated by one of the three QS systems of *P. aeruginosa*. Therefore, we focused our attention on the activation of the *rsmZ-lux* transcriptional fusion by extra-cellular extracts derived from strains mutated in each of the three QS systems. Extracts derived from strains defective in the *las*, the *rhl* and the *pqs* systems were analysed. The QS defective strain (*ΔlasI::Gm*^{R}, *ΔrhlI::Tc*^{R}, *ΔpqsA*) was also examined.

The extracts derived from the strains mutated in each of the QS systems activated the *rsmZ-lux* reporter in a similar way to the extracts derived from the wild type strain. However, the triple QS mutant extracts (*ΔlasI::Gm*^{R}, *ΔrhlI::Tc*^{R}, *ΔpqsA*) activated *rsmZ* transcription later in the growth-phase (Fig. 4). This result suggests that the production of the signal(s)

activating the Rsm/Gac system is partially modulated by the QS systems. A link between the Rsm/Gac system and the production of signal QS molecules has been previously shown [147]. Therefore, it is not surprising to find that in *P. aerugniosa* the QS systems have an effect on the production of signal molecules modulating Rsm/Gac. It would be interesting to analyse the effect of *rsmZ* activation in the triple mutant compared to the WT reporter strain PASH50.

It was also interesting to investigate whether the effect of the triple QS mutant extracts on *rsmZ* transcription was due to a general decrease in the concentrations of AHL signal molecules content. However, neither the 3OC12-HSL nor the C4-HLS molecules have an effect on the fusion (Chapter 4). The finding that PQS activates the *rsmZ-lux* fusion suggests that the effect seen on the signal production is due to the decrease in PQS content in the extra-cellular extracts of the triple mutant. However, extracts from the *pqsA* mutant induce the *rsmZ-lux* fusion in the same way as the extracts from the WT. Hence, the QS network is partially involved on the indirect modulation of Rsm/Gac signal generation. The effect and the dynamics of PQS interaction with the Rsm/Gac system are further investigated in Chapter 4.

The importance of the Rsm/Gac system in the production of the signal was also investigated. Contrary to what was previously observed in *P. fluorescens* and *P. aeruginosa* [202, 221], the signal production is independent from the Rsm/Gac system. Extracts derived from strains defective in each of the sensor kinases (LadS, RetS and GacS) were able to activate *rsmZ* transcription similarly to extracts derived from the WT. In addition, the response regulator (GacA) and the final post-transcriptional regulator (RsmA) did not affect the production of signal molecules activating the *rsmZ* promoter. Our results are difficult to reconcile the previously accepted model in which GacS/A partially regulates the production of its own

signal in a positive feedback loop. In *P. fluorescens* the Rsm/Gac system has a strong global effect on genes important for biocontrol activity [223] by allowing the bacterium to survive in the natural environment. It is possible that this post-transcriptional regulation system, which allows the bacteria to quickly adapt to changes in the environment, positively auto-regulates its activity in a pattern that is common to QS regulation. On the other hand, in *P. aeruginosa*, the QS systems are the main regulatory pathways acting on virulence genes, while two-component response systems are rather involved in promoting adaptability to different environmental conditions.

Thus, it seems possible that while *P. fluorescens* has a way to positively autoregulate Gac/Rsm system using the signal molecules, in *P. aeruginosa* this TCS is regulated in a more complex way. On one hand, the Rsm/Gac system senses for changes in the environment and modulates the synthesis of QS molecules. On the other hand, the QS system is partially involved on the signal generation modulating Rsm/Gac. This could explain why in a defective QS strain the Rsm/Gac signal generation is affected, while when mutating the components of this TCS system (such as GacS, LadS, RetS, and GacA) the effect on the signals production is not so apparent as for *P. fluorescens*.

Due to the large variety of phenotypes controlled by the Rsm/Gac system, it was not surprising to find that many HPLC fractions activate (through GacS or LadS activation) or repress (through RetS activation) *rsmZ* transcription. The diversity and multiplicity of molecules acting on the Rsm/Gac system confers complexity to its modulation. Although fractions activating *rsmZ* were found, the activating effect on the reporter strain was lost after the second round of fractionation, probably due to the dilution beyond active levels of the signal molecules during the process. For future studies it would be interesting to analyse the

HPLC fractions by using the double transcriptional-translational reporter fusion DsRedE2, which is more strongly activated by the extra-cellular extracts (Fig.11).

In parallel to the HTLC fractionating method, a TLC approach was used in order to identify signals acting on the Rsm/Gac system. An extract from the Δ*pqsA* mutant was separated by TLC and analysed using two different reporter strains. The PAKR08 (Δ*pqsA rsmZ-lux*) reporter strain in *P. aeruginosa* and the more elaborated and sensitive *P. fluorescens* CHA0 double transcriptional-translational DsRedE2 reporter. Unfortunately, it was not possible to identify any spot on the TLC plate corresponding to active molecules, although the background noise effect observed using the –lux based reporter (PAKR08) was drastically reduced when using the new fluorescence-based sensor. Further studies have to be carried out to maximise the detection method. Previous studies conducted on the GacS sensor kinase of *P. fluorescens* have shown that the periplasmic loop is not essential for GacS activity [224]. Thus, the signals are able to activate GacS by directly entering into the cytoplasmic membrane. This observation suggests that the signal molecules are highly lipophilic. This explains why the standard TLC method failed to to separate and identify the signal molecules. Hence, specific TLC plates and soluble phase for highly lipophilic compounds should be used instead.

The results obtained in liquid cultures of *P. fluorescens* CHA0 double transcriptional-translational biosensor DsRedE2 showed that this biosensor responds to extra-cellular *P. aeruginosa* extracts in a more sensitive way when compared to the PAKR08 biosensor (Fig. 11). This bioreporter strain could be used for future fractionation studies.

Finally, it was discovered that the CDA signal molecule represses small regulatory RNAs, *rsmZ* and *rsmY*, thus increasing the abundance of free RsmA and possibly promoting biofilm

dispersal. This molecule does not act on the *rsmZ* and *rsmY* transcription through modulating the activity of the sensor kinase RetS.

Previous findings linked the diffusible signal factor (DSF) to c-di-GMP turnover in *X. campestris* [239]. It is possible that CDA could act on *rsmZ* transcription through the modulation of intracellular levels of c-di-GMP. Further studies have to be conducted in order to measure the variation in c-di-GMP concentration in the strain treated with CDA. Nevertheless, it was possible to exclude the indirect effect of CDA on c-di-GMP homeostasis *via* FleQ.

The action of CDA on *P. aeruginosa* biofilm dispersal is very significant [225]. Thus, understanding how CDA acts on biofilm dispersal is essential for the development of new classes of antimicrobial compounds for the treatment of *P. aeruginosa* infections. In this study, it was shown that CDA represses *rsmZ* and *rsmY* transcription, suggesting that this molecule is involved in the more global modulation of the Rsm/Gac system. Further studies are needed to understand whether the effect of CDA on biofilm dispersal can be entirely attributed to the modulation of this post-transcriptional regulatory system.

3.5 CONCLUSIONS

The identification of the molecules modulating the Gac/Rsm pathway is crucial for the development of compounds able to target the sensor kinases at the top of this transductional cascade. In this study new sensor strains based on *rsmZ-lux* and *rsmY-lux* reporter fusions were developed with the aim to detect and identify the signal molecules activating the Gac/Rsm system in crude extracts cultures. These reporter strains, together with the fluorescence based double transcriptional-translational reporter, represent a powerful

biomolecular tool for future studies aiming to uncover the nature of the Gac/Rsm signal molecule(s). Although neither TLC nor HPLC fractionation studies allowed the identification of novel signal molecules, it was possible to identify PQS as a signal molecule activating *rsmZ* transcription (see Chapter 4 for further details).

Chapter 4

Modulation of the Gac/Rsm system by PQS

4 Modulation of the Gac/Rsm system by PQS

4.1 INTRODUCTION

In certain bacterial species, the production of virulence factors and secondary metabolites is subject to the expression of genes which are regulated both at the transcriptional and post-transcriptional level.

In *P. aeruginosa*, the Gac/Rsm two component regulatory system directly regulates the production of secondary metabolites and is involved in controlling motility. It also exerts a global effect on *P. aeruginosa* production of virulence determinants by controlling the expression of the synthase genes *lasI* and *rhlI* of the QS network [81, 141, 147, 151].

One of the genes found to be controlled by RsmA is *lecA*, which codes for the lectin LecA (also known as PA-IL) [152]. Although LecA protein levels were shown to be strongly enhanced in an *rsmA* mutant, there was no evidence supporting the hypothesis of RsmA directly binding the *lecA* mRNA. Since LecA production is regulated via the *las/rhl* QS cascade [80, 240], the formulated hypothesis was that RsmA repression was a consequence of a reduction in the levels of AHLs. This hypothesis was supported by the finding that a strain overproducing RsmA was shown to have reduced levels of 3-oxo-C12-HSL and C4-HSL [152]. However, the inability to restore the levels of lectin by exogenously adding AHLs to the RsmA-overexpressing strain, suggested that RsmA was also involved in *lecA* modulation in a QS-independent way.

The regulation of LecA has been extensively studied for its importance in biofilm development. LecA and LecB function together as adhesins, which are important for biofilm architecture, and exert a cytotoxic effect towards the respiratory epithelial cells [241-243].

The production of LecA was found to be regulated via QS, as a *lecA::luxCDABE* transcriptional fusion (the *lux* operon was inserted into the *lecA* gene at the 73rd codon in *P. aeruginosa* chromosome) was shown to be activated by RhlR and its cognate autoinducer molecule C4-HSL. The finding of a conserved *lux*-box in *lecA* promoter region suggested that RhlR could promote *lecA* transcription binding to this region [194]. The involvement of the QS system in the regulation of *lecA*, was confirmed by Diggle *et al.* (2003). PQS was shown to enhance the production of RhlR and C4-HSL, and to act on the sigma factor RpoS that is also involved in *lecA* transcriptional regulation [94].

From these data it is not possible to have a clear understanding of the way PQS regulates the transcription of target genes. A *pqsE* mutant does not produce LecA, but it produces wild type levels of PQS. This finding suggested that this metallo-ß-lactamase-like protein could act on *lecA* transcription as a mediator of PQS action [94, 104].

An analysis of transposon mutants conducted on the *lecA::lux* reporter strain constructed by Winzer (2000) revealed that MvaT is another important regulator of *lecA* expression, which acts negatively on *lecA* transcription [244]. MvaT is a transcriptional regulator belonging to a family of proteins functionally related to the H-NS proteins [245]. Moreover, it was found that the deletion of a second regulator belonging to the H-NS protein family, namely MvaU, was also causing the derepression of *lecA* transcription [246].

A more recent study, aiming to detect the determinants involved in biofilm formation, revealed that the sigma factor RpoE (AlgU) was critical for the formation of key matrix biofilm components, by indirectly acting on *lecA* transcription (Fig. 1) [247].

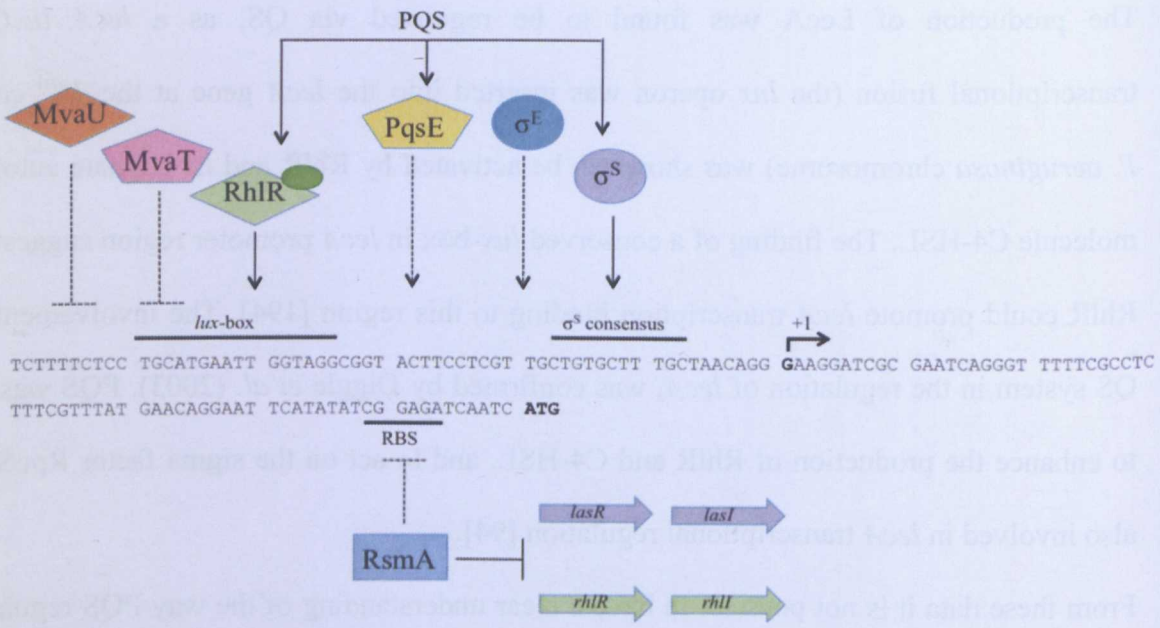


Figure 4-1. Schematic representation of *lecA* transcriptional and post-transcriptional regulation.

Dashed arrows indicate the indirect effect of the regulators (this is the case of RpoE and PqsE), that the exact binding site of the regulator to the promoter is unknown (as in the case of MvaU and MvaT), or that the regulation has not been previously proved (this is the case of RsmA post-transcriptional regulation).

Finally, it is important to note that the majority of experiments conducted to unravel *lecA* transcriptional modulation described here, were obtained using the *lecA::lux* fusion constructed by Winzer *et al.* (2000) [194]. In this reporter strain, the *lux* operon was inserted at the 73rd codon of the *lecA* gene in the chromosome of *P. aeruginosa*. Thus, the effects of the post-transcriptional regulation on this reporter cannot be excluded.

Swarming motility is another phenotype regulated by RsmA. In this case the regulation is positive, as an *rsmA* mutant is impaired in the ability to swarm [151]. Rhamnolipids are essential for swarming motility [248]. RsmA was shown to be involved in the post-transcriptional regulation of the rhamnolipid biosynthetic operon *rhlAB*. However, the lack of swarming of the *rsmA* mutant is not solely due to the reduced rhamnolipid synthesis, since additional factors could be implicated [151]. The way RsmA exerts its positive effect on the translation of *rhlA* is still unknown. In *E. coli*, the RsmA analogue CsrA has been shown to

exert positive effects at the level of the *flhDC* operon that regulates flagellar motility [249]. CsrA binds to a 5' region of the *flhDC* mRNA, stimulating its translation and stabilizing the transcript [135]. Possibly, in *P. aeruginosa* there is an equivalent mechanism by which RsmA modulates *rhlA* translation.

As previously found (Chapter 3), HPLC fractionating studies conducted on the extracellular extracts of *P. aeruginosa* PAO1 revealed that PQS is able of activating *rsmZ-lux* transcriptional fusion. This signal molecule is involved in the cell-to-cell communication mechanism known as QS, and is generally considered to control virulence gene expression at the transcriptional level. It was investigated whether PQS was able to exert its post-transcriptional control on target genes, by altering the levels of *rsmZ* sRNA.

4.2 AIM OF THE STUDY

It was interesting to analyse the mode of action of PQS on *rsmZ* transcription in order to demonstrate the role of PQS in the control of target genes such as swarming and LecA production, through the modulation of the Gac/Rsm system.

4.3 RESULTS

4.3.1 PQS induces the *rsmZ* promoter and delays *rsmY* promoter inactivation

Extracellular extracts from *P. aeruginosa* were able to activate *rsmZ-lux* fusion. In particular, HPLC fractionation of solvent-extracted supernatants revealed the presence of more than one fraction responsible for the induction. Further HPLC/mass spectrometry analysis revealed the presence of PQS in an active fraction. The induction of both *rsmZ* and *rsmY* by PQS was analysed on a *pqsA* mutant background PAKR08 (Δ*pqsA rsmZ-lux*) and PAKR09 (Δ*pqsA*

rsmY-lux). Overnight cultures of PAKR08 and PAKR09 reporter strains were diluted 1000 times in LB media with the addition of 80 μM PQS or 5% (v/v) of methanol as a negative control, and then grown in a TECAN assays (Material and Methods, 2.11). It was shown that PQS activates *rsmZ* transcription, and delays *rsmY* transcriptional inactivation (Fig. 2).

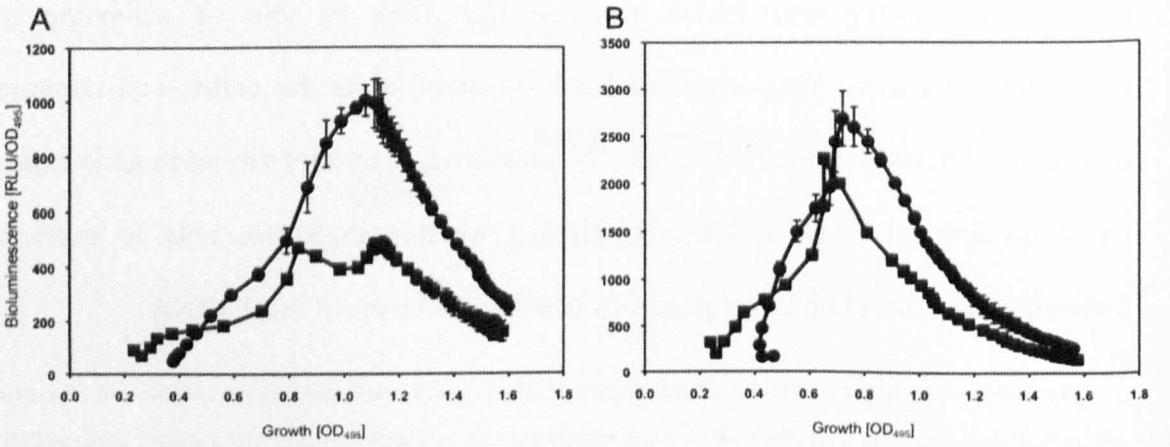


Figure 4-2. Activation of the *rsmZ-lux* and *rsmY-lux* fusions by PQS.

Effect of PQS addition to the strains PAKR08 (Δ*pqsA rsmZ-lux*) (A) and PAKR09 (Δ*pqsA rsmY-lux*) (B). 80 μM of PQS added (circles) compared to the control, 5% (v/v) methanol (squares).

4.3.2 Dose-response curve of PQS inducing the *rsmZ* promoter

Since it was not possible to further enhance *rsmY* activation (in agreement with earlier observations, see Chapter 3), the *rsmZ-lux* reporter was used to create a dose response curve for PQS induction in the reporter stain PASH50. Under the conditions used, the half-maximal effective concentration (EC_{50}) was calculated to be 13 +/- 3 μM (Fig. 3). A higher concentration of PQS was required to obtain similar induction levels when using the corresponding *pqsA* mutant reporter PAKR08 (EC_{50} 25 +/- 4 μM). The *pqsA* mutant is defective in the production of a large number of AQs molecules [114]. The results obtained here indicate that PQS is the main AQ responsible for the activation of *rsmZ*.

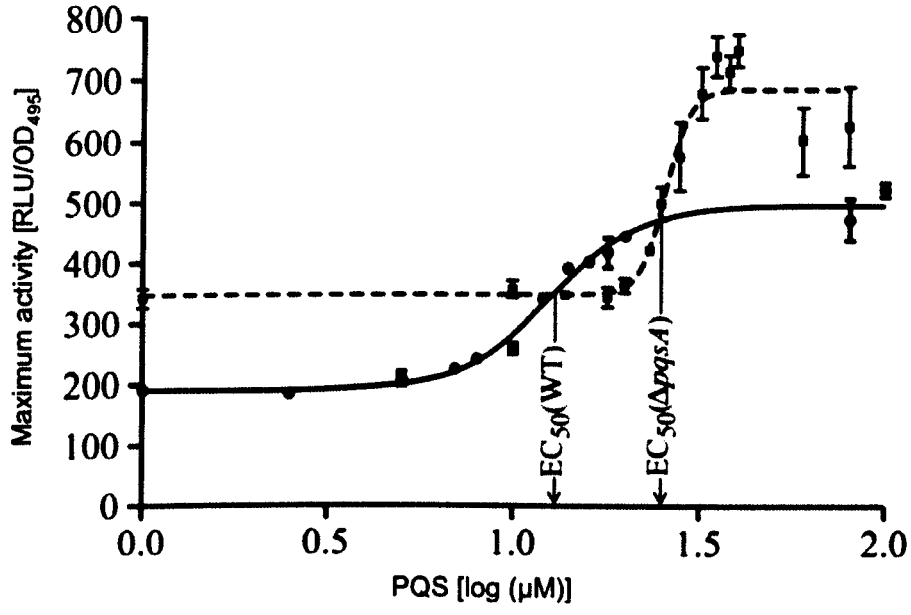


Figure 4-3. Dose response curve of the maximal activation of the *rsmZ-lux* reporter.

The maximal activity of the *rsmZ-lux* reporter at different PQS concentrations was plotted in the wild type (PASH50) (circles) and in a *pqsA* mutant PAKR08 (squares). EC_{50}= effective concentrations at which 50% of the maximal activation of each reporter system is obtained.

4.3.3 PQS induces the *rsmZ* promoter independently of PqsR, PqsE, and in synergy with the Gac two component system

PQS binds and activates the transcriptional regulator PqsR that in turn induces the expression of the *pqsABCDE* operon required for PQS biosynthesis and signaling [97]. Whilst the proteins encoded by *pqsABCD* are required for the biosynthesis of the molecule, the function of PqsE remains unclear. However, PqsE is essential for the regulation of PQS-controlled genes and can exert this function on its own [94, 104, 109, 250]. It was therefore assessed whether the induction of *rsmZ* expression by PQS was dependent on PqsR or PqsE. Thus, the ability of PQS to induce the *rsmZ* was analysed in the strains PAKR48 (Δ*pqsR rsmZ-lux*) and PAKR42 (Δ*pqsE rsmZ-lux*). Interestingly, PQS was able to activate the *rsmZ* promoter in the absence of either PqsR or PqsE, suggesting the existence of an alternative mechanism responsible for this activation (Fig. 4). Moreover, although *rsmZ-lux* response curve was

different in the PAKR08 strain (Δ*pqsA rsmZ-lux*) compared to the corresponding wild type reporter strain PASH50 (*rsmZ-lux*) (Fig. 5), a *pqsA* mutation did not affect *rsmZ* induction by PQS (Fig. 4).

Since GacA and GacS are key regulators of *rsmZ* expression, it was investigated the possibility that PQS might induce *rsmZ* transcription via the GacA/GacS two-component system. Hence, the effect of PQS were tested on the *rsmZ* promoter fusion in the strains PAKR40 (Δ*gacA rsmZ-lux*) and PAKR29 (Δ*gacS rsmZ-lux*) respectively. As expected, the activity of the fusion was very low in both of these mutants compared to the wild type. However, by adding PQS it was able to double the residual maximal activity of the reporter. This suggests that PQS acts in synergy with the GacA/GacS two-component system for the induction of *rsmZ* transcription (Fig. 4).

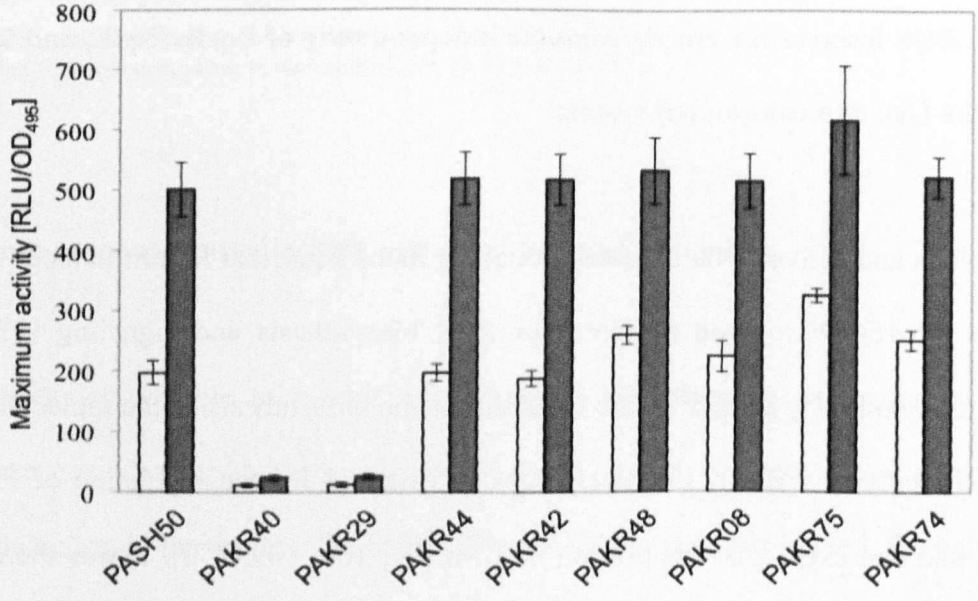


Figure 4-4. Activation of the *rsmZ-lux* fusion in different mutants by PQS.

PQS was added (in gray) at a concentration of 80 μM in the strains PASH50 (*rsmZ-lux*), PAKR40 (Δ*gacS rsmZ-lux*), PAKR29 (Δ*gacA rsmZ-lux*), PAKR44 (Δ*ladS rsmZ-lux*), PAKR42 (Δ*pqsE rsmZ-lux*), PAKR48 (Δ*pqsR rsmZ-lux*), PAKR08 (Δ*pqsA rsmZ-lux*), PAKR75 (Δ*rhlR rsmZ-lux*) and PAKR74 (Δ*lasR rsmZ-lux*). Maximal promoter activity is defined as the maximum relative light units (RLU) per optical density of the culture at 495 nm (OD_{495}) obtained over the duration of the experiment. As control (in white) methanol 5% (v/v) was added.

Furthermore, it was interesting to investigate the importance of LadS and RetS for *rsmZ-lux* activation by PQS. As expected, the *rsmZ-lux* fusion was strongly derepressed in the PAKR53 (Δ*retS rsmZ-lux*) strain (Chapter 3, Fig. 13), and hence the addition of PQS to this mutant failed to affect the maximal bioluminescence levels observed (data not shown). Induction of the fusion by PQS did not appear to be affected by the *ladS* deletion in the strain PAKR44 (Δ*ladS rsmZ-lux*) (Fig. 4). Thus, PQS induces *rsmZ* transcription in a RetS and LadS independent way.

Interestingly, when *ladS* was deleted, the expression of *rsmZ-lux* fusion was not affected. LadS does not seem to be essential for *rsmZ* activation, indicating that, under the conditions used, the main elements controlling *rsmZ* expression are GacS/GacA (in a positive way) and RetS (in a negative way). This observation is in contrast to what was previously shown in the *P. aeruginosa* PAK strain by Ventre *et al.* (2006) [155].

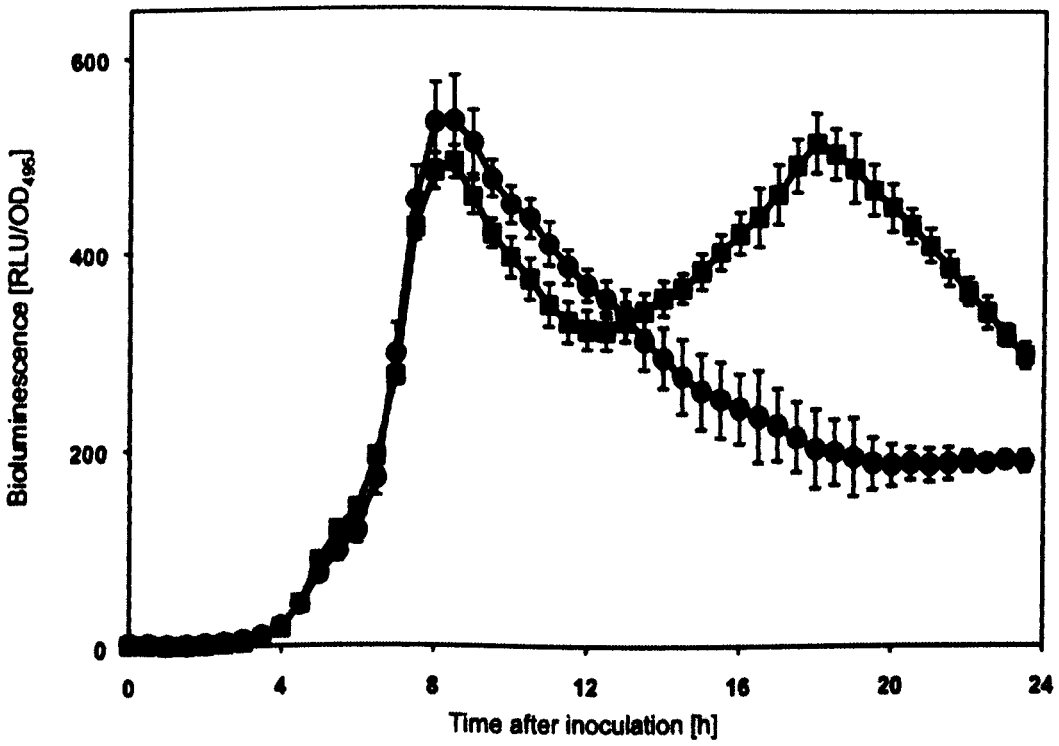


Figure 4-5. *rsmZ-lux* activity in the WT and *pqsA* mutant background.
Induction of PASH50 (*rsmZ-lux*) (squares) and PAKR08 (Δ*pqsA rsmZ-lux*) (circles).

4.3.4 PQS induces the *rsmZ* promoter independently of RhlR and LasR

Detailed analysis of the *rsmZ* promoter reveals the presence of an unreported *lux*-box located immediately upstream of the -35 site. This 20-nucleotides sequence, although not an inverted repeat, shows significant sequence similarity to the putative *luxI*, *rhlI* and *rhlA lux*-boxes (Fig. 6) [251].

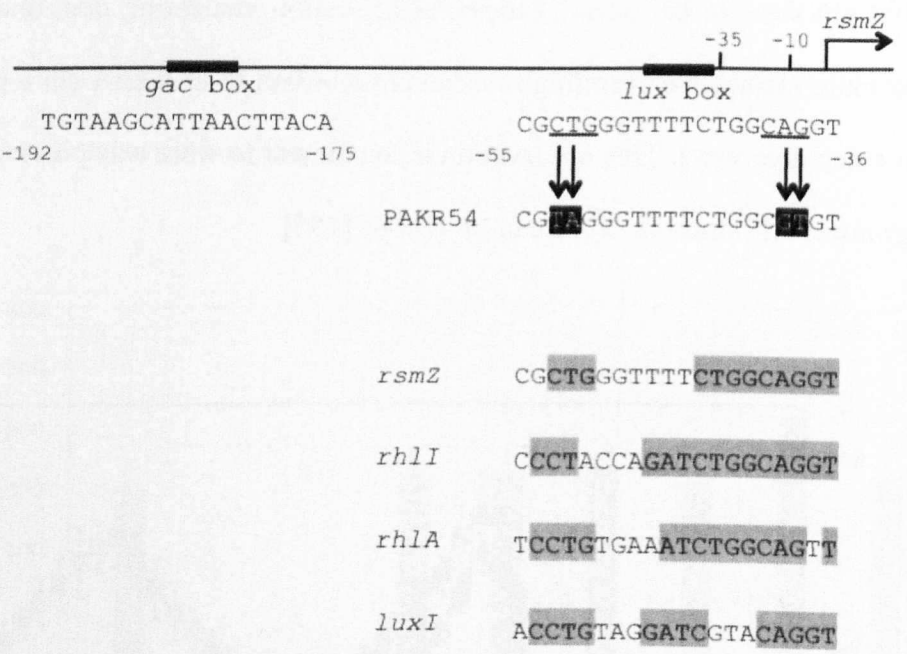


Figure 4-6.Organization of the *rsmZ* promoter.

Upstream the *gac*-box activating sequence, while immediately upstream of the -35 site the *lux*-box of which CTG and CAG nucleotides (underlined) are conserved in other promoters containing a *lux*-box (*rhlI*, *rhlA* and *luxI* promoters are compared). Of these, the 2nd and 3rd nucleotides (CT) and the 15th and 16th (AG) were mutated to give rise to the strain PAKR54 (*rsmZ'-lux*). The conserved nucleotides present in *lux*-box from other promoters are also shown (in grey).

These observations led to the hypothesis that *rsmZ* is also controlled by the RhlR and/or the LasR systems (Fig. 7). Confirming this hypothesis, multicopy overexpression of *lasR* (pKR69) or *rhlR* (pKR70) in the strain PASH50 (*rsmZ-lux*) caused a strong induction of the

rsmZ-lux reporter. However, the addition of C4-HSL and 3-oxo-C12-HSL to the strains carrying the plasmids pKR69 and pKR70 failed to increase *rsmZ* expression (Fig. 8).

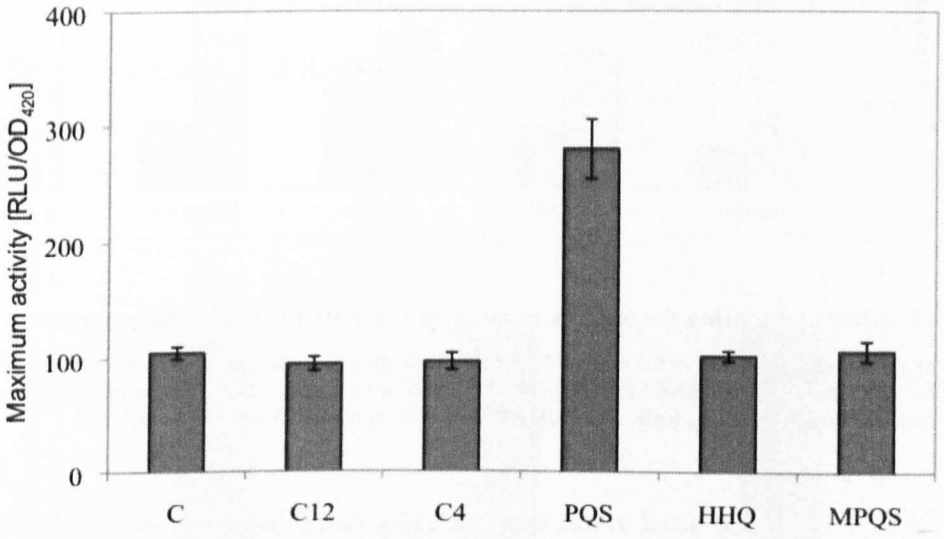


Figure 4-7. Effect of different QS signal molecules on *rsmZ-lux* activity

Impact of QS signal molecules on the PASH50 strain: C, methanol 5% (v/v); C12, 3-oxo-C12-HSL; C4, C4-HSL; PQS, HHQ; MPQS. All the molecules were added at a final concentration of 80 µM.

To investigate whether *rsmZ* activation by RhlR and LasR requires the intact *lux*-box, 4 point mutations were performed as described in Material and Methods (section 2.4.4) (Fig. 6). The plasmids pKR70 and pKR69, containing full length *rhlR* and *lasR* genes respectively, were transformed into the strain PAKR54 (*rsmZ*-lux*). In this fusion, the *lux* operon is under the regulation of the *rsmZ** promoter mutated in the putative *lux*-box. Neither RhlR nor LasR were activating the *rsmZ** promoter, suggesting that RhlR and LasR activate the *rsmZ* promoter through its *lux*-box (Fig. 8).

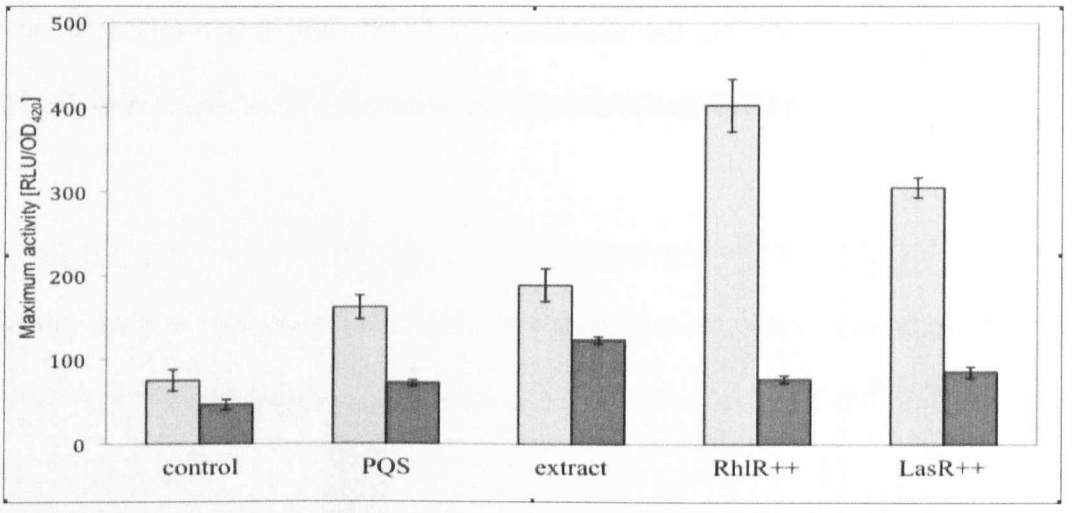


Figure 4-8. Effect of mutating the *lux*-box element on the activity of the *rsmZ* promoter.

The activity of the *rsmZ* promoter in the strain PASH50 (in light gray) was compared with the activity of the *rsmZ** promoter (mutated *lux* box) in the strain PAKR54 (dark gray) measured in the presence of *P. aeruginosa* extracellular extracts, PQS (80 μM), and when overexpressing RhlR and LasR. As a control, methanol 5% (v/v) was added.

The effect of *rhlR* and *lasR* overexpression on *rsmZ-lux* in a *gacA* (PAKR29) and *gacS* (PAKR40) mutant background were also analysed. As observed in the wild type, both in the *gacA* and *gacS* mutant background RhlR and LasR were capable of activating the *rsmZ* promoter. Therefore, these regulators acted independently from GacA and GacS, probably binding to a yet unidentified region in *rsmZ* promoter (Fig. 9).

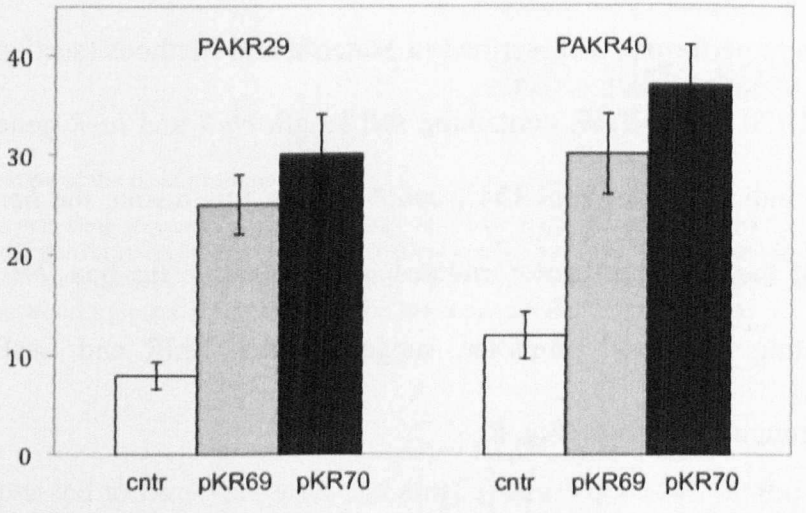


Figure 4-9. Effect of LasR or RhlR overexpression on *rsmZ-lux* activity in a *gacA* and *gacS* mutant.

The strains PAKR29 (Δ*gacA rsmZ-lux*) and PAKR40 (Δ*gacS rsmZ-lux*) were analysed in the presence of the pKR70 (RhlR++) and pKR69 (LasR++) plasmids. As a control (white bars) the strain carrying the empty plasmid to create the genetic bank (pME6000) was used.

These observations led to the hypothesis that PQS had an indirect effect on *rsmZ-lux* activation by promoting *rhlR* transcription. This hypothesis is in line with the fact that PQS modulates many genes through the activation of the *rhl* system [93]. However, both PAKR74 (Δ*lasR::Gm*^{R} *rsmZ-lux*) and PAKR75 (Δ*rhlR rsmZ-lux*) strains responded to the addition of PQS as the wild type strain (PASH50) (Fig. 10).

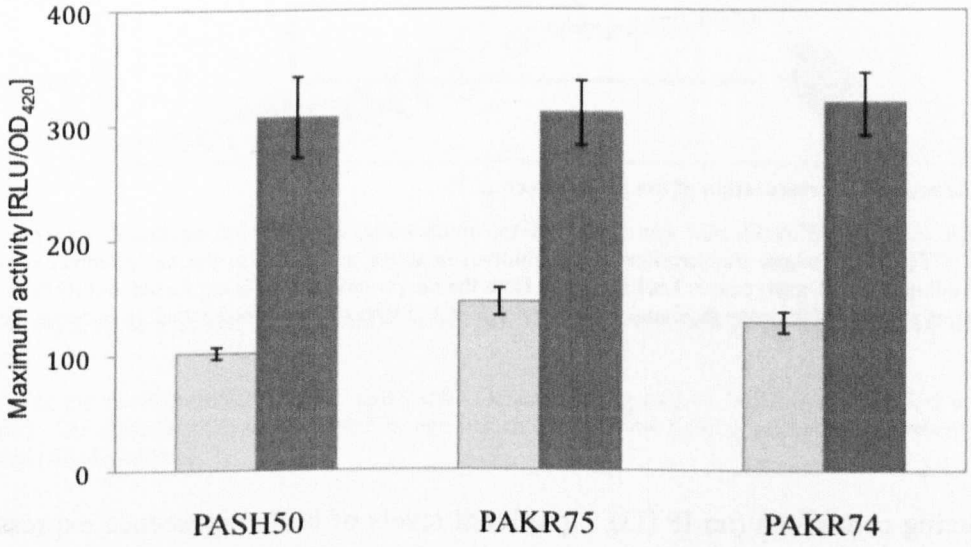


Figure 4-10. Effect of rhlR and lasR mutation on the activation of the *rsmZ* promoter by PQS.

The activation of the *rsmZ* promoter by PQS addition (80 μM) (in dark gray) was compared in the PASH50 (*rsmZ-lux*), PAKR75 (Δ*rhlR rsmZ-lux*) and PAKR74 (Δ*lasR::Gm*^{R} *rsmZ-lux*) mutant with respect to the addition of solvent methanol 5% (v/v) (light gray).

4.3.5 Construction of an *E. coli* heterologous system for the study of RhlR regulation on *rsmZ* transcription.

To further analyse whether RhlR could regulate *rsmZ* independently from the genetic context, it was analysed the effect of RhlR overexpression in a heterologous system. For this purpose, a specific plasmid pKR18 was constructed (Materials and Methods, 2.4.6). This plasmid carries the *rsmZ-lux* fusion. The *rhlR* gene is under the stringent regulation of an inducible *tac* promoter (Fig. 11). The *tac* promoter is repressed by LacI^Q, whose effect is hampered upon

the addition of IPTG. In inducing conditions (1 mM IPTG), RhlR is expressed and can bind the *rsmZ* promoter. To avoid unwanted interaction of the LuxR homologue (SdiA) with *rsmZ* [252], pKR18 was transformed in the *E. coli* RVH-E1 strain (Δ*sdiA*) [190].

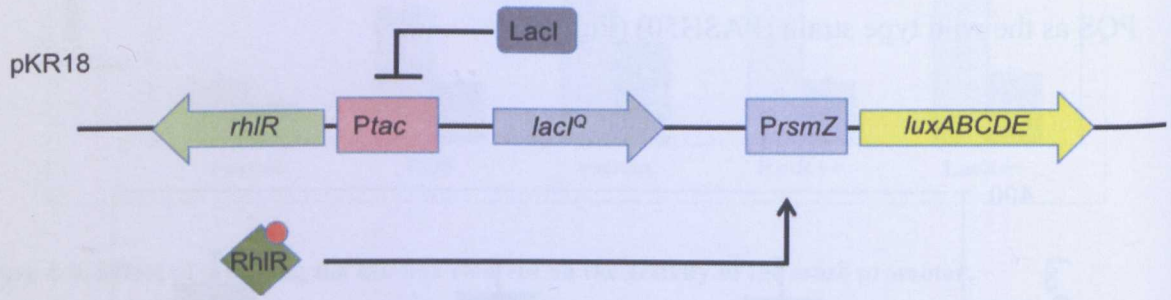


Figure 4-11. Schematic representation of the pKR18 vector.

This vector is derived from pME6032. *rhlR* was cloned into the multicloning site under the regulation of the *tac* promoter. In the absence of IPTG, LacI (whose transcription is constitutive) is active and binds to the *tac* promoter repressing *rhlR* transcription. Adding IPTG (1 mM) causes LacI to detach from the *tac* promoter, *rhlR* is expressed and RhlR is able to bind (in the presence or absence of the cognate signal molecule C4-HSL) to the *rsmZ* promoter which transcribes the *luxABCDE* operon.

In non-inducing conditions (no IPTG), significant levels of bioluminescence expression were detected. This indicates that *rsmZ* is active in the *E. coli* heterologous background. However, when inducing *rhlR* expression, the level of *rsmZ* transcription increased approximately threefold (Fig. 12). On the other hand, when the experiment was repeated using the plasmid pKR19 (*rsmZ**, *lux*-box mutated), the induction of *rhlR* failed to increase *rsmZ* expression (Fig. 12).

It was then tested whether the addition of the RhlR cognate signal molecule C4-HSL was affecting the levels of *rsmZ* transcription. No alterations of *rsmZ* expression were observed (data not shown), suggesting that, in this heterologous system, RhlR acts as a transcriptional activator of *rsmZ* through its *lux*-box in a C4-HSL independent way.

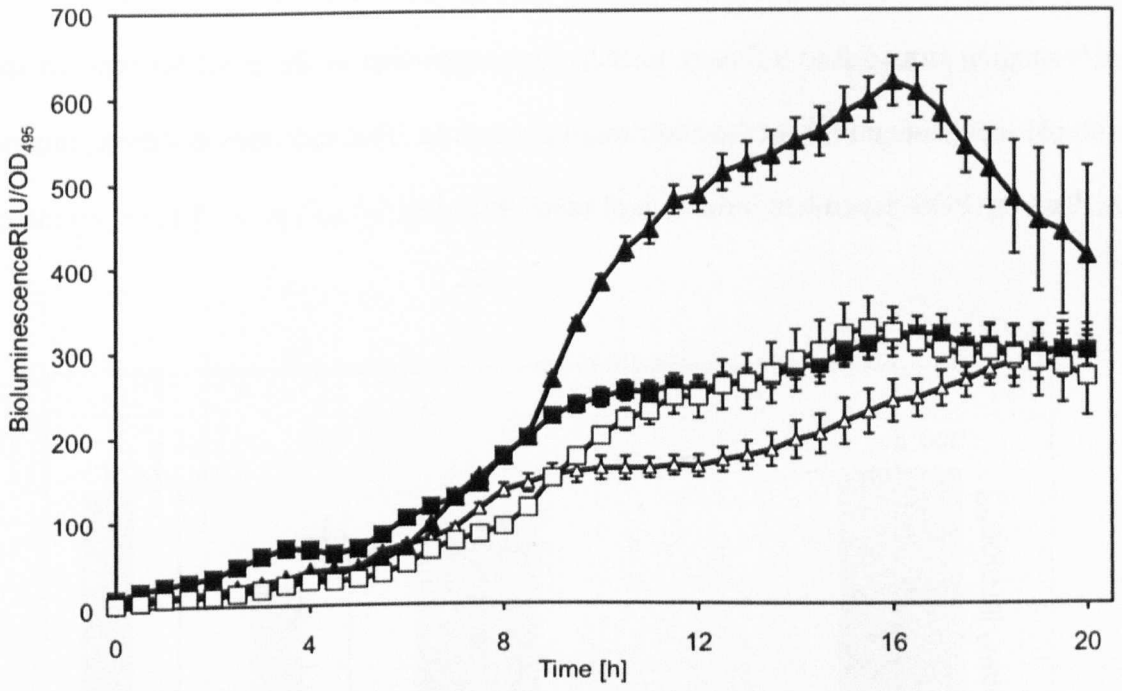


Figure 4-12. Effect of the overexpression of *rhlR* on the activation of *rsmZ-lux* in an heterologous *E. coli* system.

Activity of the *rsmZ* promoter in an *E. coli* (Δ*sdiA*) strain carrying pKR18 (*rsmZ-lux*) (triangles) or pKR19 (*rsmZ*-lux*) (squares). The experiment was conducted in non-inducing conditions (empty symbols), and when *rhlR* expression was activated (filled symbols).

4.3.6 PQS induces the *rsmZ* promoter independently from pyocyanin

Phenazines are amongst the virulence factors produced by *P. aeruginosa* in response to PQS signaling [109]. These molecules are redox-active compounds which exert their toxicity on other prokaryotes and eukaryotes [253]. Recently, the phenazine pyocyanin was suggested to act as a QS signaling molecule involved in the activation of a specific set of genes during late exponential growth phase [254]. It was interesting to assess whether PQS might induce the *rsmZ* promoter *via* increased pyocyanin production. Thus, it was analysed the effect of pyocyanin on the *rsmZ-lux* fusion. Pyocyanin was extracted from *P. aeruginosa* cultures (Material and Methods, 2.16.2) and then added to the growth medium at a concentration of 100 μM. Under physiological conditions, pyocyanin is a zwitterion [255]. Therefore, when

adding pyocyanin to the growth media, it was important to buffer the culture to a defined pH (pHs ranging from 5.0 to 9.0 were tested). The expression of the *rsmZ-lux* reporter increased with pH independently from the addition of pyocyanin. This indicates that pyocyanin does not mediate the PQS-dependent induction of *rsmZ* (Fig. 13).

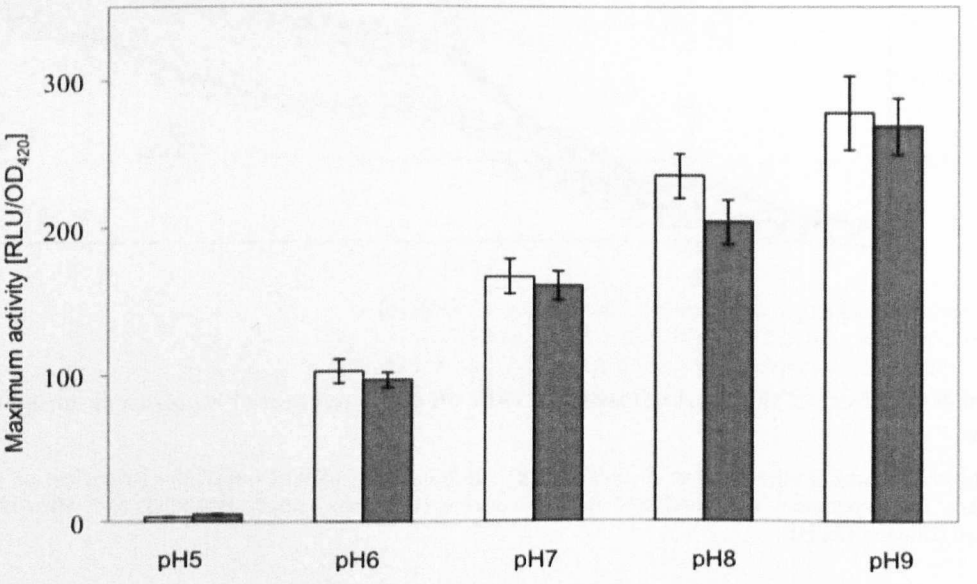


Figure 4-13. Effect of pH and pyocyanin on the activity of the *rsmZ* promoter.

Maximum bioluminescence of the PASH50 (*rsmZ-lux*) strain when adding water (white bars) or pyocyanin (gray bars) in different pH buffered media.

In order to exclude the possibility that phenazines were involved in *rsmZ* modulation, the *rsmZ-lux* transcriptional fusion was introduced in a strain unable to produce phenazines. In *P. aeruginosa* PAO1, the production of phenazines is controlled by two operons, namely *phz1* and *phz2*. The mutant defective in both operons PAKR76 (Δ*phz*1/2 *rsmZ-lux*) was created and then tested for its ability to respond to PQS. PAKR76 reporter was still able to respond to PQS addition (Fig. 14). It was concluded that pyocyanin and in general phenazines are not necessary for the physiological induction of *rsmZ* by PQS.

Recent studies showed that pyocyanin exerts its signaling function through the activation of the transcriptional factor SoxR [256]. This is a redox-responsive regulator that activates the

transcription of genes containing a specific upstream binding site (*sox*-box). However, an analysis of *rsmZ* promoter showed that this box is not present, suggesting that the response regulator SoxR is not involved in PQS action on *rsmZ* transcription. To exclude this hypothesis, a *soxR* mutant carrying the *rsmZ-lux* fusion should be tested for PQS activation.

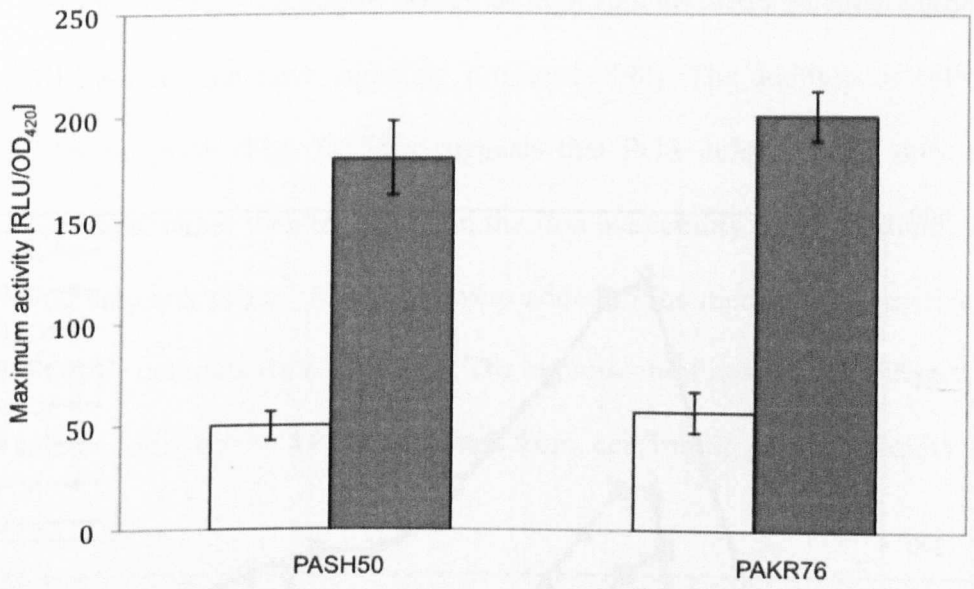


Figure 4-14. Effect of phenazines mutant on the activation of *rsmZ-lux* by PQS.

Activity of the *rsmZ-lux* fusion in the PASH50 (*rsmZ-lux*) and PAKR76 (Δ*phz1*/2) strains in the presence (gray bars) of PQS (80 μM). As a control, methanol 5 % (v/v) was added (white bars).

4.3.7 PQS induces the *rsmZ* promoter independently from the formation of membrane vesicles

Recently, the role of PQS in the formation of membrane vesicles (MVs) was described [257]. PQS, unlike HHQ, can interact strongly with the membrane lipids promoting the formation of the MVs. These vesicles are involved in important biological processes, such as delivering to the neighbouring cells PQS and other antimicrobial quinolones [257]. HHQ was shown to have no effect on *rsmZ-lux*. Moreover, it is believed that this molecule is not involved in MVs formation. Thus, it was interesting to investigate if the effect of PQS on *rsmZ* promoter

activation was due to its physiological effect on the lipid membrane, rather than acting as a signal molecule. For this purpose, PQS derivatives that interact with the outer membrane lipids were tested for their ability to induce *rsmZ* expression. A range of PQS derivatives with altered acyl side-chain lenght were tested; PQS acyl side-chain is composed of seven corbon atoms, for this reason we refer to it as C7-PQS. Among all the analogues tested, only C3-PQS had a slight inducing effect on the *rsmZ-lux* fusion (Fig. 15).

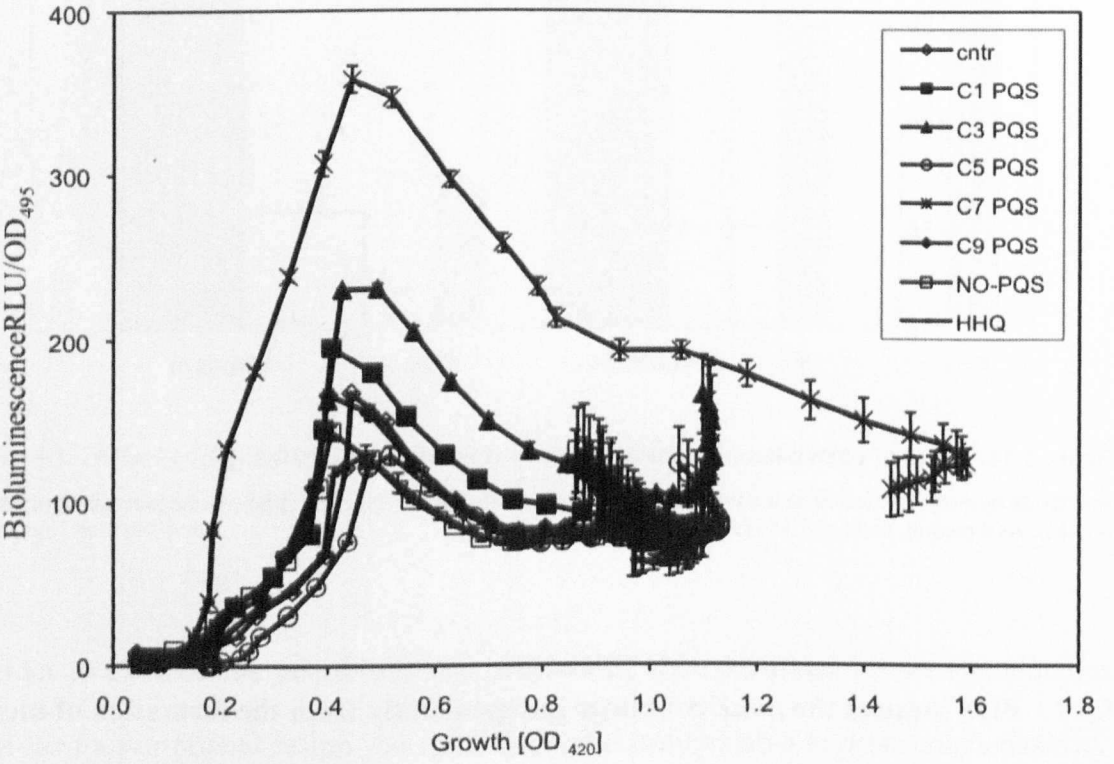


Figure 4-15. Effect of different PQS analogues on the *rsmZ-lux* fusion.

The activity of PASH50 (*rsmZ-lux*) after the addition of PQS (C7-PQS) or different PQS analogues varying in the acyl chain length (from 1 to 9 carbons) or in the ring substitutions (NO group). 80 μM of each molecule were added. As a control 5% (v/v) methanol was added.

4.3.8 PQS induces the *rsmZ* promoter independently from its iron chelating activity

Previous studies have revealed that PQS is able to chelate iron, thus facilitating siderophore-mediated iron delivery into the cell [98]. Therefore, it was important to understand if PQS was acting on *rsmZ* transcription through its signaling properties, or by modulating the iron availability in the cell. To evaluate the importance of iron chelation for the PQS-dependent regulation of *rsmZ*, the *rsmZ-lux* induction was examined after the addition of methyl PQS (MPQS). This molecule is structurally similar to PQS and chelates iron with a similar affinity. However, MPQS does not have signaling properties [98]. The addition of MPQS did not affect *rsmZ* transcription (Fig. 7). This suggests that PQS induces *rsmZ* only through its signaling properties, rather than by acting on the iron availability in the medium. To confirm this, saturating amounts of Fe^{3+} (100 μM) were added to the media, in alternative 80 μM of preformed PQS-Fe complex (in a 3:1 ratio). The expression of *rsmZ* is not affected neither by iron in its soluble form, nor by its PQS-chelated form, confirming previous results (Fig. 16).

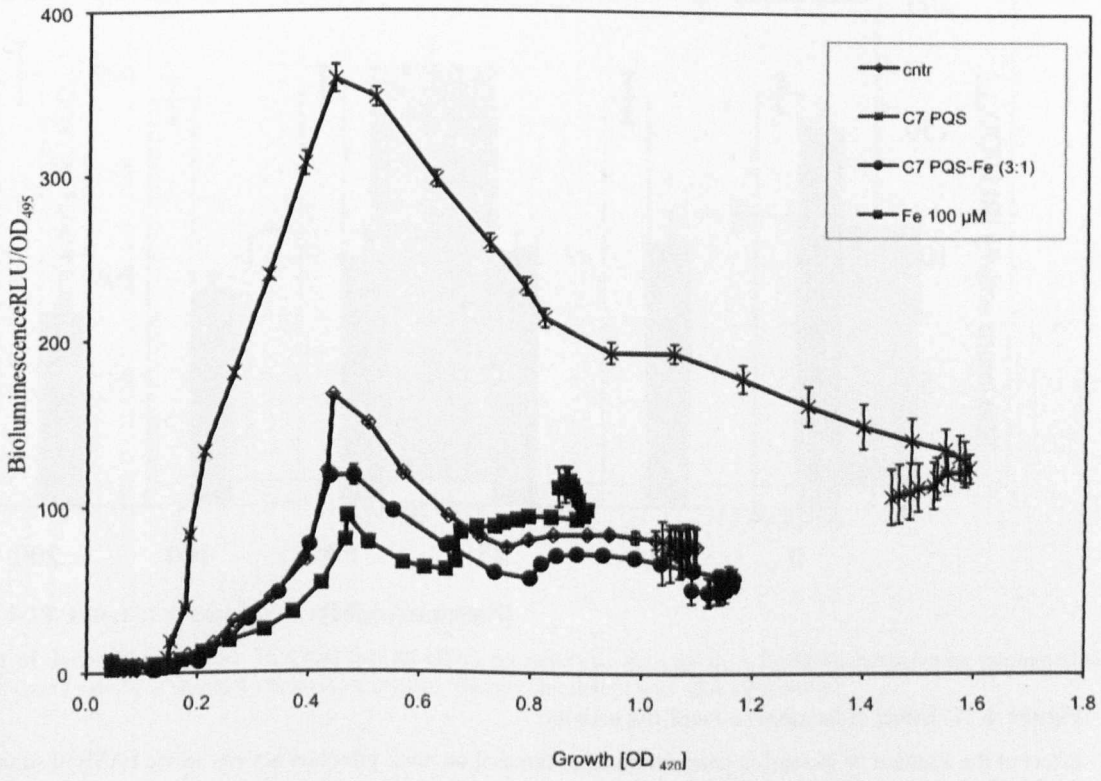


Figure 4-16. Effect of iron and of iron-PQS complex on the activation of *rsmZ-lux*.

Activity of *rsmZ-lux* fusion when adding PQS (80 μM), PQS chelated with iron (3:1 ratio) or only iron at physiological concentration (100 μM). As a control 5% (v/v) methanol was added.

4.3.9 Farnesol inhibits *rsmZ* transcription

Farnesol is a compound produced by many organisms involved in inter- and intraspecific interactions [258, 259]. This volatile sesquiterpene was recently shown to inhibit the production of PQS by repressing the *pqs* operon [260]. For this reason, it was decided to investigate the effect of farnesol on the expression of *rsmZ*. Interestingly, farnesol reduced *rsmZ-lux* activity in a WT strain (Fig. 17). When adding farnesol to the Δ*pqsA* mutant this effect was not evident, supporting the hypothesis that farnesol modulates *rsmZ* transcription by repressing PQS production.

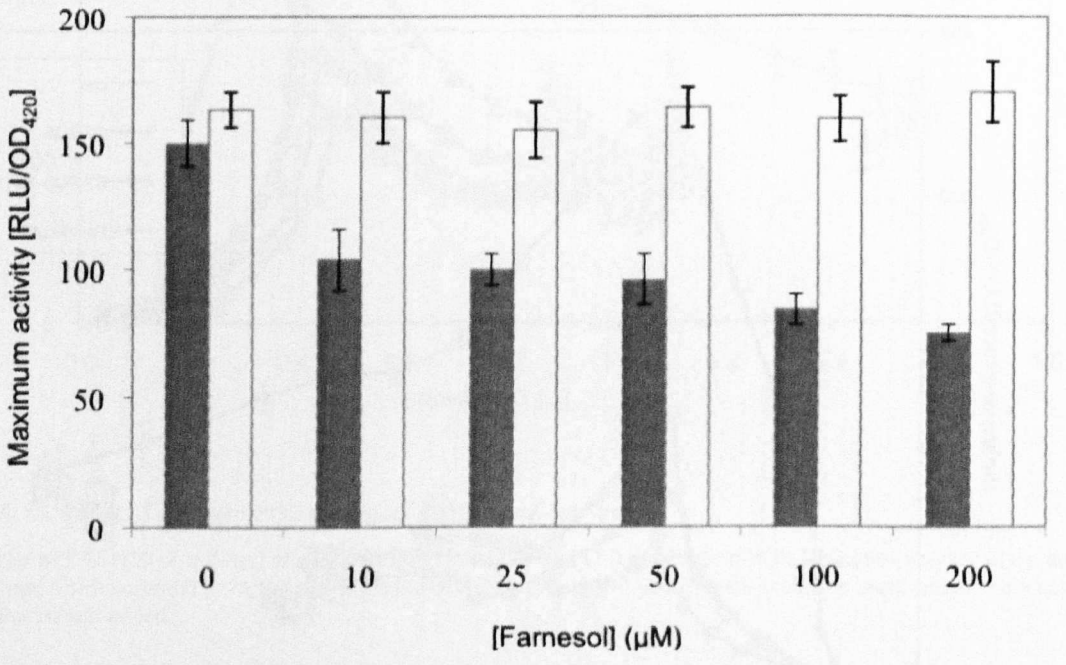


Figure 4-17. Effect of farnesol on *rsmZ-lux* activity.

Effect of the addition of increasing concentrations of farnesol on *rsmZ* promoter activity in the PASH50 strain (gray) and in the PAKR08 (Δ*pqsA rsmZ-lux*) mutant background (white).

4.3.10 Dynorphin promotes *rsmZ* transcription

The ability of *P. aeruginosa* to intercept the opioid compounds produced by the host, and to integrate them into the quorum sensing circuit, has been recently reported [261]. During stress conditions, the host organism releases opioids. *P. aeruginosa* has developed the ability to sense the dynorphin opioid produced by mammalian intestinal cells, enhancing its virulence. Recently, the synthetic analogue U-50488 was shown to directly induce the production of multiple virulence factors and in particular of the PQS signal molecule. Therefore, the effect of dynorphin on the *rsmZ-lux* fusion was explored. As expected, this molecule was shown to activate the *rsmZ-lux* fusion in a PQS-dependent way. Indeed, when adding dynorphin U-50488 to PAKR08 (Δ*pqsA rsmZ-lux*), the effect on *rsmZ* was not evident (Fig. 18).

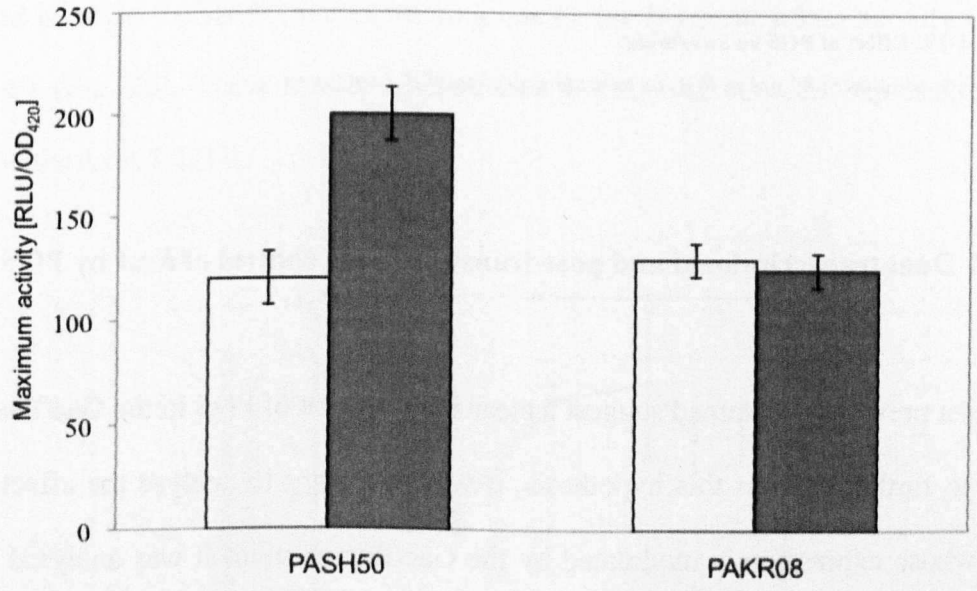


Figure 4-18. Effect of dynorphin on *rsmZ-lux* activity.

Addition of dynorphin 100 μM (in gray) has an effect on *rsmZ-lux* activity in a PASH50 background compared with a PAKR08 (*pqsA rsmZ-lux*) mutant strain. As a control 5% (v/v) methanol was added (in white).

4.3.11 PQS inhibits swarming motility

It is known that in *P. aeruginosa*, an *rsmA* defective strain does not swarm [151]. Hence, since PQS induces *rsmZ* transcription, addition of PQS should result in a reduction in swarming caused by the titration of RsmA by RsmZ. To investigate this, the effect of the addition of 80 μM of PQS, HHQ or MPQS on *P. aeruginosa* PAO1 swarming phenotype was tested (Materials and Methods section 2.1). As predicted, whilst MPQS and HHQ have little or no impact on swarming, PQS shows a substantial inhibition of this phenotype (Fig. 19).

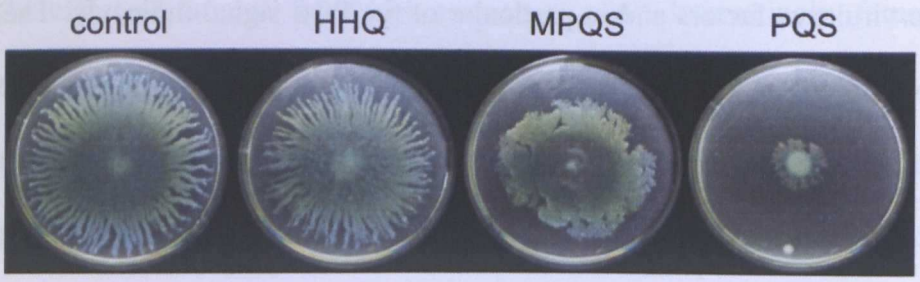


Figure 4-19. Effect of PQS on swarming.
Effect of the addition of 80 μM of PQS on swarming motility of *P. aeruginosa*.

4.3.12 Dual transcriptional and post-transcriptional control of *lecA* by PQS.

The data previously obtained suggest a clear involvement of PQS in the Gac/Rsm pathway. In order to further support this hypothesis, it was necessary to analyse the effect of PQS on a gene whose expression is modulated by the Gac/Rsm system. It was analysed the impact of PQS on *lecA* expression by constructing both transcriptional and post-transcriptional fusions. The complex regulation of *lecA* can be divided into transcriptional and post-transcriptional regulation (Fig. 1). The expression of this gene has been extensively studied for its importance in biofilm development [241].

4.3.12.1 RsmA directly controls the expression of lecA at the post-transcriptional level

Using a *lecA-lux* transcriptional reporter and Western blot analysis, the global post-transcriptional regulator RsmA was found to exert a negative control on the production of the LecA (PA-IL) galactophilic lectin [94, 152]. However, the mechanism by which RsmA regulates the expression of *lecA* remained to be elucidated, as it was not clear whether this control was direct or through another regulator. To investigate whether RsmA directly controls *lecA* mRNA translation, the 5' untranslated transcribed region (UTR) of this gene was translationally fused to *lacZ* (*lecA'-'lacZ*) and transcribed from a constitutive kanamycine resistance gene promoter (P_{Km}). This resulted in the generation of the plasmid pMM44 (P_{Km} *lecA'-'lacZ*). When this plasmid was transformed into the wild type PAO1, the ß-galactosidase activity remained constant during the first 5 hrs of incubation, until the end of the exponential phase, after which the reporter was induced (Fig. 20). However, when the same plasmid was introduced in strain PASK09 (overexpressing *rsmA*), the ß-galactosidase activity remained at basal levels (Fig. 20). These results indicate that RsmA controls *lecA* expression by acting directly on the *lecA* 5' UTR.

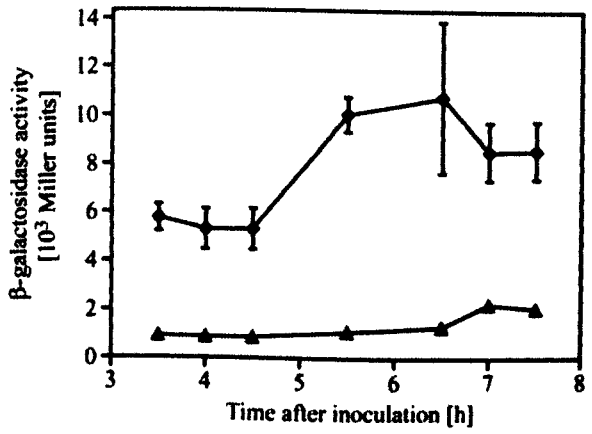


Figure 4-20. Regulation of a translational P_{Km}-*lecA'-'lacZ* reporter fusion by RsmA.

Plasmid pMM44 carrying the translational reporter fusion was introduced in the wild type *P. aeruginosa* PAO1 (diamonds) and in strain PASK09 overexpressing *rsmA* (triangles). ß-galactosidase activity (in Miller Units) was followed in time.

Previous studies demonstrated that RsmA interacts directly with *lecA* 5' UTR. These studies were performed by RNA electrophoretic mobility shift assays, as described elsewhere (S. Kuehne, PhD thesis). Briefly, transcripts corresponding to the 5' UTR of *lecA* from the +1 nucleotide to the start codon were produced *in vitro*, incubated with increasing amounts of purified RsmA and subsequently resolved by non denaturing polyacrylamide gel electrophoresis. The complexes were electro-transferred onto a nylon membrane and the RNA was detected using a *lecA* 5' UTR DIG-labeled DNA probe. Increasing amounts of RsmA caused an RNA mobility shift, demonstrating that RsmA directly interacts with the *lecA* 5' UTR. The 115-nt 5' UTR of the *carA* gene (PA4758), encoding a putative carbamoyl-phosphate synthase, was used as a negative control. No alteration in the RNA mobility patterns of *carA* was observed, confirming that the binding of RsmA to the *lecA* mRNA is specific.

4.3.12.2 PQS exerts its control of lecA expression both at the transcriptional and the post-transcriptional level

At the transcriptional level, the *lecA* gene is controlled by two QS signal molecules, C4-HSL and PQS [94, 194]. The fusion used to study *lecA* transcriptional regulation was made by inserting the *luxCDABE* operon at the 73rd codon of *lecA* in the chromosome of *P. aeruginosa* [194]. Consequently, there was a possibility that the 219 nucleotides encompassing these first 73 codons could be subjected to an unexpected post-transcriptional regulatory effect. In order to exclusively assess the transcriptional control of the *lecA* promoter, a new *lecA-lux* transcriptional fusion was used. In this fusion, the 5' UTR region of the *lux* operon was directly fused to the +1 *lecA* transcription start nucleotide (M. Messina, PhD thesis).

To study the post-transcriptional modulation of *lecA*, a second fusion was created. This construct encompassed the 5' UTR of *lecA* and the 18th first codons (C. Pustelny, PhD thesis).

Contrary to the construct used previously by Winzer *et al* (2000), the new *lecA-lux* constructs were made using the mini-CTX::*luxCDABE* transcriptional reporter delivery system carried by pHKBS1, which effectively integrates the fusions in the chromosome at the CTX attachment site [198]. In the fusion used by Winzer *et al.* (2000), the *lecA* coding sequence was replaced by a double homologous recombination with the *lecA-lux* fusion, resulting in a LecA-negative strain.

Both the P*lecA*'$_{(18\ codons)}$-*lux* and the P*lecA*$_{(+1)}$-*lux* were cloned into pHKBS1, resulting in the pCP2 and pMM84 plasmids, respectively. These plasmids were then introduced into the wild type strain PAO1 to generate the PACP2 and PAMM84 strains, respectively (Fig. 21).

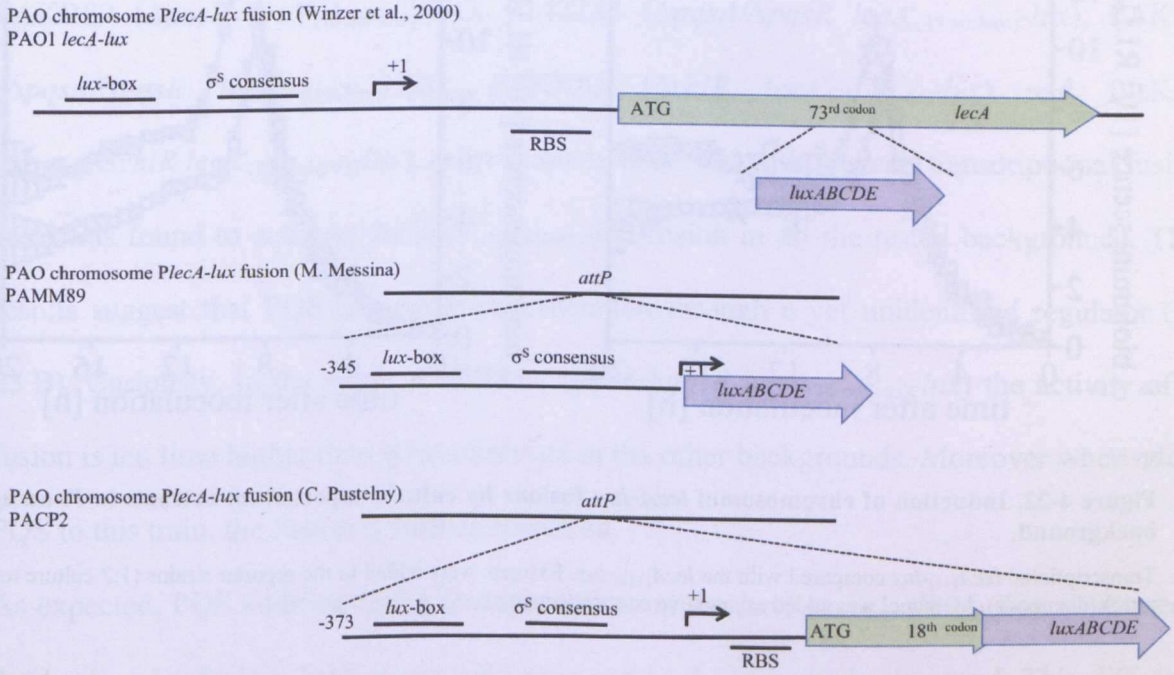


Figure 4-21. Schematic representation of the different *lecA* fusions utilized in this study.

In line with previous studies, both new fusions were induced by PQS and by a C4-HSL-PQS mixture [194]. Interestingly, the induction of the *lecA*'$_{(18\ codons)}$-*lux* by the culture supernatant was higher than that of the *lecA*$_{(+1)}$-*lux*, indicating the existence of post-transcriptional control

of *lecA* expression by culture supernatants (Fig. 22). In the attempt to identify the molecule in the culture supernatant responsible for *lecA'*$_{(18\ codons)}$*-lux* activation, the HPLC-derived fractions previously found to activate *rsmZ* was tested (Chapter 3). Fraction 6 (the one containing PQS) was found to activate the *lecA'*$_{(18\ codons)}$*-lux* fusion, suggesting that PQS is one of the molecules responsible for *lecA* post-transcriptional activation. These results reinforce the hypothesis that the control of *lecA* expression by PQS and RsmA can occur at the translational level, and that the key elements of this regulation are present within the region encompassing the 5' UTR and the first few codons of *lecA*.

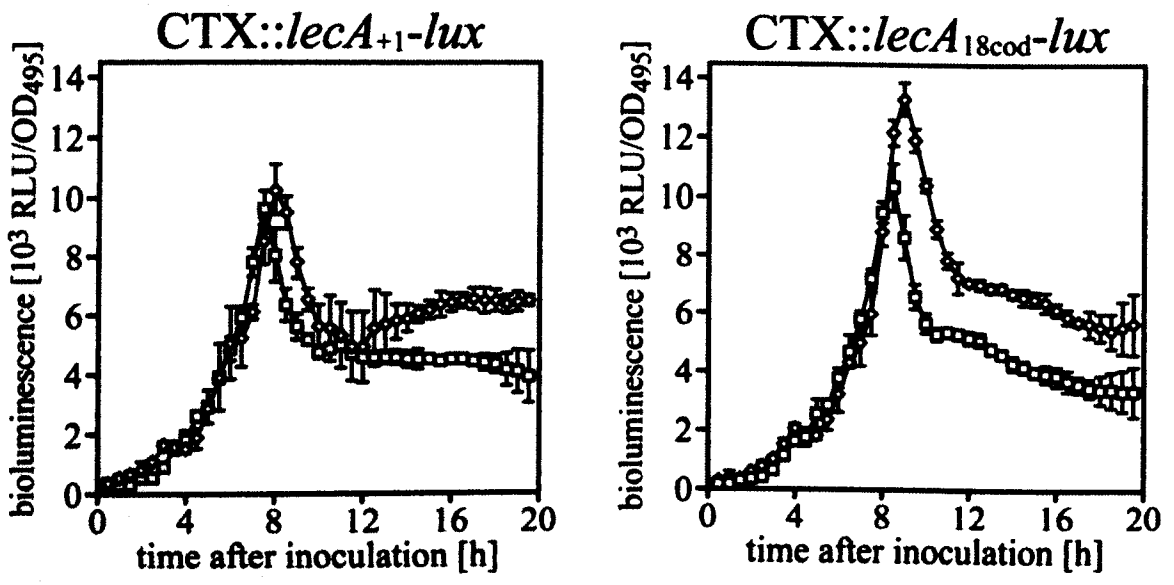


Figure 4-22. Induction of chromosomal *lecA-lux* fusions by culture supernatant extracts in *P. aeruginosa* background.

Transcriptional *lecA*$_{(+1)}$*-lux* compared with the *lecA*$_{(+18)}$*-lux*. Extracts were added to the reporter strains (1:2 culture to extract ratio) (diamonds). Methanol was added as negative control (squares).

4.3.12.3 Study of lecA regulation by the pqs system and by its own product

It is known that quorum sensing regulates lectin production. More specifically, the expression of *lecA* is induced directly by RhlR and indirectly by PQS [94, 194]. In order to understand

the way PQS was modulating *lecA,* it was analysed the *lecA* transcriptional (*lecA*$_{(+1)}$-*lux*) and translational (*lecA*'$_{(18\ codons)}$-*lux*) fusions in different mutant backgrounds (Fig. 23A and 23B). In particular, it was tested the effect of PQS in the wild type background PAMM84 (*lecA*$_{(+1)}$-*lux*) and in the mutants PAKR60 (Δ*pqsA lecA*$_{(+1)}$-*lux*), PAKR61 (Δ*pqsE lecA*$_{(+1)}$-*lux*), PAKR62 (Δ*pqsR lecA*$_{(+1)}$-*lux*), PAKR63 (Δ*pqsA*/Δ*pqsR lecA*$_{(+1)}$-*lux*), PAKR64 (Δ*pqsA*/Δ*pqsE lecA*$_{(+1)}$-*lux*), PAKR65 (Δ*rhlR lecA*$_{(+1)}$-*lux*) and PAKR87 (Δ*pqsA*/Δ*rhlR lecA*$_{(+1)}$-*lux*). PQS was found to activate the *lecA*$_{(+1)}$-*lux* fusion in all the tested backgrounds, suggesting that PQS transcriptional activation of *lecA* is dependent on a yet unknown regulator (Fig. 23 A).

To analyze the post-transcriptional effect of PQS on *lecA*, the translational fusion (*lecA*'$_{(18\ codons)}$-*lux*) was inserted into the wild type (PACP2) and into different backgrounds, giving rise to the PAKR88 (Δ*pqsA lecA*'$_{(18\ codons)}$-*lux*), PAKR68 (Δ*pqsE lecA*'$_{(18\ codons)}$-*lux*), PAKR89 (Δ*pqsR lecA*'$_{(18\ codons)}$-*lux*), PAKR84 (Δ*pqsA*/Δ*pqsR lecA*'$_{(18\ codons)}$-*lux*), PAKR85 (Δ*pqsA*/Δ*pqsE lecA*'$_{(18\ codons)}$-*lux*), PAKR83 (Δ*rhlR lecA*'$_{(18\ codons)}$-*lux*) and PAKR86 (Δ*pqsA*/Δ*rhlR lecA*'$_{(18\ codons)}$-*lux*). As previously observed with the *lecA* transcriptional fusions, PQS was found to activate the *lecA*'$_{(18\ codons)}$-*lux* fusion in all the tested backgrounds. These results suggest that PQS acts on *lecA* translation through a yet unidentified regulator (Fig. 23 B). Curiously, in the strain PAKR85 (Δ*pqsA*/Δ*pqsE lecA*'$_{(18\ codons)}$-*lux*) the activity of the fusion is ten time higher than what observed in the other backgrounds. Moreover when adding PQS to this train, the fusion is further enhanced.

As expected, PQS addition had a stronger effect on the *lecA*'$_{(18\ codons)}$-*lux* fusion compared to the *lecA*$_{(+1)}$-*lux* fusion, both in the wild type and in the mutants background. This difference can be explained by the presence in the *lecA*'$_{(18\ codons)}$-*lux* fusion of the RBS of *lecA*, which is positively regulated by PQS.

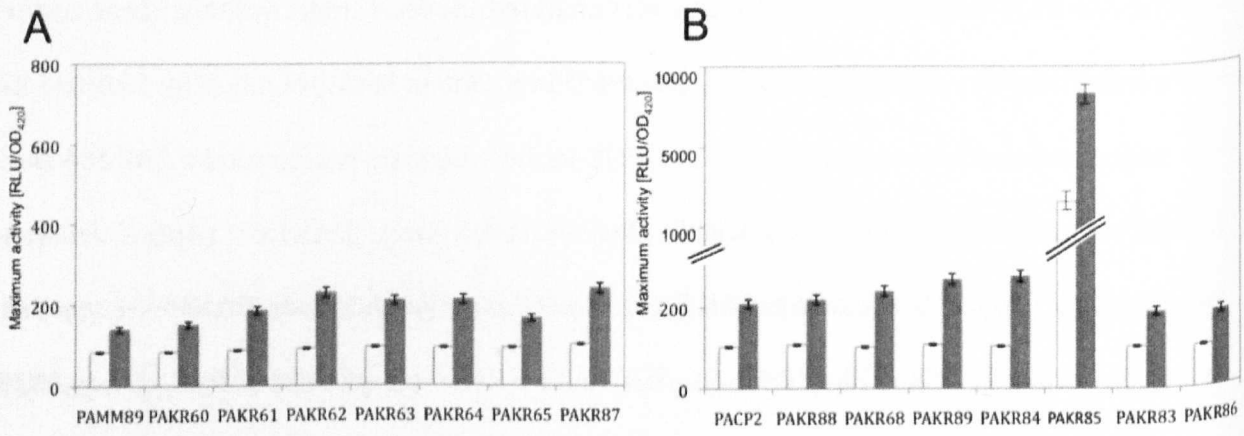


Figure 4-23. Effect of PQS addition to different mutant backgrounds on transcriptional and translational *lecA-lux* fusions.

(A) PQS (80 μM) was added to different mutant backgrounds to the $lecA_{+1}$-*lux* fusion (in grey). As a control, 5% (v/v) MeOH was added (in white). (B) PQS (80 μM) was added to different mutant backgrounds to the $lecA_{+18cod}$-*lux* fusion (in grey). As a control, 5% (v/v) MeOH was added (in white).

It was also observed that the levels of *lecA-lux* expression were significantly higher in the first fusion used by Winzer *et al.* [194], which was obtained by gene replacement insertion of the *luxCDABE* operon at the 73rd codon of *lecA* in the chromosome, thus creating a *lecA*-defective mutant. For this reason, it was studied the role of lectin in the modulation of its own expression. For this purpose, the strain PAO1 *lecA-lux* was transformed with a plasmid expressing *lecA* (pLECA). When the expression of *lecA* is restored, the levels of *lecA-lux* expression are significantly reduced, suggesting that *lecA* could modulate its own expression. However, *lecA* expression is not restored to the levels observed using the $lecA'_{(18\ codons)}$-*lux* and $lecA_{(+1)}$-*lux* fusions (still 20 times higher) (Fig. 24). This can be explained by the difference in the constructs used to create the *lecA-lux* fusions.

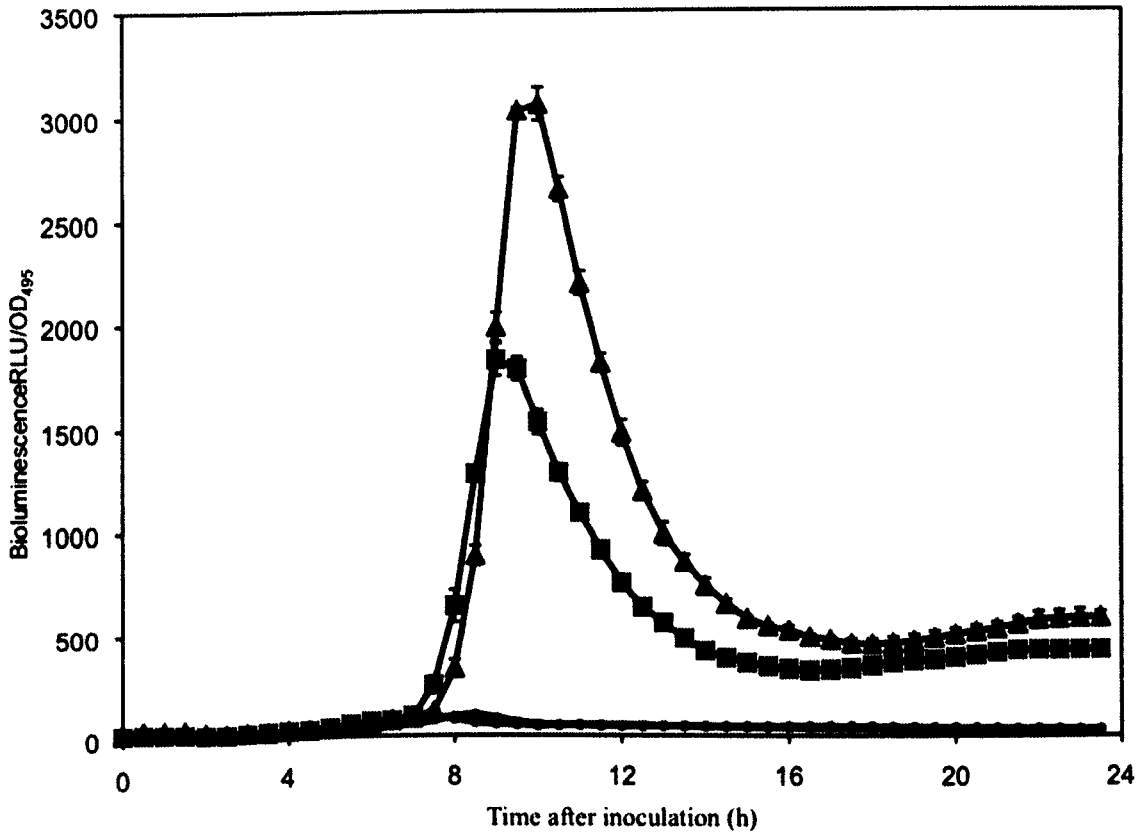


Figure 4-24. Effect of lectin overexpression on *lecA-lux* transcriptional fusion.

The *lecA-lux* fusion created by Winzer *et al* (2000) (triangles) was complemented with the pLECA plasmid overexpressing *lecA* (squares). PAPCP2 ($lecA'_{(18\ codons)}$-*lux*) and PAMM89 ($lecA_{(+1)}$-*lux*) fusions are also shown (line and circles, respectively).

4.3.12.4 Study of lecA post-transcriptional regulation by PQS

These results suggest that PQS activates an important switch in the post-transcriptional control of *lecA* expression. Thus, a *pqsA* mutant (unable to produce this signal molecule) should show a reduction in *lecA* translation. To investigate this, pMM44 (P_{Km} *lecA'-'lacZ*) was introduced into the PAO*pqsA* (Δ*pqsA*) strain. Deletion of *pqsA* resulted in reduced ß-galactosidase activity (Fig. 25A) when compared to the parent strain. Furthermore, addition of 80 μM PQS to this PAO*pqsA* (Δ*pqsA*) increased ß-galactosidase activity up to two folds, confirming the positive post-transcriptional control of *lecA* expression by PQS (Fig. 25B).

As a negative control, the pMM11 (P_{Km} *lacZ'-'lacZ*) plasmid was used. This plasmid contained the *lacZ* coding sequence and its 5' UTR, under the transcriptional control of P_{Km} constitutive promoter. pMM11 was transformed into the wild type and the PAO1*pqsA* background. The addition of PQS did not have an effect in the ß-galactosidase activity (data not shown).

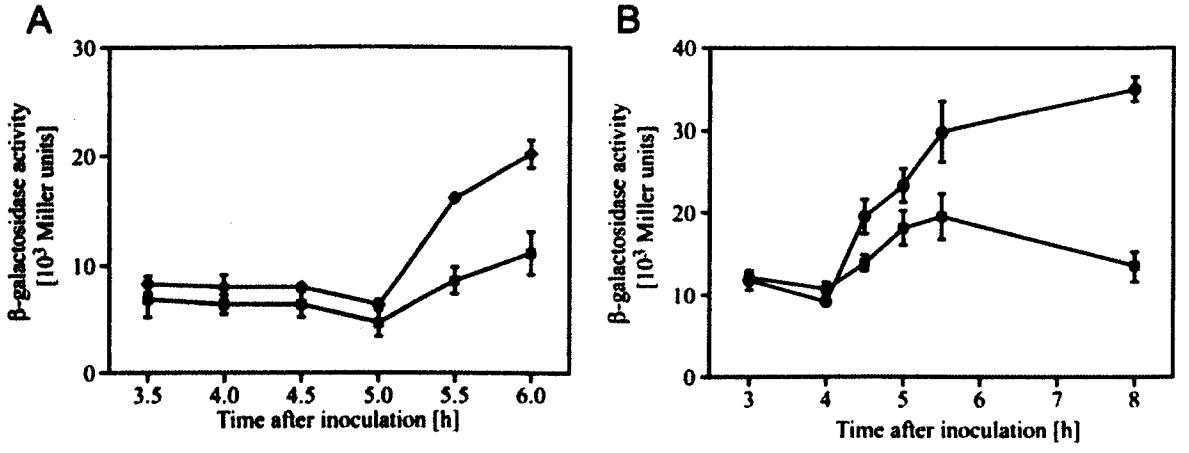


Figure 4-25. Effect of PQS on a P_{Km}-*lecA'-'lacZ* translational reporter fusion.

(A) pMM44 carrying the translational reporter fusion was introduced in the wild type *P. aeruginosa* PAO1 (diamonds) and its Δ*pqsA* derivative (squares). ß-galactosidase activity (in Miller Units) was followed in time. (B) As before, using the strain ΔpqsA/pMM44. The medium was supplemented with either methanol (squares) or PQS (80 μM) (circles).

4.3.12.5 PQS post-transcriptional action on lecA, through rsmZ and rsmY.

PQS seems to act positively on *lecA* post-transcriptional regulation, and in opposition with the negative regulation exerted by RsmA. The suggested model was that PQS induced the transcription of the regulatory sRNAs (RsmY and RsmZ), which would in turn titrate out RsmA. The concentration of free RsmA in the cell would decrease, preventing the action of this protein on *lecA* mRNA. Indeed, a deletion of either *rsmY* or *rsmZ* in the Δ*pqsA* background, PAKR101 (Δ*pqsA/rsmY*) and PAKR102 (Δ*pqsA/rsmZ*), abolished PQS post-transcriptional regulation of the translational reporter carried by pMM44 (Fig. 26).

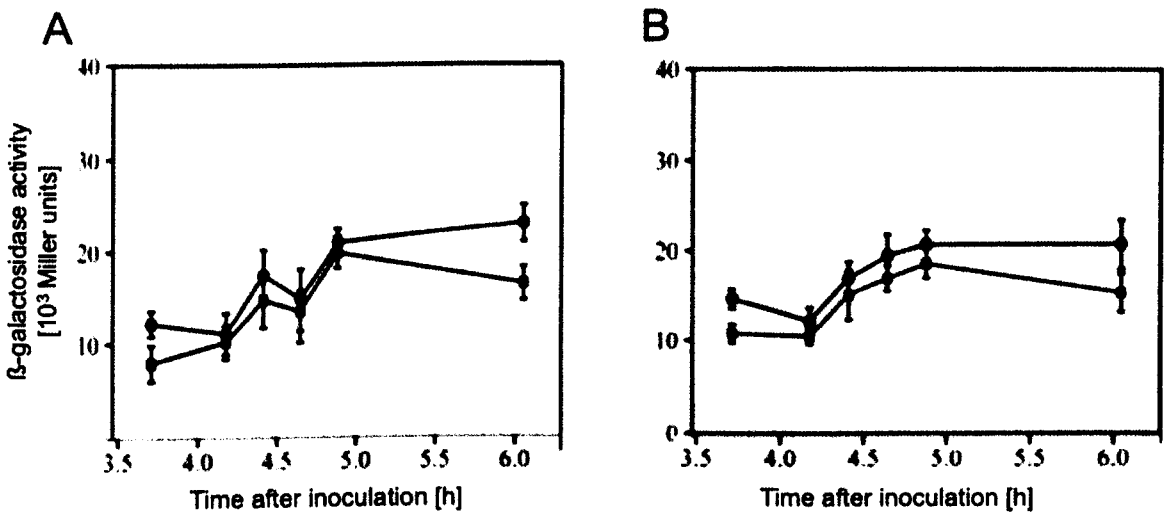


Figure 4-26. Effect of PQS on a P_{Km}-*lecA'-'lacZ* translational reporter fusion in the Δ*rsmZ* and Δ*rsmY* background.

pMM44 carrying the translational reporter fusion was introduced in the double (A) PAKR102 (Δ*pqsA/rsmZ)* or (B) PAKR101 (Δ*pqsA/rsmY)* mutant strain. ß-galactosidase activity (in Miller Units) was followed in time after the addition of PQS (circles) or methanol solvent control (squares).

4.3.12.6 PQS post-transcriptional action on swarming is not dependent on rsmZ and rsmY.

To carry out a more general analysis of PQS action on phenotypes regulated by RsmA, it was decided to investigate if PQS was acting on swarming through the induction of *rsmZ* and *rsmY* expression. The effect of PQS addition on swarming phenotype was tested on the double mutants PAKR101 (Δ*pqsA/rsmY*) and PAKR102 (Δ*pqsA/rsmZ*) background. In both cases, PQS is still able to inhibit swarming. Hence, its effect appears to be unrelated to its modulation of the Rsm/Gac system, and is due to a mechanism that remains to be elucidated (Fig. 27).

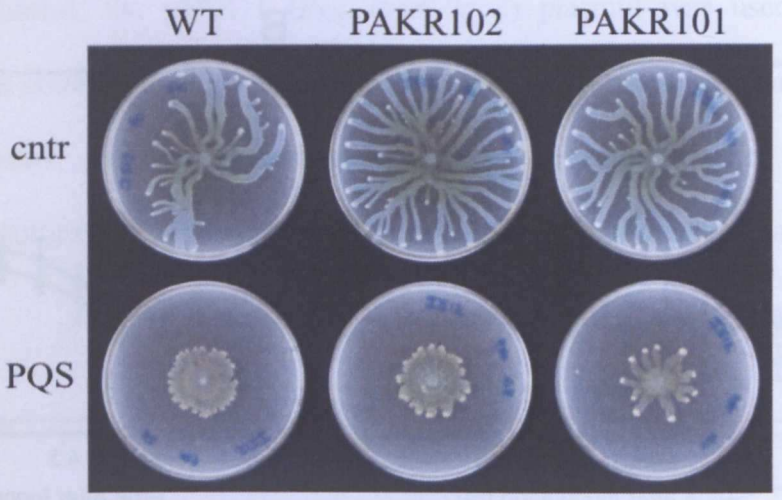


Figure 4-27. Effect of PQS on swarming in the wild type, the double PAKR101 (Δ*pqsA*/*rsmY*) and PAKR102 (Δ*pqs*/*rsmZ*) background.

PQS was added to a final concentration of 80 μM to the swarming plates. As a control, 5% (v/v) methanol was added.

4.3.12.7 Epistatic analysis on PQS and rsmA expression

In order to demonstrate that PQS exerts its effect on *lecA* expression through *rsmZ* and not by negatively modulating *rsmA* expression, epistatic analysis were carried out.

Previous microarray analysis conducted on the PQS regulon indicates that PQS does not affect *rsmA* mRNA levels [262, 263]. It was performed a western blot analysis comparing the levels of RsmA produced in the wild type and in the *pqsA* mutant, with the latter being supplemented with 80 μM of PQS. The results indicate that PQS does not affect RsmA protein levels. Thus, its effect on *rsmZ* transcription is not through the modulation of RsmA protein levels (Fig. 28).

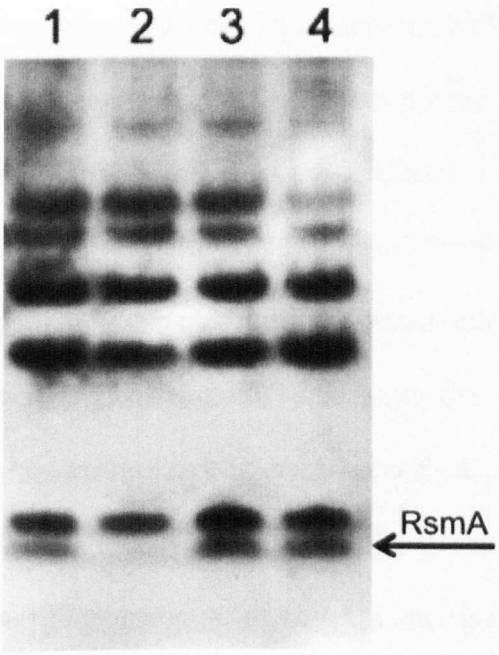


Figure 4-28. Effect of PQS on RsmA protein levels.

SDS-PAGE analysis of *P. aeruginosa* protein extracts derived from (1) PAO1, (2) PASH03, (3) PAOpqsA (*ΔpqsA*) and (4) PAOpqsA with the addition of 80 μM of PQS in the growth media.

4.4 Discussion

The finding that PQS had an effect on *rsmZ-lux* activation led us to the hypothesis that PQS had a role on post-transcriptional regulation. Thus, it was decided to investigate whether PQS signal molecule was involved in the modulation of the Gac/Rsm system.

Although the addition of 10 μM of PQS to the medium did not induce the homologous *rsmZ-lacZ* fusion in *P. fluorescens* CHA0, the effect of this molecule on *P. aeruginosa* had not been previously tested. PQS has been reported to be produced by *P. aeruginosa* at concentrations ranging from 6 to 25 μM [92, 94, 111], hence the concentrations of synthetic PQS that induce the *rsmZ-lux* fusion used in this study (EC_{50} = 13-25 μM depending on the endogenous production of PQS) are well within the physiological range.

Firstly, the possibility that PQS action on *rsmZ-lux* was through its iron chelating potential or through its interaction with the membrane lipids was excluded. Then, the identification of the

regulator responsible for PQS activation of *rsmZ* transcription was carried out. This analysis was among the regulators known to respond to PQS (PqsR, PqsE, RhlR),.

It was also interesting to understand the hierarchy of PQS modulation. Although functional GacS and GacA proteins were shown to be required for more than 90% of the full expression levels of *rsmZ*, PQS was able to double the residual activity of the *rsmZ* promoter in the *gacS*, *gacA* and *ladS* mutants, suggesting that PQS acts independently of and synergistically with the Gac system. In particular, it was observed that LadS was not affecting *rsmZ* transcription. Unfortunately, we still cannot provide an explanation for this data, which disagree with previous experiments. However, it needs to be considered that earlier experiments showing LadS modulation of *rsmZ*, were conducted on the *P. aeruginosa* PAK strain [155]. Therefore, it is possible that the genetic diversity of *P. aeruginosa* PAO1 and PAK may account for the dissimilar *rsmZ* modulation.

Apart from the AQs, it was also evaluated the potential of the AHLs, 3-oxo-C12-HSL and C4-HSL to modulate *rsmZ* expression. The Gac/Rsm system has been previously shown to control QS by modulating the Las and Rhl systems and the amounts of AHLs produced over growth [147, 152]. However, evidence for the inverse regulation, i.e. the post-transcriptional regulation of the Rsm system by the Las and/or Rhl systems, has not been found. Here it was shown that the overexpression of *lasR* or *rhlR* from multicopy plasmids induced the *rsmZ-lux* reporter, whereas deletion of the chromosomal *lasR* or *rhlR* had no significant effect on the induction of the *rsmZ* fusion by PQS. The positive impact of RhlR and LasR overproduction on the activity of the *rsmZ* promoter in the absence of AHLs is interesting. Given that *rsmZ* contains a *lux* box that is required for RhlR, LasR-dependent activation, it is possible that the two LuxR homologues are capable of acting in the absence of AHLs, although this remains to be confirmed.

Interestingly, disruption of the *lux* box also made the reporter unresponsive to exogenous PQS, and it is important to note that the *rsmY* promoter seems to lack this regulatory region.

However, the presence of an intact *lux* box was not required for substantial induction of the *rsmZ* promoter by spent culture extracts. This suggests the existence of additional activating signal molecules, which could act via the Gac two component system on different regions of the promoter.

The effect of RhlR overexpression on *rsmZ-lux* was also analysed in the heterologous *E. coli* system. The results suggest that RhlR acts on *rsmZ* promoter in a direct way, independently from the genetic context, and that this regulation is dependent on the integrity of the *lux* box.

It was also investigated whether PQS was modulating *rsmZ* through the activation of phenazines and in particular of pyocyanin. This metabolite has been suggested to function as the terminal signal of a sequential QS cascade involving the ordered activation of the Las, Rhl and PQS systems. This cascade results in the expression of a sub-set of genes designated as the PYO stimulon [254]. Although pyocyanin was not found to activate *rsmZ* promoter, it was interesting to observe the effect of pH on *rsmZ-lux* fusion.

In *E coli*, the function of BarA and UvrY (the orthologues of GacS and GacA) depends on the pH of the growth medium. Indeed, the expression of *rsmZ* analogues *csrB* and *csrC* is reduced under acidic conditions [264]. Although a similar effect of acidic pH on the expression of *rsmZ* was observed, our results are too preliminary to conclude that pH is involved in *rsmZ* regulation in *P. aeruginosa*. It is also probable that pH modulates the light output of the *lux* reporter by affecting the presence of the substrates required for the bioluminescence.

Finally, it was investigated the role of PQS on post-transcriptional regulation. The proposed hypothesis was that this molecule could indirectly modulate RsmA target genes such as *lecA* by modulating the amount of free RsmA.

RsmA negatively controls the production of the galactophilic lectin LecA and represses a chromosomally-encoded transcriptional *lecA-lux* fusion. This repression can be overcome by supplying PQS to the medium [94, 152].

Firstly, it was demonstrated that RsmA directly binds to the *lecA* mRNA leader sequence. This confirms that RsmA exerts its negative effect on *lecA* at the post-transcriptional level (S. Kehune, PhD thesis). A post-transcriptional P_{Km}-*lecA'-'lacZ* fusion was also shown to be negatively regulated by RsmA overexpression.

Secondly, it was shown that PQS controls *lecA* expression both at the transcriptional (using the $lecA_{+1}$-*lux* transcriptional fusion) and post-transcriptional level (using the P_{Km}-*lecA'-'lacZ* post-transcriptional fusion).

At the transcriptional level, PQS seems to activate the $lecA_{(+1)}$-*lux* independently from RhlR, PqsR and PqsE. This observation is in disagreement with previous data (Fig. 1). Thus, it seems possible that PQS transcriptional activation of *lecA* depends on a yet unidentified regulator, and is unrelated to the *rhl* or to the *pqs* systems.

Curiously, when using the fusion $lecA'_{(18\ codons)}$-*lux*, PqsE seems to act in synergy with PqsA for the repression of the fusion. Indeed, in the strain PAKR85 (Δ*pqsA*/Δ*pqsE* $lecA'_{(18\ codons)}$-*lux*) the fusion is highly active (10 times more than in the other backgrouds). This activation is further increased when adding PQS.

At the post-transcriptional level, it was shown that PQS regulation of *lecA* is mainly through the modulation of *rsmZ* transcription, since *rsmY* transcription is not affected by PQS. However, the presence of a functional *rsmY* gene is required for PQS to fully induce the P_{Km}-*lecA'-'lacZ* translational fusion. As *rsmY* promoter is one of the most highly expressed in *P. aeruginosa* [228], a deletion of this gene will itself have a strong impact on the RmsA-sRNA balance in the cell. This effect cannot be overcome by the induction of *rsmZ* by PQS.

It was also show that PQS does not affect RsmA protein levels, hence the possibility that PQS acts through the negative modulation of *rsmA* expression can be discarded. Our data is confirmed by previous microarrays analyses, which indicate that PQS does not affect *rsmA* mRNA levels [113, 262].

Altogether, the above results strongly support the hypothesis that *rsmZ*, in concert with *rsmY* and in opposition to *rsmA*, is the principal gene mediating the post-transcriptional control of gene expression by PQS. In particular, it was shown that RsmA and PQS have opposite effects on LecA production: whilst RsmA acts as negative regulator acting on *lecA* 5' UTR, PQS has a positive effect, both on its transcription and indirectly on its translation rates (Fig. 27).

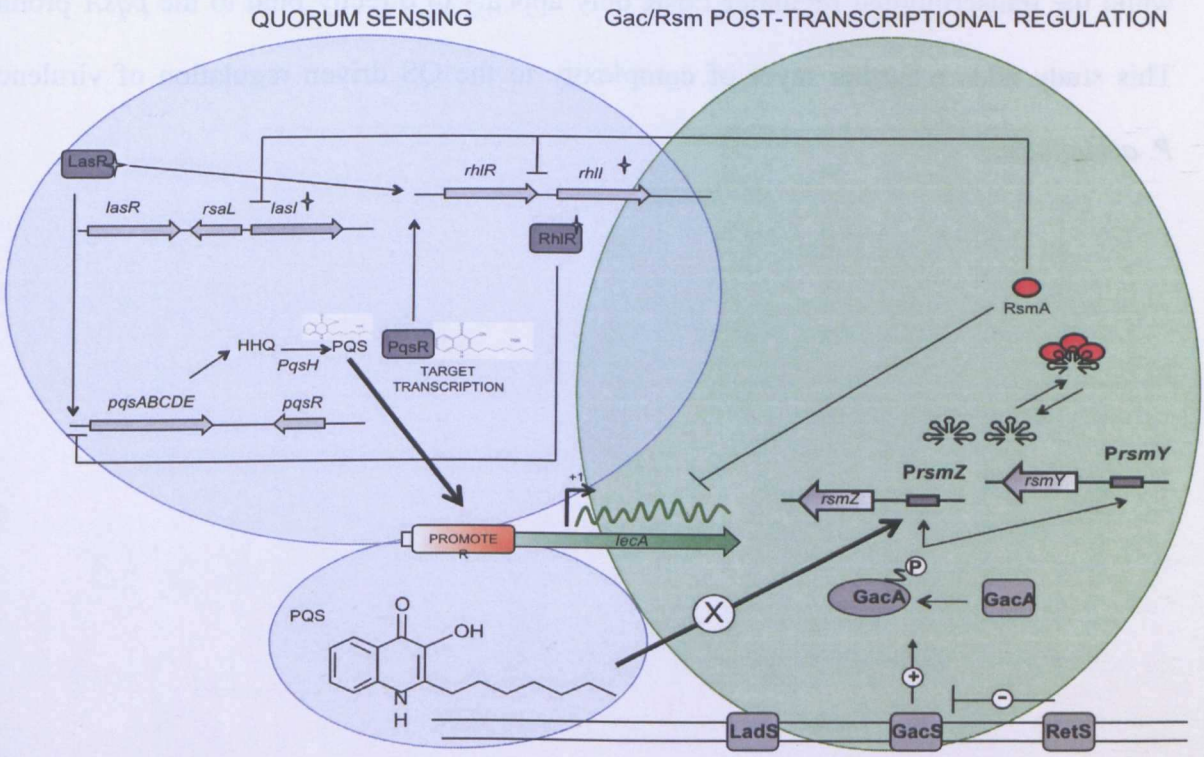


Figure 4-29. Schematic representation of PQS action on both the QS and the Gac/Rsm regulatory systems.

4.5 CONCLUSIONS

In this chapter, we present a better understanding of the global control of virulence in *P. aeruginosa*. The importance of this study resides on the fact that we demonstrate that QS

signal molecule PQS controls global gene expression at the post-transcriptional level. In particular, we showed that the expression of virulence factors such as *lecA* is positively controlled by PQS at the post-transcriptional level, and that this regulation is essentially occurring via RsmZ. Moreover, it was established that *rsmZ* expression is enhanced by PQS in synergy with the Gac system, although the intermediate regulator by which PQS regulates *rsmZ* transcription remains unknown.

To date, the molecular basis by which PQS controls virulence remains obscure. This is mainly because PqsE, an enzyme of the metallo-beta-lactamase superfamily, still remains unknown, while the transcriptional regulator PqsR only appears to directly bind to the *pqsA* promoter. This study adds a further layer of complexity to the QS driven regulation of virulence in *P. aeruginosa.*

Chapter 5

Study of *rsmZ* transcriptional regulation

5 Study of rsmZ transcriptional regulation

5.1 INTRODUCTION

The GacS/GacA two component system is conserved in different bacteria, as it regulates a broad range of virulence and stress response factors [265]. GacS/GacA homologues are also found in *Erwinia* and *Vibrio fisherii* species, VarS/VarA in *Vibrio cholerae*, BarA/UvrY in *E. coli*, BarA/SirA in *Salmonella*, and LetS/LetA in *Legionella*.

When activated by phosphorylation, the GacA response regulator promotes the transcription of small regulatory sRNAs, which in turn bind and reduce the activity of the free post-transcriptional regulators belonging to the RsmA/CsrA family. This two component system modulates the expression of the target genes through the regulation of small regulatory RNAs (sRNAs) [136, 223, 228, 266-268]. To date, there has been no evidence showing the direct transcriptional regulation of target genes by this two component system (independently from the sRNAs).

In *P. aeruginosa*, the GacS/GacA TCS acts exclusively through the modulation of *rsmZ* and *rsmY* expression [226]. Studies conducted on the *rsmZ* and *rsmY* promoter regions allowed for the identification of two conserved regions, which may represent the upstream activation site (UAS) recognized by the phosphorylated form of GacA [202, 228, 269]. Transcriptional *rsmZ* and *rsmY* fusions lacking the UAS region were shown to lose their activity in a similar way to what observed when mutating the GacA response regulator [226]. These results confirmed the importance of the UAS sequences for GacA activation, and suggested that the phosphorylated GacA binds these sequences.

Similar sequences have been found in the promoter region of the regulatory sRNAs of *Vibrio fischeri*, *P. fluorescens* and *L. pneumophila* [202, 268, 269].

The difference in length and structure of *rsmZ* and *rsmY* promoters suggests that they are differently regulated. Indeed, it was recently demonstrated that two members of the H-NS family of global regulators, namely MvaT and MvaU, could bind to an A-T rich region located 174-bp between the UAS and the transcriptional starting site of the *rsmZ* promoter. This A-T rich region is not present in *rsmY* [226]. MvaT and MvaU act as repressors. This explains the higher activity of the *rsmY* promoter when compared to the one of *rsmZ* [228]. On the other hand, the HptB signaling pathway was shown to control exclusively *rsmY* through a yet unidentified sigma factor [160]. In details, HptB is the histidine-containing phosphotransfer module of a hybrid system, and its phosphorylation is activated by three hybrid sensors, namely PA2824, PA1611 and PA1976 [140]. When HptB is active, it represses the PA3346/PA3347 cascade, inhibiting the release of a yet unknown σ factor that promotes *rsmY* transcription [160].

Although the GacS/GacA system acts solely through the modulation of *rsmZ* and *rsmY* transcription, these two sRNAs are regulated in a different way by complex networks (HptB) or regulators (MvaT and MvaU). These further layers of regulation of *rsmZ* and *rsmY* may have evolved to allow for a more precise response to specific environmental conditions.

5.2 AIM OF THE STUDY

In the previous chapter, it was analysed the effect of the PQS signal molecule on *rsmZ* transcriptional activation. The results presented in this chapter are aimed at the identification of new regulators of *rsmZ* and *rsmY* transcription, and in general at the discovery of new pathways modulating this post-transcriptional regulatory system.

For this purpose, three different approaches were used. On one hand, a series of promoter pull down experiments were performed on *rsmZ* and *rsmY* promoters, which allowed for the direct identification of regulators binding *in vitro* to the promoters. On the other hand, we used the

genetic bank and transposon mutagenesis approach to identify new regulators acting *in vivo* on the *rsmZ* promoter.

5.3 RESULTS

5.3.1 Construction and screening of a genetic bank

Three independent genetic banks were generated using the chromosomal DNA from *P. aeruginosa* PAO-L strain (Materials and Methods, section 2.5), and were used to transform the strain *P. aeruginosa rsmZ-lux* (PASH50). A total of 6000 colonies were screened for increased or decreased light production compared to the PASH50 strain with the empty pME6000 plasmid.

If we consider the size of *P. aeruginosa* PAO1 chromosome, the number of clones screened and the average size of the cloned chromosomal fragment, we can calculate that by screening 6000 clones we are covering 94% of the genome, as determined by Poisson's distribution

$$p = 1 - (1 - (l/g))^n,$$

where p is the probability of cloning a particular fragment (which corresponds to the genomic coverage), g is the size of the genome (6270 Kb), l is the average size of the chromosomal fragments cloned (2-4 Kb, l = 3), and n is the number of mutants screened [270, 271].

5.3.2 RhlR and LasR enhance *rsmZ* transcription

Using the genetic bank approach, we identified two clones with enhanced light production. The chromosomal fragment cloned into the plasmids pKR69 and pKR70 contained the *lasR*

and *rhlR* coding regions, respectively (Fig. 1).

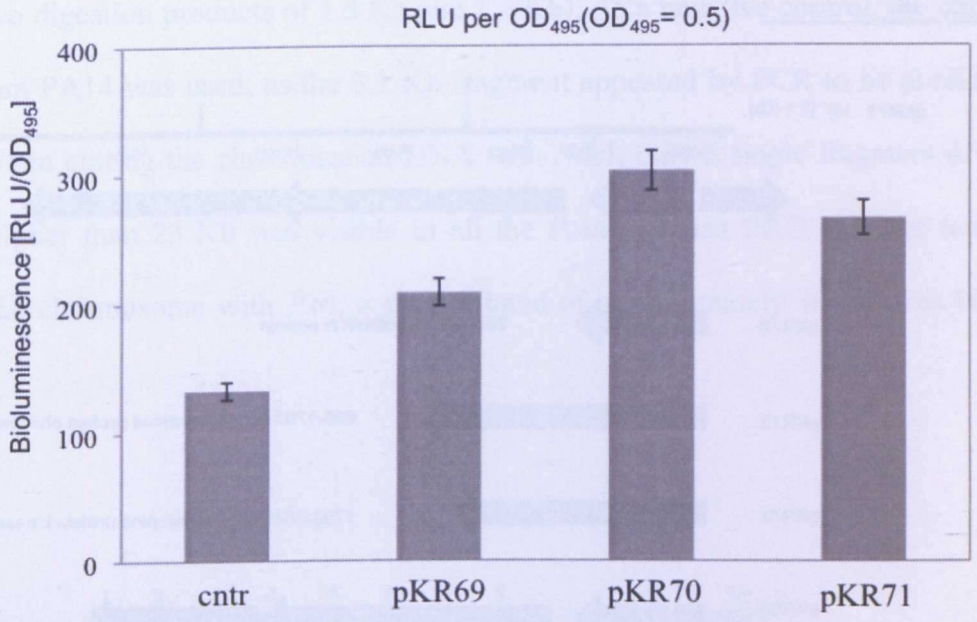


Figure 5-1. Effect of overexpression of genetic bank fragments on *rsmZ* transcription.

Activity of *rsmZ-lux* fusion at OD_{495} = 0.5 in PASH50/pME6000 (*rsmZ-lux*) (cntr), and with the plasmids pKR69 (*lasR*), pKR70 (*rhlR*) and pKR71 (*upf*). Error bars are standard deviation between three independent experiments.

5.3.3 An unknown fragment of PAO1 genome promotes *rsmZ* transcription

A third clone carrying the plasmid pKR71 derived from the genetic bank, was found to greatly enhance *rsmZ* promoter activity (Fig. 1). The plasmid pKR71 was sequenced and analysed using the *P. aeruginosa* Genome Sequence database (http://www.pseudomonas.com). However, the 3.1 Kb fragment cloned in this plasmid did not correspond to any known region. Further analysis was conducted using the BLAST programs available from the NCBI web site (http://www.ncbi.nlm.nih.gov/). The analysis revealed that this fragment had not been previously sequenced and it contains three ORFs potentially coding for a hypothetical protein homologue to the Pmen_2122 from *P. mendocina* ymp, a

novel serine/threonine phosphatase and a novel serine/threonine protein kinase (Fig. 2).

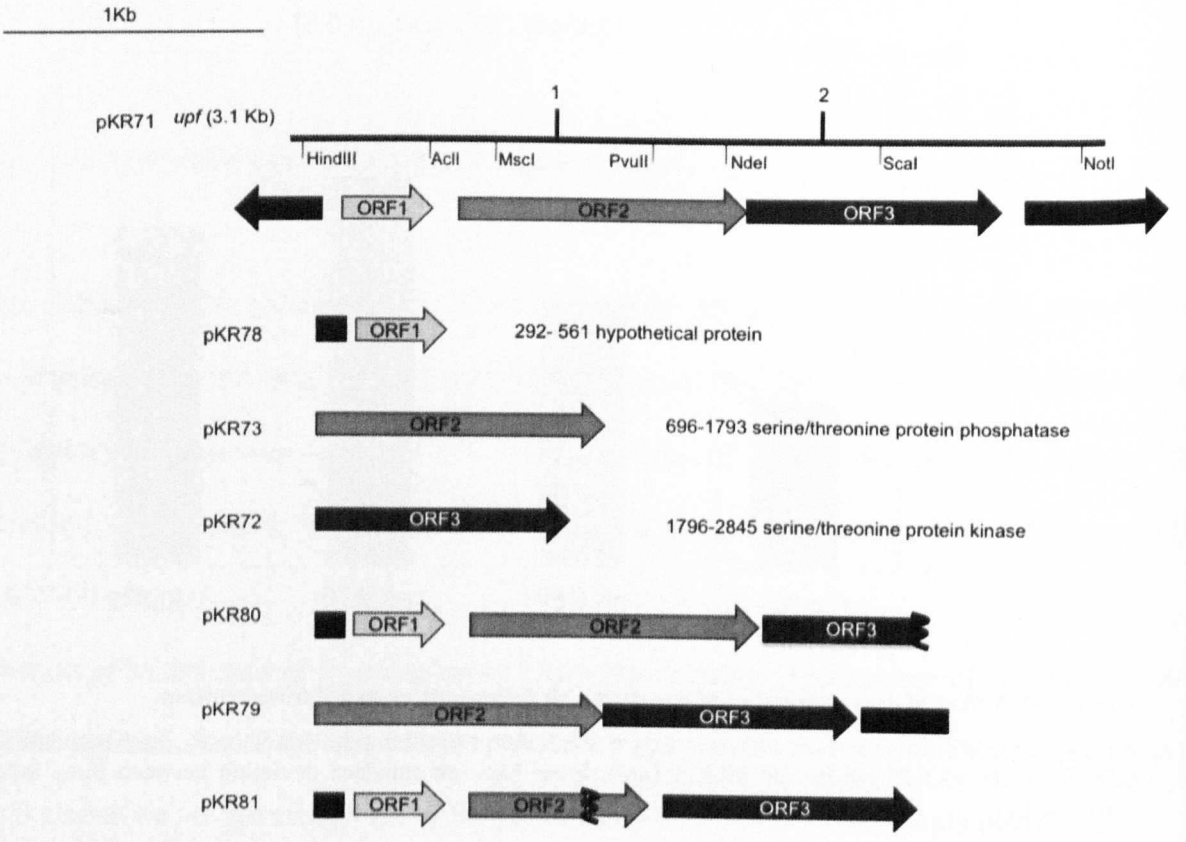


Figure 5-2. Organization of ORFs in *upf* fragment from pKR71.

The *upf* region identified with the genetic bank approach is a 3.1 Kb fragment which carries three open reading frames (ORFs). The second and the third ORF were cloned separately in the expression vector pME6032, giving rise to the plasmids pKR73 and pKR72. Moreover, the first ORF and each combination of the three ORFs were cloned into the pME6000 vector, giving rise to pKR78, pKR80, pKR79 and pKR81.

Further PCR analysis, which was conducted using primers specific to the unknown fragment, revealed that this sequence was present not only in the PAO1-L strain (from which the genetic bank fragments were derived) but also in the strains PAO1-N and PAO1-DSM.

To confirm this, a Southern blot analysis was performed on different strains of *P. aeruginosa*. The 3.1 Kb sequence was excised from pKR71 and used to produce a DIG-labeled probe (Materials and Methods, section 2.9). This probe was tested against the chromosome of the

different PAO1 sublines derived from the laboratory collection (PAO-N, PAO-L, PAO-DSM) (Fig. 3). The chromosomes derived from these strains were extracted and digested with two restriction enzymes, namely *Pst*I (non cutter) and *Nde*I (cuts the cloned fragment once, giving rise to two digestion products of 1.5 Kb and 1.6 Kb). As a negative control, the chromosomal DNA from PA14 was used, as the 3.1 Kb fragment appeared by PCR to be absent from this strain. When cutting the chromosomal DNA with *Nde*I, only a single fragment of molecular weight higher than 23 Kb was visible in all the *P. aeruginosa* PAO sublines tested. When cutting the chromosome with *Pst*I, a shorter band of approximately 10 Kb was visible (Fig. 3).

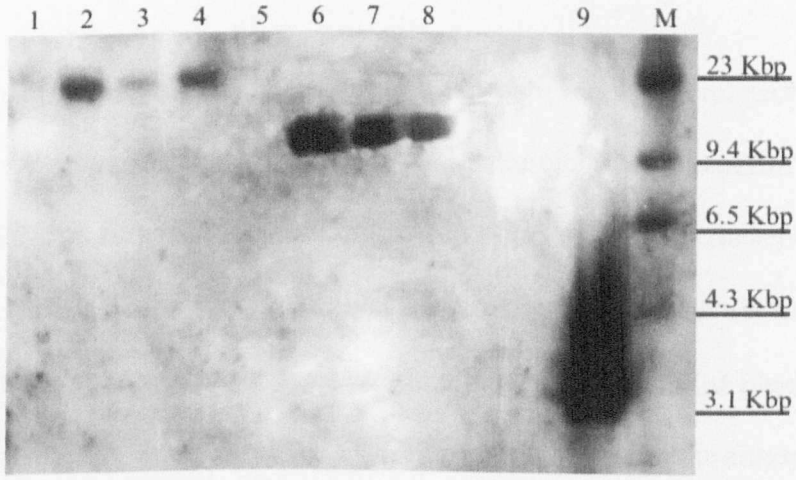


Figure 5-3. Southern blot of genome from different *P. aeruginosa* PAO1 strains, using *upf* frament as a probe.

The *upf* region identified with the genetic bank approach. Lanes 1 and 5 represent bands obtained using the genomic from PA14, while Lanes 2 and 6 represent bands obtained using the genomic from PAO1-N; lanes 3 and 7 represent bands obtained using the genomic from PAO1-DSM; lanes 4 and 8 represent bands obtained using the genomic from PAO1-L. M represents the marker (DNA Molecular Weight Marker II, DIG-labeled, Roche).

Previous studies conducted on the analysis of different *P. aeruginosa* clinical isolates showed that the PAO1 genome is highly diversified among different strains. In the *P. aeruginosa*

genome, there are specific segments called regions of genomic plasticity (RGP), which are characteristic of each strain. The RGP include regions of at least four contiguous ORFs and have been acquired by horizontal transfer. The RGP regions are thought to confer greater diversity to *P. aeruginosa*, enabling it to adapt and to survive in a wide range of niches [272]. In some cases, these DNA segments derive from prophages that are integrated into the chromosome. The organization of the ORFs phosphatase/kinase/kinase observed in the 3.1 Kb fragment is similar to the organization of the genes in some prophages integrated into the bacterial chromosome [273]. For this reason, we decided to call this 3.1 Kb region the Unknown Phage Fragment (*upf*).

5.3.4 The *upf* is not present in the clinical isolates tested

It was interesting to investigate if the *upf* region present in strain PAO1 was also found in *P. aeruginosa* strains in chronicle infection. Therefore, we performed a Southern blot analysis to study the presence of the *upf* region in eight clinical isolates from cystic fibrosis patients. The strains are derived from pediatric (isolates number 1, 4, 7 and 9) and adult patients (isolates number 14, 23, 35 and 73). The *upf* region was not detected in any of these isolates, suggesting that it is strain-specific.

5.3.5 The entire *upf* is needed for the induction of *rsmZ* promoter

To analyse which of the three complete ORFs were responsible for the activation of the *rsmZ-lux* transcriptional fusion, the second and third ORFs, coding for a threonine/serine phosphatase and a kinase respectively, were amplified and then cloned into the inducible expression vector pME6032, giving rise to pKR73 (ORF 2) and pKR72 (ORF 3). The hypothesis was that the kinase or the phosphatase could modulate the GacA/GacS

phospohorilation state. pKR73 and pKR72 were transformed into the PASH50 (*rsmZ-lux*) strain to assess the induction of bioluminescence.

However, neither ORF2 nor ORF3 restored *rsmZ* promoter activation, suggesting that two or more ORFs might cooperate for the activation of *rsmZ* transcription. Thus, we decided to test all the ORFs combinations (Materials and Methods, 2.4.5). The resulting vectors are pKR78 (1 ORF), pKR80 (1 and 2 ORFs), pKR79 (2 and 3 ORFs) and pKR81 (1 and 3 ORFs) (Fig. 2). These vectors were transformed into PASH50 and tested for *rsmZ-lux* activation. None of the ORFs combination tested was capable of acting on *rsmZ* transcription, suggesting that the entire *upf* was required for the activation of *rsmZ*.

5.3.6 The *upf* acts on *rsmZ* independently from GacA and GacS

We were interested in understanding whether the *upf* region was acting on *rsmZ* activation in a GacA or GacS dependent way. However, when transforming the pKR71 plasmid into the strains PAKR29 (Δ*gacA rsmZ-lux*) or PAKR40 (Δ*gacS rsmZ-lux*), we could still observe an induction of *rsmZ* transcription by *upf*, suggesting that this activation is GacA/GacS independent (Fig. 4). Although *upf* region codes for a serine/threonine phosphatase/kinase, its effect is not due to a modulation of the GacA or GacS phosphorylation. Further studies have to be performed in order to understand if the *upf* fragment modifies the phosphorilation state of another component of this TCS system, such as RetS or LadS.

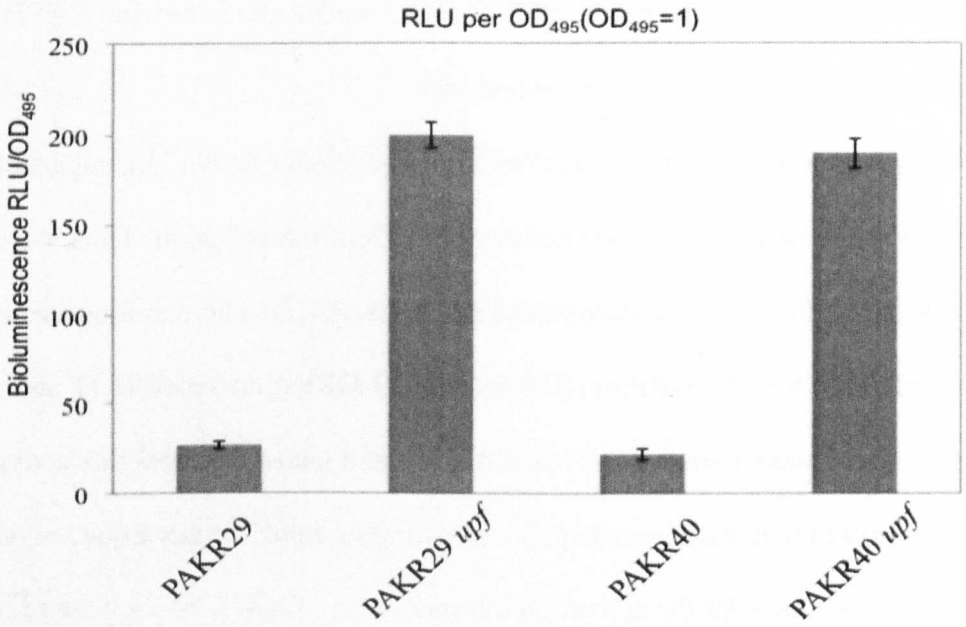


Figure 5-4. Impact of *upf* overexpression on *rsmZ-lux* reporter fusion in the *gacA* and *gacS* mutant background.

The vector pKR71 carrying the *upf* fragment was introduced in the PAKR29 (Δ*gacA rsmZ-lux*) and PAKR40 (Δ*gacS rsmZ-lux*) strains. The maximal promoter activity is defined as the maximum relative light units (RLU) per optical density of the culture at 495 nm (OD_{495}) obtained over the duration of the experiment. As a control, the empty pME6000 vector was introduced into the PAKR29 and PAKR40 strains. The error bars are standard deviation between three independent experiments.

5.3.7 Screening of a transposon library for mutants with altered *rsmZ-lux* expression

The results obtained using the genetic bank approach prompted us to develop a transposon library in the PASH50 strain (*rsmZ-lux*) to screen for colonies with enhanced or reduced bioluminescence phenotype. For this purpose, 10 random transposition experiments were carried out (derived from 10 different conjugations between PASH50 and the strain containing the transposon delivery vector pLM1). A total of 4000 transposon mutants derived from PASH50 (*rsmZ-lux*) were screened for altered bioluminescence. The statistical probability to inactivate a given gene in the strain PAO1 (5570 genes) is equal to $P= 1- (1-\underline{f})^N$, where $\underline{f}$ represents the genomic fraction (1/5570) and N is the number of screened clones [270]. This equation is valid if we assume that the probability of a transposon insertion is

identical for each gene. Thus, after 4000 clones were screened we statistically covered 50% of the genome. The site of mini-Tn5 insertion was identified by a plasmid rescue technique (Materials and Methods, section 2.5) [196].

5.3.8 *wspF* mutant enhances *rsmZ* transcription

After screening for 4000 colonies, it was identified one clone (PAKR26) exhibiting increased bioluminescence compared to PASH50. The flanking regions of the transposon, corresponding to the interrupted gene, were recovered and sequenced. The transposon had inserted into the *wspF* coding region.

WspF is a methyl esterase that reduces the activity of the WspR c-di-GMP cyclase [274, 275]. When knocking out *wspF*, WspR is always active, and the intracellular concentration of c-di-GMP in the cell increases. This mutation causes a very distinct phenotype characterized by a super wrinkled colony morphology[275]. The effect of a *wspF* mutation on c-di-GMP levels was confirmed by LC/MS and by phenotypic analyses (M. Messina, PhD Thesis).

To be certain that the effect on *rsmZ* activation was due to the *wspF* mutation and not to a polar effect, an in-frame *wspF* mutant PAKR50 (Δ*wspF rsmZ-lux*) was contructed. The activation of *rsmZ* in this strain was two fold more active compared to PASH50, confirming the result obtained with the transposon mutagenesis (Fig. 5).

It was investigated whether the effect observed on *rsmZ* expression was due to the alteration of c-di-GMP levels. For this reason, it was tested whether the *rsmZ* activation levels could be restored by decreasing the concentration of c-di-GMP in the cell. For this purpose, we overexpressed the phosphodiesterase PA2567 in the PAKR50 (Δ*wspF rsmZ-lux*) background. Phosphodiesterases (PDEs) are known to hydrolyse c-di-GMP, thus decreasing the intracellular concentration of this signal molecule. The plasmid expressing PA2567 (pMM-

B10) was transformed into PAKR50 (Δ*wspF rsmZ-lux*). Interestingly, the overexrpessed PA2567 strain showed reduced activity of the *rsmZ-lux* compared to the levels obtained with PASH50 (*rsmZ-lux*) (Fig. 5). Thus, the mutation on the *wspF* gene causes the activation of *rsmZ* transcription by acting on the levels c-di-GMP in the cell.

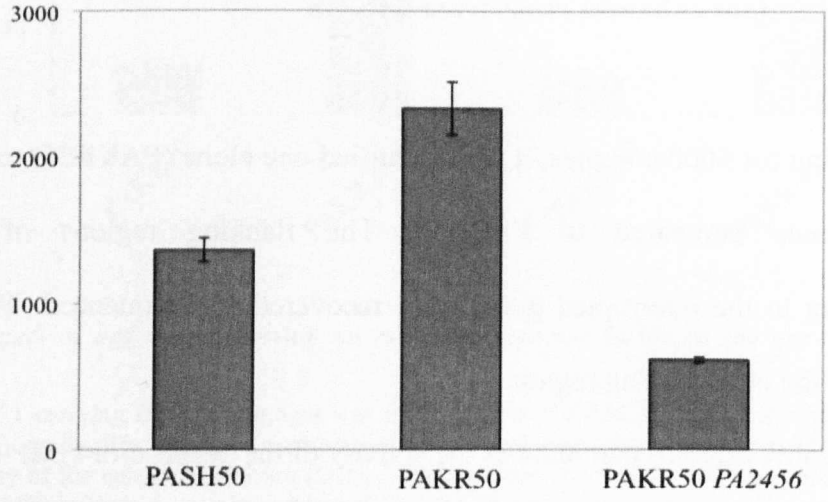


Figure 5-5. Effect of c-di-GMP level alteration on *rsmZ-lux* fusion.

The bioluminescence of PASH50 (*rsmZ-lux*) and PAKR50 (Δ*wspF rsmZ-lux*) when overexpressing PA2567. The measurements were obtained at OD_{495} = 0.4. Error bars are standard deviation between three independent experiments.

Finally, it was interesting to study if c-di-GMP was able (through the modulation of *rsmZ*) to act on final target genes controlled by the Gac/Rsm post-transcriptional regulatory system. For this reason, a translational fusion *lecA'-'mCherry* was constructed, giving rise to pMM43 (M. Messina, PhD Thesis). This plasmid was then transformed into the PA2567 inducible strain PASH51 (Materials and Methods, section 2.4.2). By inducing (1 mM IPTG) PASH51, we promoted the transcription of PA2567, thus decreasing the levels of c-di-GMP in the cell. On the other hand, in non-inducing conditions (no IPTG) pASH51 is a Δ*PA2567*. Hence, we expect higher levels of c-di-GMP in the cell.

Our hypothesis was that PASH51/pMM43 in inducing conditions (low c-di-GMP levels) would have low levels of *rsmZ* transcript and, thus, low levels of *lecA* tranlation. In this case,

RsmA would be mostly in its free form (not titrated by *rsmZ*), able to inhibit *lecA* translation. This situation would be reversed when PASH51/pMM43 was grown in non-inducing conditions. The results obtained using the *lecA'-'mCherry* fusion confirmed this hypothesis (Fig. 6).

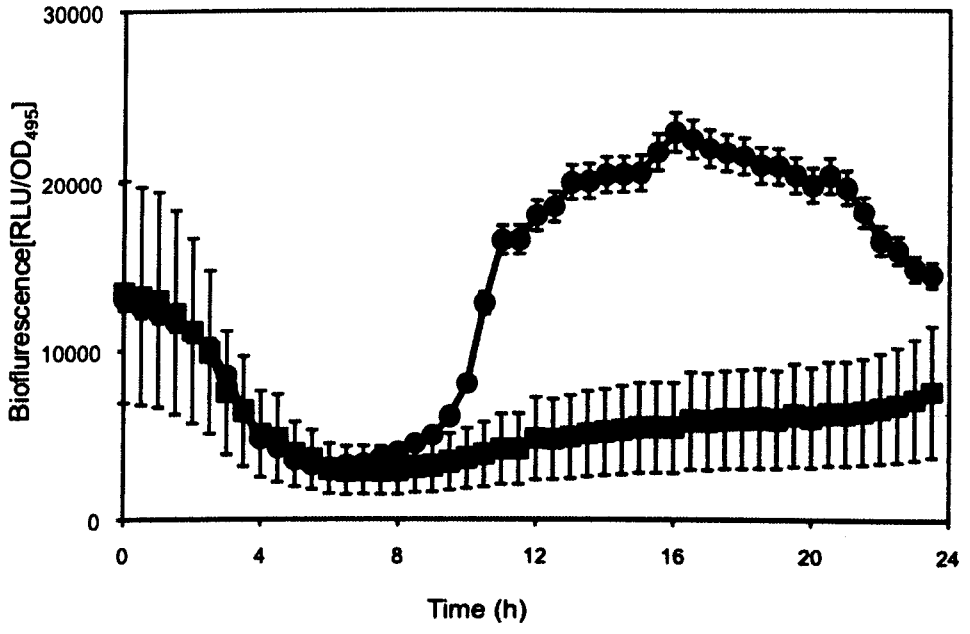


Figure 5-6. Effect of PA2567 overexpression on *lecA'-'mCherry* fusion.

Comparison of the fluorescence derived from the strain PASH51 (PA2567 inducible strain) carrying the pMM43 plasmid (*lecA'-'mCherry*) when overexpressing PA2567 (IPTG 1mM, squares) and in the PA2567 mutant conditions (no IPTG, circles). Error bars are standard deviation between three independent experiments.

5.3.9 Promoter pull-down

We were also interested in conducting an in *vitro* study of *rsmZ* and *rsmY* regulation using the promoter pull-down technique. This method allows for the identification of regulators (present in a protein extract) able to bind to a biotinylated (BIO) promoter DNA fragment that has been previously mobilized on a streptavidin column. After an elution step, the proteins bound to the promoter can be recovered and analysed by SDS-page. The bands corresponding

to the proteins can be then cut and analysed using MALDI-TOF analysis. This analysis gives us a prediction of a protein identity. The P-score is the probability that the observed match is a random event. Protein scores greater than 78 are considered significant ($p<0.05$).

5.3.10 Promoter pull down with the *rsmZ*-BIO promoter region

The promoter pull down experiment on the *rsmZ*-BIO or the *rsmZ**-BIO (mutated in the *lux*-box) promoters were conducted using proteins from crude cell extracts from *P. aeruginosa* PAO1-N cultures collected at different time points in the growth curve (OD_{600} 0.5, 1 and 2.5). When necessary, different signal molecules such as agmatine (the AguR binding molecule, refer to section 5.3.11), PQS and AHLs were added to the growth media.

To amplify the *rsmZ* or the *rsmZ** promoter fragments we used the RVPrsmZ and the FWBIOPrsmZ primers. The latter was biotinylated at the 5' end (Sigma). The SDS-PAGE bands derived from the promoter pull-down experiment using crude extracts collected at different growth phases (middle exponential, end exponential and stationary phase) were differing only in intensity. At OD_{600} of 1 (when the *rsmZ* promoter is highly induced) some bands became more visible. The addition of PQS (80 μM), 3-oxo-C12-HSL (10 μM) and C4-HSL (10μM) signal molecules to the growth media previous to the protein extraction did not significantly change the band pattern (data not shown).

Here, we present the results obtained using the *rsmZ*-BIO and *rsmZ**-BIO promoters and protein crude extracts derived from coltures of *P. aeruginosa* (OD_{600} = 2.5). Both promoters used produced the same protein band pattern. Nine bands were identified (Fig. 7). From these, only eight gave a high MALDI-TOF P score, as described below (Tab. 1).

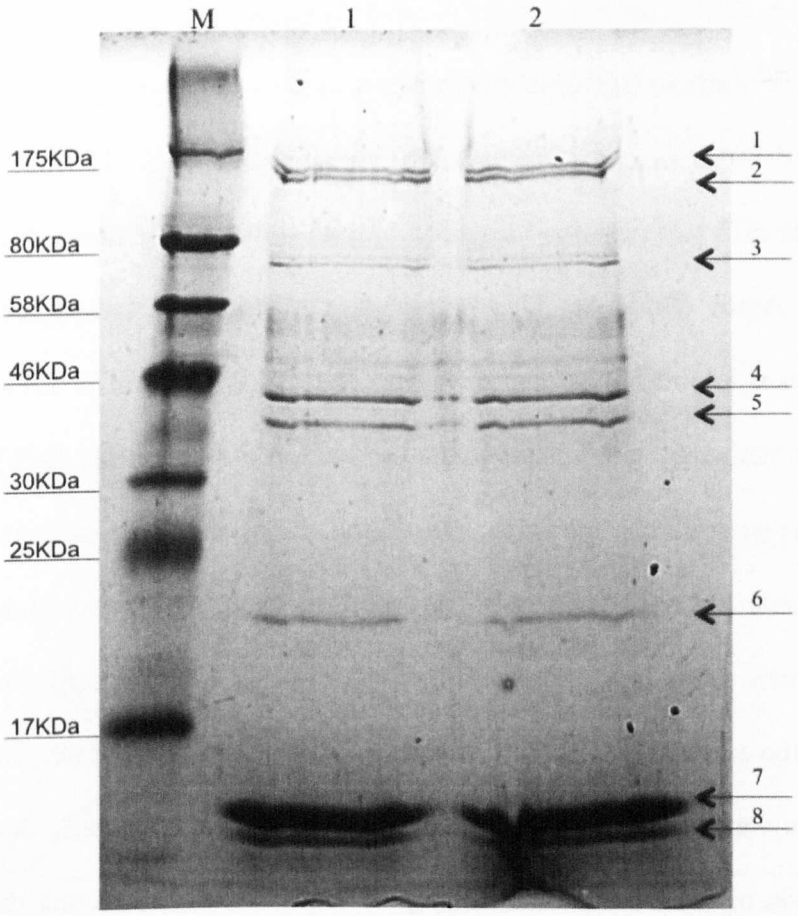


Figure 5-7. SDS-PAGE bands derived from promoter pull-down experiment using protein extracts from PAO1 cell lysate and *rsmZ*BIO.

Bands derived using the cell lysate from PAO1, and the *rsmZ* promoter (1) or the *rsmZ** promoter (*lux-box* mutated) (2). Seven bands were analysed by MALDI-TOF. M protein marker (Promega).

	Gene	Protein description	P value
Band 1	PA4270	RNA polymerase ß-subunit	130
Band 2	PA0779	ATP-dependent Lon protease	98
Band 3	PA3831	Leucyl aminopeptidase PepA	192
Band 4	PA4238	DNA-directed RNA polymerase α-subunit RpoA	133
Band 5	PA0294	Transcriptional regulator AguR	153
Band 6	PA4232	Single-strand DNA binding protein	96
Band 7	PA4315	Transcriptional regulator MvaT	84
Band 8	PA2667	Transcriptional regulator MvaU	103

Table 1. Protein from PAO wild type, found to bind to *rsmZ*-BIO promoter in the pull down experiment

5.3.11 AguR binds to the *rsmZ* promoter

Amongst the different proteins identified by pull-down experiments, we were able to identify the protein AguR (Band 5). This regulator is a transcriptional repressor that binds to the *aguBA* operon inhibiting its transcription. The *aguBA* operon is involved in the agmatine biosynthetic pathway, which leads to the production of polyamines [276].

Polyamines (spermidine, spermine, putrescine, cadaverine) are ubiquitous molecules whose role in bacteria pathogenesis has been recently proven. AguR was found to interact with the *rsmZ* promoter from late exponential phase through to stationary phase. Moreover, when incubating the extracellular protein extracts with the *rsmZ** promoter (*lux*-box altered), AguR was still recovered in the pull down experiment. This result suggests that AguR does not bind to the *lux*-box of the *rsmZ* promoter (Fig. 7). Furthermore, extracting the crude proteins from *P. aeruginosa* cultures grown at different temperatures (37° C and 42° C) and in different media (LB and CAA) did not alter AguR binding to *rsmZ*-BIO.

The binding of AguR to *aguBA* operon is antagonized by agmatine, the precursor of polyamines [276]. This is a typical negative feedback loop: when the concentration of polyamines are low in the cell, agmatine binds to AguR impairing its binding to the *aguBA* operon. This causes an increase in the synthesis of the polyamines. It was investigated the possibility that AguR binding to *rsmZ* could be inhibited by the addition of agmatine. However, when this compound was added (1 mM) to the growth media prior to the protein extraction, the band corresponding to AguR was still appearing in the SDS-PAGE.

5.3.12 AguR overexpression shifts *rsmZ* transcription to a later growth phase

To study the effect of the overexpression of *aguR* on *rsmZ* transcription pKR14 was

contructed. This plasmid contains the *aguR* inducible gene (plasmid derived from pME6032). This construct was inserted into the PASH50 (*rsmZ-lux*) strain. When *aguR* was overexpressed (1 mM IPTG), the expression of *rsmZ* was shifted from the exponential to stationary phase of growth (Fig. 8). Interestingly, AguR has been shown to bind to a specific *agu*-box, which is not present in the *rsmZ* promoter [276].

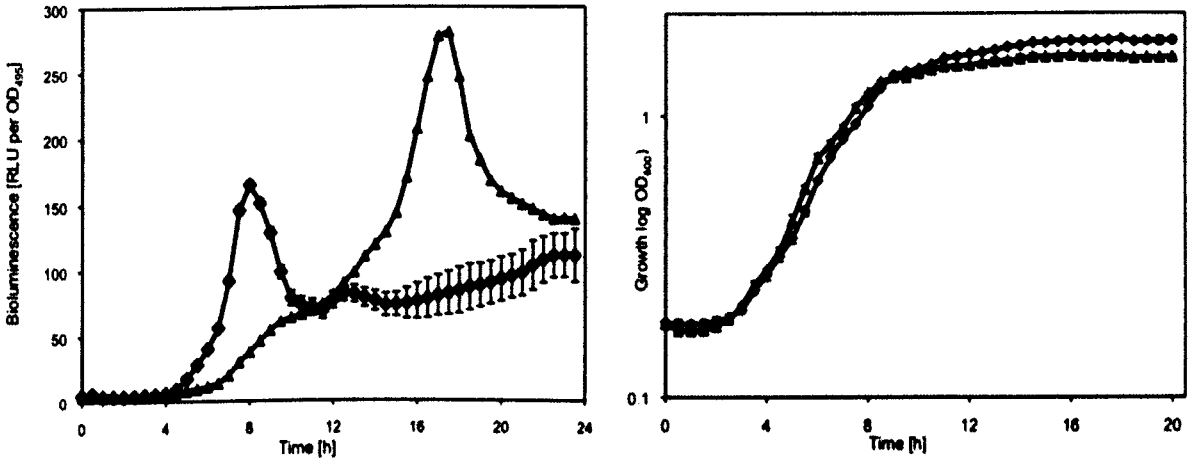


Figure 5-8. Effect of *aguR* overexpression on *rsmZ-lux* transcriptional fusion and on growth.

Bioluminescence (left graph) and growth (right graph) of the PASH50 (*rsmZ-lux*) strain carrying the *aguR* inducible vector pKR14. No IPTG added (diamonds); IPTG added (triangles). Error bars are standard deviation between three independent experiments.

5.3.13 Effect of an *aguR* mutation on *rsmZ* transcription and on several phenotypes

To examine the impact of AguR on the Gac/Rsm cascade, an in-frame *aguR* mutant was contructed harboring the *rsmZ-lux* fusion (PAKR56). The transcription of *rsmZ* in the PAKR56 strain was subjected to a modest increase (by 10 %) compared to the activity of the *rsmZ-lux* fusion in the PASH50 strain. The effect of *aguR* mutation was not as significant as expected, suggesting that, in the conditions tested, AguR was not affecting *rsmZ* transcription. For this reason, it was decided to test the PAKR56 strain for different phenotypes such as swarming, biofilm, growth and antibiotic resistance. No significant phenotype difference

between the mutant and the wild type strain was observed.

5.3.14 Agmatine negatively affects *rsmZ* transcription

Due to the importance of the agmatine molecule for the modulation of AguR activity [276], we decided to investigate if this molecule and its polyamine derivatives, namely spermine and spermidine, were capable of preventing AguR modulation of *rsmZ*. Adding agmatine to the PASH50 (*rsmZ-lux*) strain caused a reduction of *rsmZ* activity (Fig. 9). This effect was not observed in the PAKR56 (Δ*aguR rsmZ-lux*) background, suggesting that agmatine binding to AguR increases its capability to repress *rsmZ* transcription. On the other hand, spermine and spermidine did not have any effect on *rsmZ* transcription (data not shown).

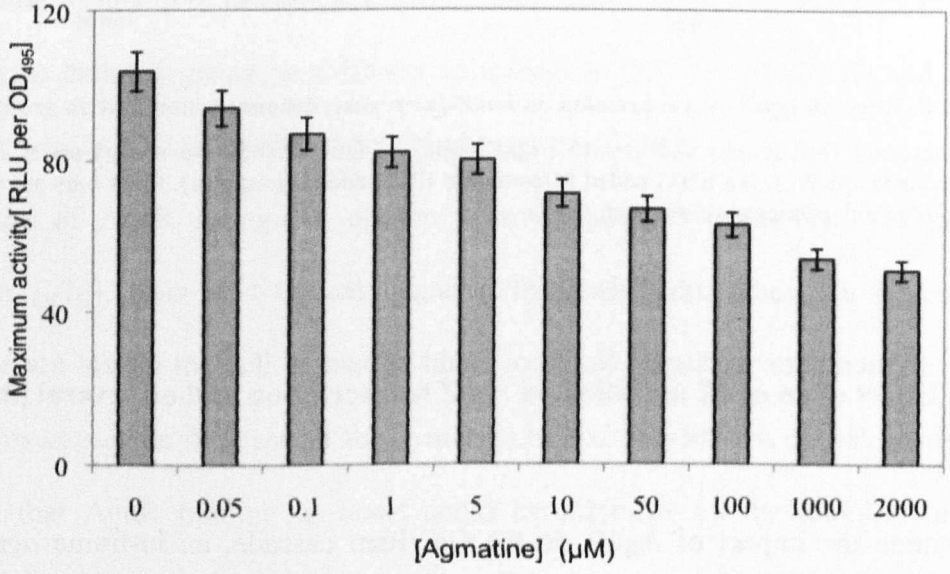


Figure 5-9. Effect of agmatine addition on *rsmZ-lux* transcriptional fusion.

Maximal activation of the *rsmZ-lux* fusion in the wild type after the addition of increasing concentrations of agmatine. Maximal promoter activity is defined as the maximum relative light units (RLU) per optical density of the culture at 495 nm (OD_{495}) obtained for the duration of the experiment. Error bars are the standard deviation between three independent experiments.

5.3.15 AguR purification

The finding of AguR binding to the *rsmZ* promoter is very interesting. To our knowledge, there are no studies demonstrating the direct binding of a regulator to the *rsmZ* promoter region. In order to demonstrate AguR direct interaction with the *rsmZ* promoter, we decided to purify AguR and to conduct DNA mobility shift analysis. AguR was cloned in the pET18b+ vector resulting in pETaguR (the resulting protein carries a 6-histidine tag at the C-terminal). The plasmid was then introduced into *E. coli* BL21 (pLysS) for overexpression. AguR was always found in the insoluble fraction (Material and Methods 2.6). To purify AguR in its soluble form, we added 1 mM of agmatine to the induction culture, corresponding to the physiological concentration found in *P. aeruginosa* (Fig. 10) [277]. To our knowledge, this is the first time AguR has been purified in the soluble form.

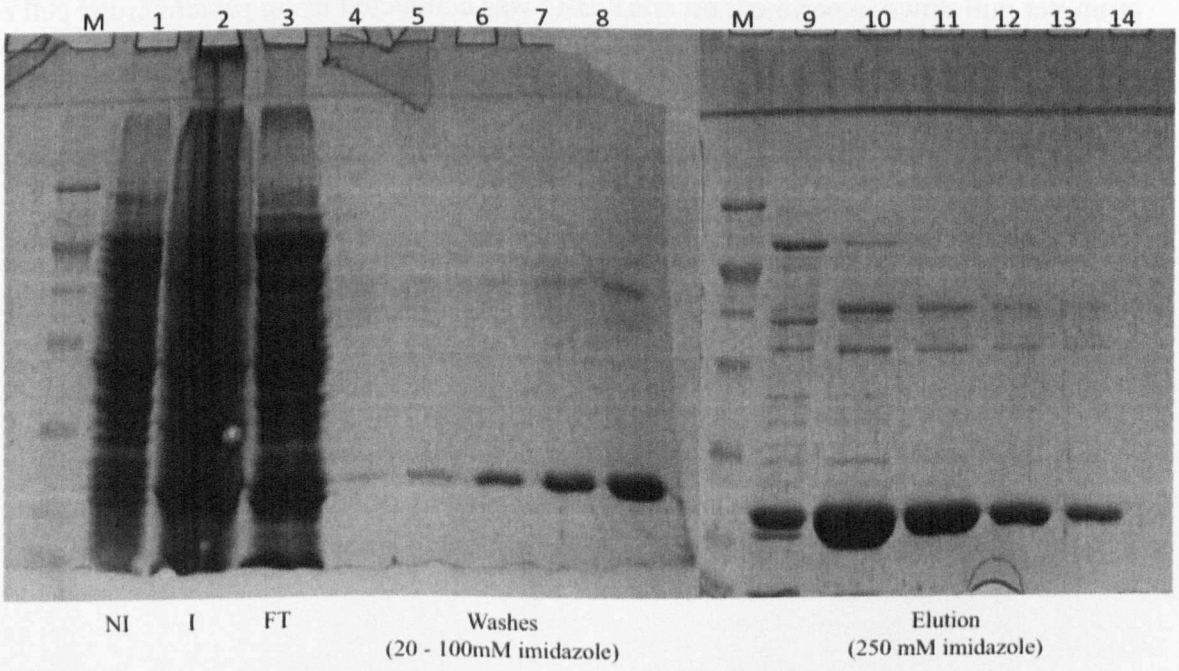


Figure 5-10. Purification of AguR-6His.

SDS gel containing non induced (lane 1) and induced (lane 2) protein extracts from *E. coli* BL21(pLysS) carrying the plasmid for the overexpression of AguR (pETaguR). Lane 3, flow through; from lane 4 to lane 8, washes with 20 to 100mM imidazole. From lane 9 to lane 13, elution using 250 mM imidazole. M, protein marker (Promega).

5.3.16 Electrophoresis mobility shift assay of AguR on the *rsmZ* promoter

Unfortunately, although it was possible to purify AguR in the soluble fraction, it was not possible to observe a shift when incubating the *rsmZ*-DIG probe with increasing concentrations of AguR. At the moment, we are conducting further experiments using rediolabelled *rsmZ*-P^{32} probe.

5.3.17 Promoter pull down using *rsmY*-BIO

The results obtained with the promoter pull down experiments conducted on the *rsmZ* promoter encouraged us to use the same approach for the *rsmY* promoter. The primers FWBIOPrsmY and RVPrsmY were used to amplify the byotinilated promoter of *rsmY*. The promoter pull down experiment on *rsmY*-BIO was conducted using protein crude cell extracts from *P. aeruginosa* PAO1-N coltures collected at different time points in the growth curve (OD_{600} 0.5, 1 and 2.5) (Fig. 11). It was not possible to identify bands corresponding to transcriptional regulators (Tab. 2).

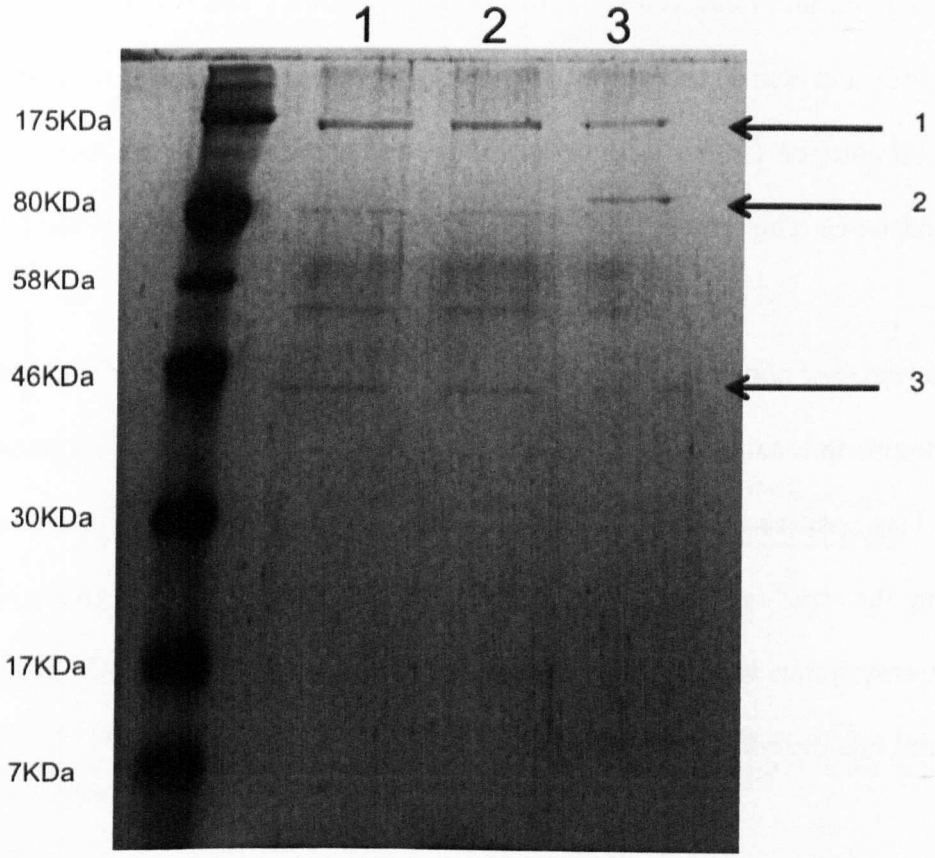


Figure 5-11. SDS gel bands derived from promoter pull-down experiment using PAO1 cell lysate protein extracts and *rsmY*BIO.

Bands derived using the cell lysate from PAO1 OD_{600} of 0.5 (1), OD_{600} of 1 (2) and OD_{600} of 2.5 (3). The *rsmY*-BIO promoter was used for this experiment. M protein marker (Promega).

	Gene	Protein description	P value
Band 1	PA4270	RNA polymerase ß-subunit	102
Band 2	PA0779	ATP-dependent Lon protease	130
Band 3	PA3831	Leucyl aminopeptidase PepA	90

Table 2. Protein from PAO wild type strain to bind to *rsmY*-BIO promoter in the pull down experiment

5.3.18 RsmA mediated control of *rsmZ* transcription through GacA

Previous studies showed that *P. fluorescens* RsmA (and its homologue RsmE) positively

regulates *rsmZ* and *rsmY* transcription. Moreover, RsmA and RsmE were found to stabilize both RsmZ and RsmY transcripts, increasing their half-lifes [278]. Similarly, in *E. coli* the RsmA homologue CsrA positively regulates the expression of the small regulatory RNAs *csrB* and *csrC*. This control was shown to be indirect through CsrS and CsrA modulation [279].

In *P. aeruginosa* numerous studies have shown how RsmA positively controls *rsmZ* and *rsmY* at the transcriptional level, although the mechanism of action is not yet understood (Fig. 12) [151]. This positive regulation of *rsmZ* by RsmA was also observed in our laboratory. When inserting the *rsmZ-lux* fusion in an RsmA inducible strain PASK10 (giving rise to PAKR24), *rsmZ* transcription was strongly enhanced by RsmA induction (1 mM IPTG), while it was repressed in a Δ*rsmA* mutant condition (no IPTG) (Fig. 13).

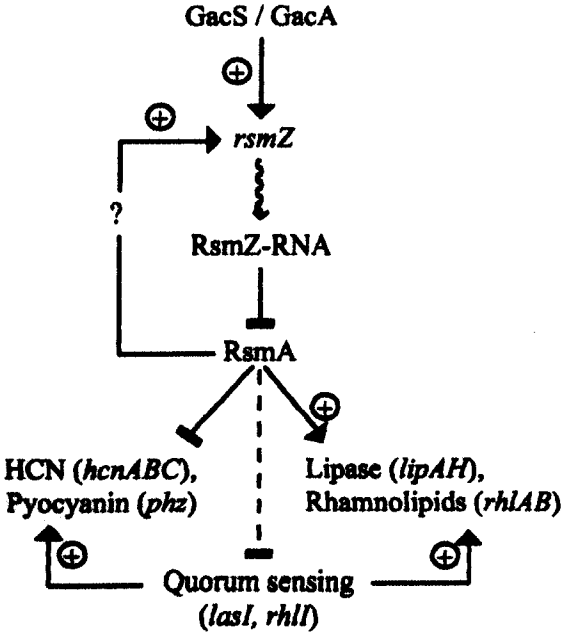


Figure 5-12. Model of the GacA/RsmA signal transduction pathway in *P. aeruginosa* PAO1.

The expression of *rsmZ* is positively regulated both by GacA and RsmA (Figure extracted from Heurlier *et al.*, 2004).

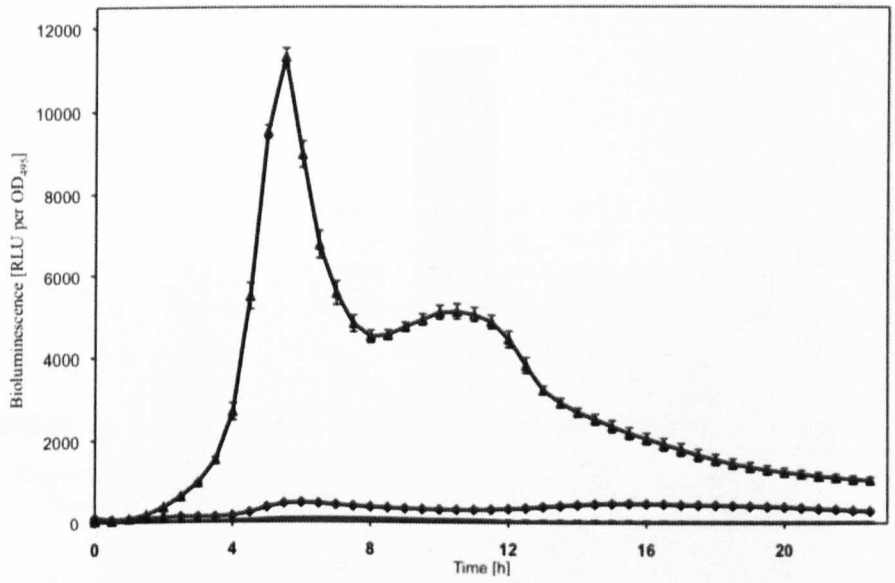


Figure 5-13. Effect of *rsmZ-lux* transcriptional fusion.

Bioluminescence of the *P. aeruginosa* PASH50 (*rsmZ-lux*) (diamonds) compared to the one of PAKR24 (PASK10 *rsmZ-lux*) in absence of IPTG (line) and in inducing conditions (triangles). Error bars are standard deviation between three independent experiments.

Studies conducted on *P. aeruginosa* PAO1 showed that RsmA action on *rsmZ* transcription was independent from GacA activation [151]. The effect of RsmA on the activation of *rsmZ* transcription was not evident in a *gacA* mutant strain. When a plasmid overexpressing RsmA (pSK61) was transformed into the PAKR29 (Δ*gacA rsmZ-lux*) background, *rsmZ* expression could not be enhanced to the levels observed when pSK61 was transformed into the PASH50 (*rsmZ-lux*) strain. Thus, our results suggest that RsmA induction of *rsmZ* expression is GacA dependent (Fig. 14).

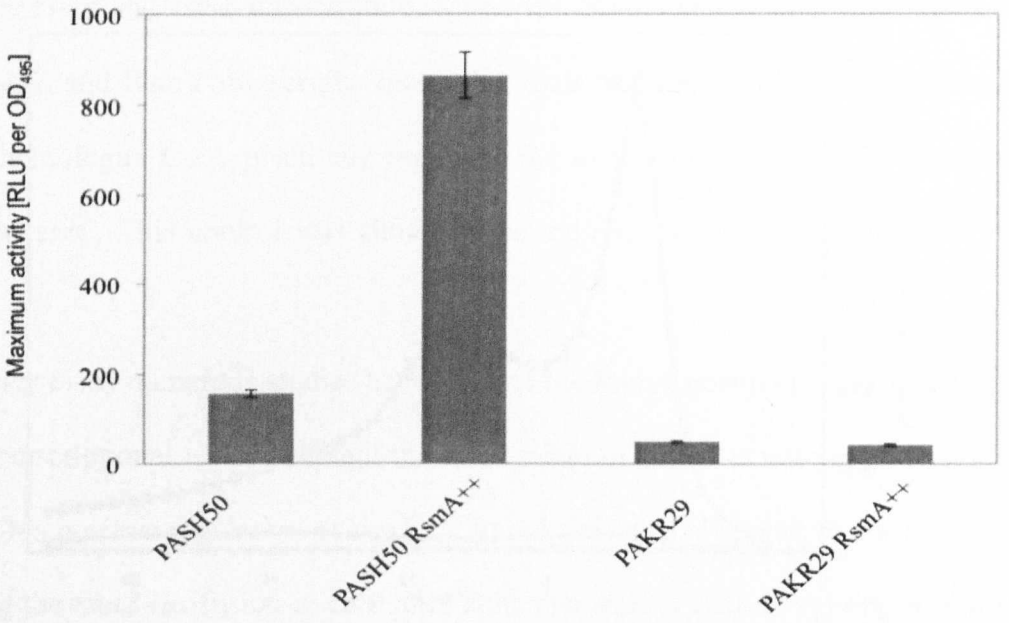


Figure 5-14. Effect of *rsmA* overexpression on *rsmZ-lux* transcriptional fusion.

Maximal activation of the *rsmZ-lux* fusion in the PASH50 (*rsmZ-lux*) and in the PAKR29 (Δ*gacA rsmZ-lux*) background. The strains were either carrying the empty pME6031 plasmid, or the plasmid overexpressing *rsmA* (pSK61). Maximal promoter activity is defined as the maximum relative light units (RLU) per optical density of the culture at 495 nm (OD_{495}). Error bars are standard deviation between three independent experiments.

5.3.19 Small ribosomal subunits linked to RsmA activation of *rsmZ*

The particularly high activation of *rsmZ* by RsmA prompted us to perform a pull-down analysis on *rsmZ*-BIO using the protein crude extracts derived from the PASK10 strain (RsmA inducible mutant). The aim was to identify regulators (induced by RsmA) acting in synergy with GacA for *rsmZ* activation. The protein crude extracts were collected from a non-induced PASK10 culture (RsmA mutant) at OD_{600} 0.5, and from two induced PASK10 cultures (RsmA overexpressing conditions) at different time points of the growth curve (OD_{600} 0.5 and OD_{600} 1).

The extracts derived from PASK10 coltures collected at $OD_{600} = 0.5$ (when *rsmZ-lux* fusion is maximally activated) give rise to two defined bands of approximately 30 KDa (Fig. 15). These bands are not visible when the protein extracts are derived from a non induced

PASK10, or from an induced PASK10 at OD_{600} = 1 (Tab. 3).

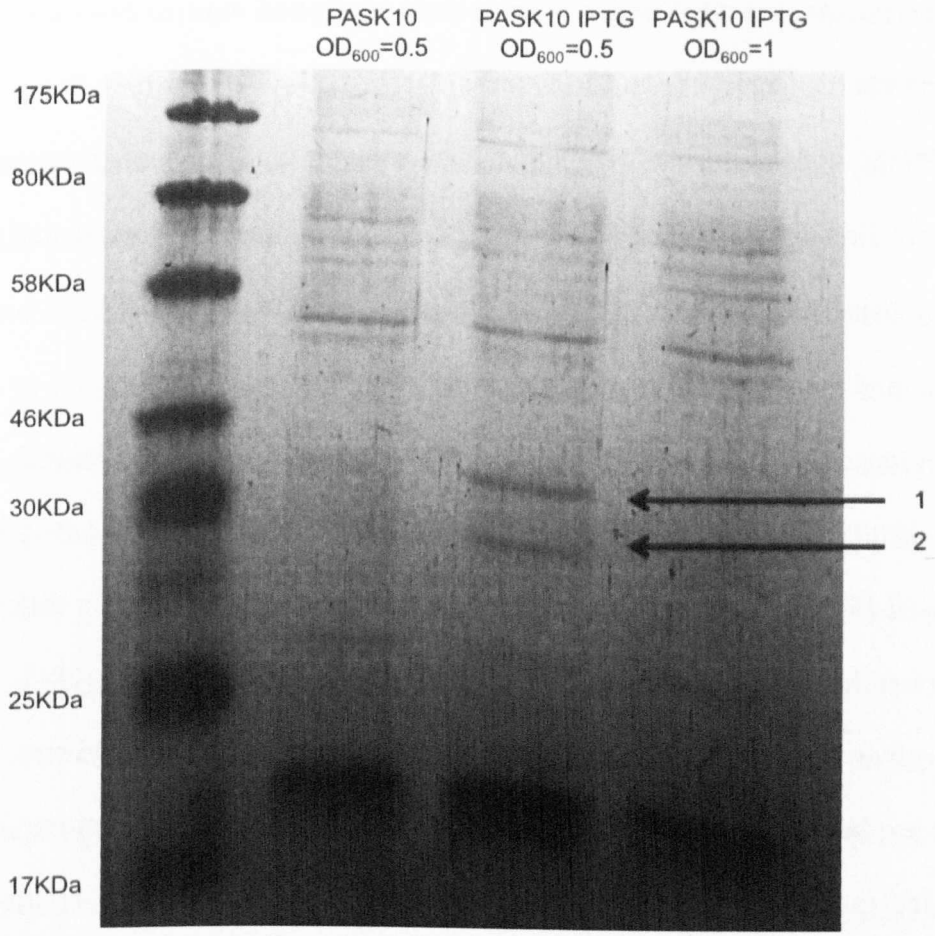


Figure 5-15. SDS-PAGE gel derived from the promoter pull-down experiment using RsmA overexpressing strain PASK10 cell lysate protein extracts and *rsmZ*BIO.

Bands derived using the cell lysate from PASK10 in Δ*rsmA* background (no IPTG added) at OD_{600} = 0.5, and in the *rsmA* overexpressing conditions (1mM IPTG added) at OD_{600} = 0.5 and OD_{600} = 1. M protein marker (Promega).

	Gene	Protein description	P value
Band 1	PA3656	30S ribosomal protein S2 RpsB	146
Band 2	PA4257	30S ribosomal protein S3 RpsC	81

Table 3. Protein from PASK10 induced and non induced cultures, found to bind to *rsmZ*-BIO promoter in the pull down experiment

5.4 DISCUSSION

The experiments presented in this chapter were conducted in order to discover new pathways acting on the regulation of *rsmZ* and *rsmY* transcription.

Through the construction of a genetic bank and through random transposon mutagenesis regulators directly or indirectly controlling *rsmZ-lux* expression were identified. On the other hand, by using the promoter pull down technique, new regulators directly binding *in vitro* to the *rsmZ* and *rsmY* promoters were identified.

P. aeruginosa PAO1 was first isolated in 1954 from a wound in Melbourne, Australia [280], and by 2000 the complete genome of the strain PAO1, of the subline PAO1-UW, had been sequenced [5]. Later studies showed that *P. aeruginosa* strains kept in different laboratories had diversified their genomes, giving rise to diverse sublines [281]. The ability of *P. aeruginosa* to thrive and to adapt quickly to different conditions and environmental niches can be explained by the incredible genomic plasticity of this bacterium. Thus, it was not surprising to find a 3.1 Kb region in the chromosome of our PAO1-N strain, which is not present in the sequenced strain PAO1-UW. This 3.1 Kb fragment was promoting *rsmZ* transcription. Although the mechanism behind this activation has not being elucidated, it appears that the whole 3.1 Kb sequence is essential to induce *rsmZ* expression. Moreover, the mechanism by which the *upf* region acts on *rsmZ* does not seem to depend neither on GacA, nor on GacS, suggesting that the proteins encoded by the *upf* region might promote *rsmZ* transcription through a yet unidentified pathway.

The *upf* fragment was recently found to be part of a genomic island named RGP42 (12 Kb) present in the PAO1-DSM and MPAO strains but absent in the sequenced strain PAO1-UW [281]. Apart from the serine/threonine phosphotransferases, this genomic island contains genes encoding typical phage proteins (integrase and coat proteins) [281]. It is possible that

the serine/threonine phosphotransferases included in the RGP42 region confer to the phage the ability to survival in these bacterial strains. On the other hand, when the bacteria are exposed to different stimuli the prophage region may not be maintained and can be easily lost. This hypothesis has been confirmed by *in vivo* studies conducted on the strains containing the RGP42 region. These bacteria were more virulent than the sequenced PAO1-UW strain [281]. We can thus speculate that the original *P. aeruginosa* PAO1 must have carried RGP42, while the sequenced subline derived from this strain has lost this sequence.

An analysis of the chromosomal DNA derived from cystic fibroses patients colonised with *P. aeruginosa* in the chronic phase of infection showed that the *upf* region is not present. A possible explanation is that in the lungs of CF patients during chronic infection, the *upf* fragment does not contribute to the fitness of the bacteria and is thus lost. On the other hand, under laboratory growth conditions, *upf* enhances the fitness of the strain and is retained in the genome. This hypothesis is supported by the observation that the strain PAO1-DSM (which contais the RGP42) has been shown to have a higher fitness compared to the PAO1-UW (ΔRGP42) when grown in laboratory conditions (LB at 37 °C aerobically) [281]. Finally, we were able to draw a map of the fragments resulting from the restriction analysis (Fig. 16).

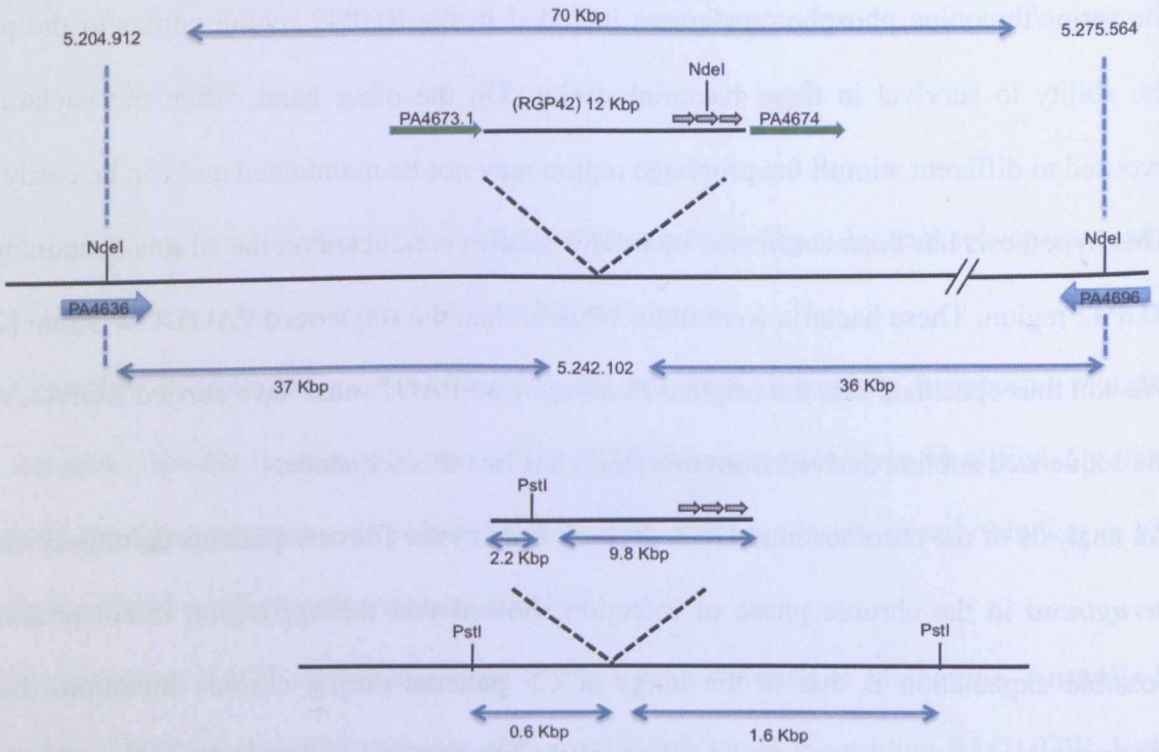


Figure 5-16. Restriction analysis of *upf* fragment digested by *Nde*I and *Pst*I.

In silico analysis conducted on the flanking regions of the sequenced *P. aeruginosa* PAO1 chromosome that encloses the 12 Kb deletion and the *upf* fragment (between PA4673.1 and PA4674) [281]. When digesting the *P. aeruginosa* PAO sublines with *Nde*I, we should observe two fragments of 47 Kb and 71 Kb. On the other hand, when digesting with *Pst*I, only one band of 14 Kb should be visible.

Using the genetic bank approach, we showed that *rsmZ* transcription is enhanced by RhlR and LasR. This regulation was confirmed by the presence of a *lux*-box in the *rsmZ* promoter (Chapter 4).

The transposon approach provided new evidence for the link between the global post-transcriptional regulation system Gac/Rsm and c-di-GMP signaling. C-di-GMP concentrations in the cell have been proven to affect *rsmZ* transcription; higher levels of this signaling molecule (Δ*wspF* background) activate *rsmZ* transcription, whilst low concentrations of c-di-GMP (Δ*wspF* harboring the PDEs protein PA2567) restore *rsmZ* to wild type levels. The finding that c-di-GMP can modulate the target gene *lecA* translation supports the hypothesis of a link between c-di-GMP and post-transcriptional regulation. When

artificially decreasing c-di-GMP levels by overexpressing the PDE PA2567, the transcription of *rsmZ* is reduced. The amount of free RsmA increases, exerting its post-transcriptional regulation on *lecA*. Hence, it affects *lecA'-'mCherry* activity.

By using the promoter pull down approach we have identified AguR, a new regulator that directly binds to the *rsmZ* promoter. In *vivo* studies conducted on a strain overexpressing *aguR* show that this regulator dramatically shifts the *rsmZ* peek of expression later in the growth curve. AguR is responsible for the polyamine biosynthesis in *P. aeruginosa* [282]. Its function seems to be unrelated to the Gac/Rsm post-transcriptional regulation system.

However, recent reports have linked polyamines (spermine, spermidine, putrescine and cadaverine) to bacteria biofilm formation [283] and other important functions, such as virulence and survival in the host. These studies were conducted in several human pathogens such as *Yersinia pestis*, *Vibrio cholerae* and *Pseudomonas mirabilis* [284-286]. Moreover, the modulation of type III secretion system by polyamines in *P. aeruginosa* was recently proven [287]. In *P. aeruginosa* PA14, agmatine can modulate biofilm formation depending on the growth phase [282]. This discovery, combined with our observation that high concentrations of agmatine can affect *rsmZ* transcripion in an AguR dependent way, suggests that in *P. aeruginosa* agmatine could be involved in biofilm regulation through the modulation of *rsmZ* transcription. The hypothesis is that *P. aeruginosa* is able to sense this signal and trigger a response. AguR could be the response regulator modulating the RsmA post-transcriptional regulation system, which affects biofilm and other phenotypes.

In an *aguR* mutant, the activity of the *rsmZ-lux* fusion is not visibly affected. One possible explanation is that, since a wide range of regulators, modulates the Gac/Rsm system, by removing a single regulator, the overall effect on *rsmZ* expression is negligible. Moreover, in the laboratory conditions tested, AguR does not seem to be important for *rsmZ* regulation.

Through the promoter pull down approach we also identified other interesting proteins that interact with the *rsmZ* and the *rsmY* promoters, such as the Lon protease. Recently, the

involvement of the Lon protease in the regulation of the QS signaling system in *P. aeruginosa* has been demonstrated. In particular, this ATP-dependent protease was found to repress the expression of the LasR/LasI system by degrading LasI, the synthethase involved in the production of the signal molecule 3OC12-HSL [288]. The finding that Lon protease was binding to the *rsmZ* and *rsmY* promoters suggests that this protein is involved in the degradation of a regulator, acting on both *rsmZ* and *rsmY*, thus having a general effect on the Gac/Rsm cascade. This hypothesis would be in accordance to the finding that Lon protease is involved in degrading TraR, the LuxR-type protein of *A. tumefaciens* [289].

In addition, the two H-NS family global transcriptional regulators, MvaT and MvaU, were found to bind the *rsmZ* promoter. These are small DNA-binding proteins, which have been shown to be involved in the regulation of QS responses and in biofilm formation in *P. aeruginosa* [208, 246]. MvaT and MvaU associate with regions of the chromosome where the percentage (A+T) is high [290]. The region between the GacA binding site and the *rsmZ* transcriptional start site is highly A+T rich. Hence, it is possible that these two proteins act on the *rsmZ* promoter in this region. Brencic *et al.* recently confirmed this finding, showing that MvaT and MvaU were acting as repressors of the *rsmZ* transcription [226].

We also obtained interesting results from the promoter pull down analysis of the cultures derived from *P. aeruginosa* strain overexpressing RsmA (PASK10 +IPTG). When RsmA expression was induced, two ribosomal proteins were recovered, bound to *rsmZ:* S2 RpsB and S3 RpsC. These proteins are part of the small ribosomal subunit.

There are many studies on the eukaryotic cells showing that the ribosomal protein S3 (RpS3) is linked with transcriptional regulation. Although the ribosome is a protein-synthesizing machine, numerous ribosomal proteins have been shown to have a bifunctional role [291, 292]. In particular, the mammalian RpS3 protein is involved in translation, DNA repair and apoptotic pathways [293, 294]. Studies suggested that S3 has an extraribosomial function in mammalian cells as a regulator of gene expression, providing evidence that the CHOP

transcriptional factor interacts with the S3 ribosomal protein [295]. More recently, the finding that mammalian RpS3 is a key subunit of the transcriptional complex NF-κB [296] implied a possible secondary function of ribosomal proteins in eukaryotes. In accordance with these observations, it can be hypothesized that the S3 ribosomal protein might also be involved in transcriptional regulation in bacteria. In particular, S3 might regulate *rsmZ* transcription in *P. aeruginosa*. The proposed hypothesis is the following: when RsmA is artificially overexpressed (PASK10 +IPTG), it binds to mRNAs in a non-specific way. This causes the ribosomes to stall, thus releasing some subunits. S3 could form a complex with a yet unidentified transcriptional regulator, promoting *rsmZ* transcription. The small regulatory RNA RsmZ would titrate RsmA, allowing the ribosome to proceed with translation. Although there are no studies on mammalian cells showing the impact of RpS2 on translation, the same hypothesis can be applied to this ribosomal protein.

The RsmA effect on *rsmZ* transcription, previously observed in *P. aeruginosa*, has been suggested to be indirectly via GacA. Thus, it was interesting to investigate if the positive regulation of *rsmZ-lux* by RsmA was due to an enhanced production of the signal molecule, which would then activate the GacS/GacA system. However, when extracting extracellular signal molecules from a strain over expressing RsmA, the effect on *rsmZ-lux* activity was similar to the one from extracts derived from the wild type strain. We thus tested the possibility that RsmA could act through GacA. We found that, in the absence of GacA, RsmA was not able of inducing the *rsmZ* promoter, suggesting that the RsmA effect on *rsmZ* is due to the positive effect of RsmA on the *gacA* transcript. This hypothesis is supported by previous studies on CsrA, the homologue of RsmA in *E. coli*, which was found to act as a positive regulator by stabilizing and thus increasing the translation of certain target mRNAs [135]. The fact that the mRNA of *gacA* was found in the microarray analysis conducted on the RNAs copurified with RsmA supports this hypothesis (S. Kuehne PhD Thesis).

5.5 CONCLUSIONS

Using a variety of different approaches, we managed to identify new regulators modulating *rsmZ* and *rsmY* transcription. Some of the regulators found to directly bind to the *rsmZ* promoter, such as AguR, have not been previously linked to the Gac/Rsm pathway. On the other hand, the finding of MvaT and MvaU confirmed previous data.

Other regulators were shown to modulate *rsmZ* expression, such as LasR and RhlR. This provided evidence for the existence of the *lux*-box in the *rsmZ* promoter, which was then found to be important for PQS induction of *rsmZ* transcription (Chapter 4).

Cyclic di-GMP was also found to promote *rsmZ* transcription. This observation may provide us with an important link between this intra-cellular signal molecule and the regulation of biofilm through the Gac/Rsm post-transcriptional pathway.

Finally, these methods allowed us to identify a previously uncharacterized DNA region of *P. aeruginosa* genome (*upf*), whose presence in multicopy activates *rsmZ* transcription. Our results form a platform for further studies on global post-transcriptional regulation by QS and internal signal molecules such as c-di-GMP.

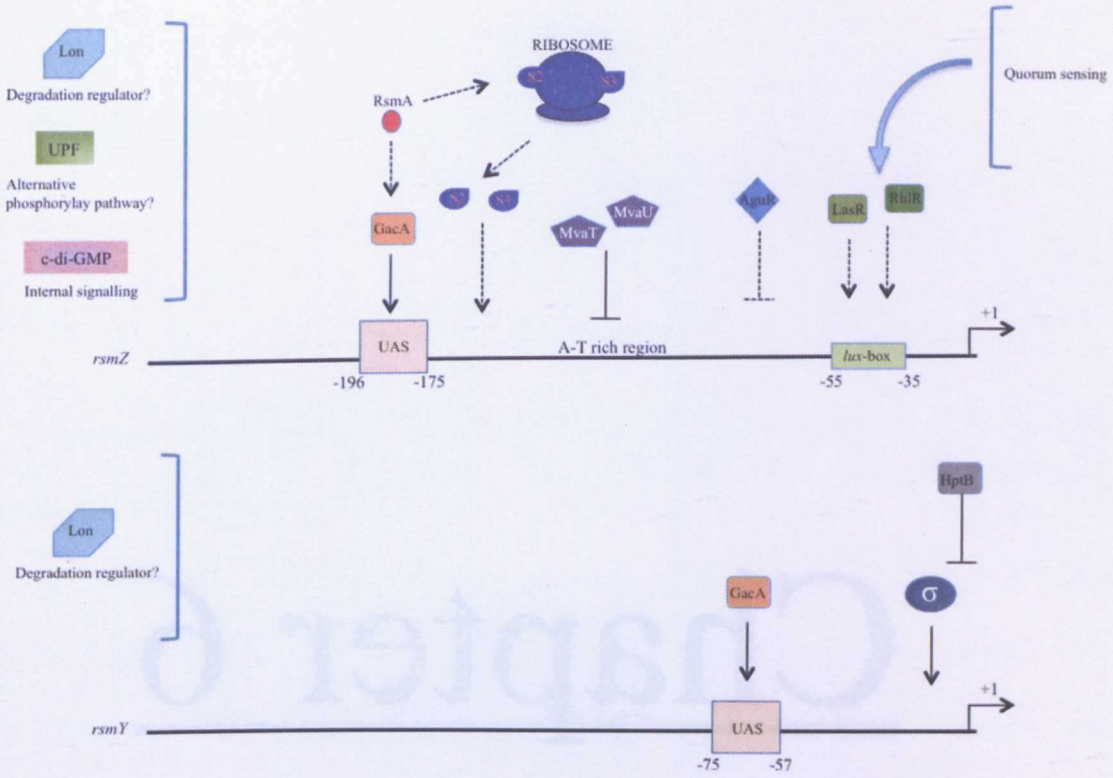


Figure 5-17. Representation of the regulators and pathways involved in *rsmZ* and *rsmY* transcriptional modulation.

Full arrows represent regulators whose interaction with the promoters has already been described. Dashed arrows are the regulators and signal molecules found to be involved in the *rsmZ* and *rsmY* transcriptional regulation. In brackets molecules, proteins or coding sequences that have an effect on *rsmZ*/*rsmY* transcription, through an unknown pathway.

Chapter 6

QS Inhibition by synthetic molecules

6 QS Inhibition by synthetic molecules

6.1 INTRODUCTION

In *P. aeruginosa*, AHL and AQs signal molecules are essential for QS-mediated gene expression of multiple virulence determinants such as secondary metabolites, type III secretion machinery and expression of stationary-phase genes [6, 194]. Further research showed that 3-oxo-C12-HSL and PQS may also function directly as virulence factors by interacting with the host eukaryotic cells modulating inflammation and immune responses. These findings suggest a critical role of these molecules in the pathogenesis of *P. aeruginosa* infection [19, 162, 165, 297, 298]. Further studies showed that 3-oxo-C12-HSL interferes with the eukaryotic immune response by down regulating human lymphocyte proliferation [297] and by attenuating the oxidative burst caused by polymorphonuclear leukocytes (PMNs) [299]. The immune suppressive ability of 3-oxo-C12-HS was further demonstrated by Pritchard *et al* (2005), by successfully administrating this molecule for the treatment of an autoimmune disease on diabetic mice [163, 297].

On the other hand, *in vivo* studies conducted on the activity of higher dosage of 3-oxo-C12-HSL (100 μM) showed that 3-oxo-C12-HSL has pro-inflammatory activity and directly interferes with the eukaryotic transcriptional regulation for the production of interleukin-8. Interleukines are pro-inflammatory cytokines which activate or chemo-attract leukocytes to the site of infection [164, 300]. Moreover, it was shown that high dosage of 3-oxo-C12-HSL promoted apoptosis in macrophages and neutrophils and had vasorelaxant properties on blood vessels [298].

The discordant data presented on the role of 3-oxo-C12-HSL on the immune system can be explained by the different dose of signal molecule used for the studies. However, there is no doubt on the biological activity of this signal molecule in immunological assays.

Further experiments conducted on *P. aeruginosa* showed that PQS is involved in the modulation of the immune system. In particular, PQS was shown to inhibit T cell proliferation [106, 301].

These experiments, together with the fact that both AHL and AQs signal molecules were detected in infected CF sputum at a concentration comparable to the one used in immunological assays *in vitro* [103], support the hypothesis that these molecules function in synergy as immune modulators [11, 94, 103, 114]. During the first stages of the infection, the signal molecules produced allow *P. aeruginosa* to evade from the host immune system. Once a higher cell population density is reached, the QS response is triggered activating the production of virulence factors. This response is coordinated with the wave of activation of the immune system caused by the higher levels of signal molecules produced, sufficient to trigger pro-inflammatory and tissue-damaging determinants. Therefore, the study of QS signal molecule analogues as a way to attenuate virulence and to disrupt the interference with the immune system is crucial for the development of novel therapeutic agents.

As it was illustrated, it is not yet clear how these signal molecules act on the eukaryotic immune system. The most direct way of searching for molecules capable of interfering with the natural QS signal molecules is to produce a range of synthetic analogues so that a single or combination of structural components of the molecule are modified. Using this approach, Chhabra *et al.* (2003) studied the overall effect of 3-oxo-C12-HSL as an immune suppressor. They constructed synthetic 3-oxo-C12-HS analogues, which were altered in their acyl-chain or lactone ring, and found that the optimal bioactivity of the molecule is dependent on an intact homoserine lactone ring with an acyl side-chain of C11 or greater [184].

In view of the fact that far more is known on the mechanism of action of the 3-oxo-C12-HSL on QS system activation through LasR, more specific approaches were used to screen for QSI compounds. QS antagonists were generated by taking the 3-oxo-C12-HSL molecule and altering its ring and acyl-chain structure [302]. In the majority of studies, these analogues failed to have a potent antagonistic effect, or the concentration required to obtain significant inhibition was too high from a therapeutic perspective (in the 100 μM range).

A different approach was to modify natural inhibitors such as the furanones. However, this strategy has gathered little success. Finally, a more general method to search for QS inhibitors was to look for the active compound responsible for the QSI activity from herbal and fungal sources such as carrot, soybean, water lily, tomato, pea seedlings, habanero and garlic [180, 303].

6.2 AIMS OF THIS CHAPTER

The present study was undertaken in order to determine whether 3-oxo-C12-HSL synthetic analogues, previously tested for immune modulatory activity (R. Gopal, PhD thesis), could interfere with LasR-mediated regulation resulting in agonistic or antagonistic effects. For this purpose, two reporter fusions were constructed using the promoters of two genes known to be regulated by LasR: *lasB* and *lasI*.

The finding of one or more molecule(s) with a strong antagonistic effect on LasR activity and a high immune modulator potential would be the first step for the development of new QSI molecules.

6.3 RESULTS

6.3.1 Construction of biosensors to screen for quorum sensing inhibitors compounds

In order to test the ability of compounds to interfere with QS, a series of biosensors were constructed which consist of a transcriptional fusion between the promoter of a QS regulated gene (*lasB* or different portions of the *lasI* promoter) and the *luxABCDE* operon from *Xenorhabdus luminescens* [198]. The background strain for the construction of these reporters was *P. aeruginosa* PAKR94 (Δ*lasI*::Gm^R). By choosing this mutant it could be possible to artificially add a specific concentration of 3-oxo-C12-HSL, which would activate the reporter to half of its maximum activity (K_{ind}). It would be possible to have a clearer understanding of the efficiency of the tested compounds for QS inhibition.

The *lasB-lux* and *lasI-lux* fusions were inserted into the chromosome of PAKR94 in the unique *attB* site of prophage CTX insertion. The sensor strains were called PAKR02 (Δ*lasI*::Gm^R *lasB-lux*), PAKR38 (Δ*lasI*::Gm^R *lasI1-lux*), PAKR46 (Δ*lasI*::Gm^R *lasI2-lux*), PAKR39 (Δ*lasI*::Gm^R *lasI3-lux*), PAKR47 (Δ*lasI*::Gm^R *lasI4-lux*), PAKR66 (Δ*lasI*::Gm^R *lasI5-lux*) and PAKR67 (Δ*lasI*::Gm^R *lasI6-lux*), (Material and Methods, section 2.4.2).

6.3.2 Determination of the K_{ind} for the biosensors

It is known that the biosensor strains do not produce the signal molecule 3-oxo-C12-HSL. The concentration of 3-oxo-C12-HSL at which the activity of the promoter was half of its maximum (K_{ind}) was therefore determined for these strains. The concentration of 3-oxo-C12-HSL would then be used in the competition assay (between QSI compounds and 3-oxo-C12-HSL, for the binding to LasR). In the assay, it was maintained a specific concentration of 3-oxo-C12-HSL (corresponding to the K_{ind}), adding a range of concentrations of the antagonist compound. Using this method, it could be assessed the strength with which the tested molecules would compete for the binding of the regulator.

6.3.2.1 Determination of K_{ind} for the lasB-lux reporter

The ability of the AHL derivatives to act as activators or as inhibitors of LasR was analysed by measuring the activity of the *lasB* promoter, which is transcriptionally regulated by LasR and RhlR [240]. For this purpose, a strain PAKR02 (*lasI*::Gm^R *lasB-lux*) reporter fusion was used. Since the biosensor strain lacks the ability to produce 3-oxo-C12-HSL, due to the disruption of the *lasI* synthase gene, it was firstly necessary to determine the K_{ind} values. For this purpose, a range of 3-oxo-C12-HSL concentrations was tested. The dose response curve for the *lasB-lux* reporter was obtained as follows. The reporter strain was incubated with increasing amounts of 3-oxo-C12-HSL and the maximum peak of activation of the reporter (measured as the bioluminescent activity) was plotted against the logarithm of the concentration of 3-oxo-C12-HSL added (Materials and Methods, 2.12). The K_{ind} for the fusion for 3-oxo-C12-HSL was calculated to be 5 μM (Fig 1).

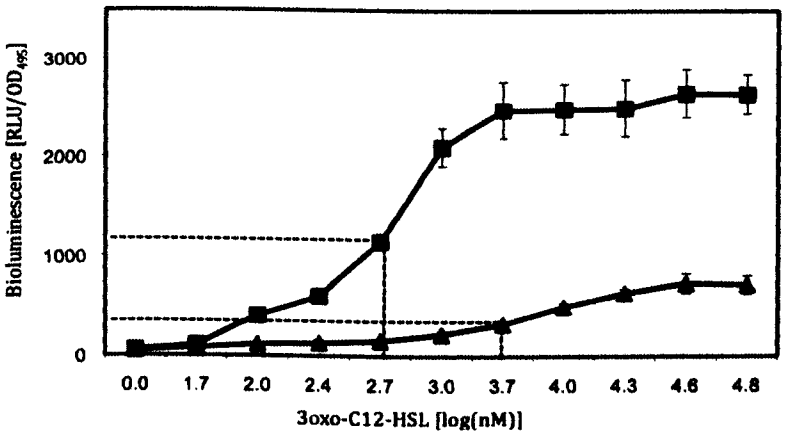


Figure 6-1. Dose response curve to 3-oxo-C12-HSL for *lasI-lux* and *lasB-lux* reporter fusions.

Comparison of the expression of two biosensors in the PAKR02 (Δ*lasI*::Gm^R *lasB-lux)* background (triangles) and PAKR47 (Δ*lasI*::Gm^R *lasI4-lux*) (squares) in the presence of increasing concentration of 3-oxo-C12-HSL. The half maximal expression for PAKR02 and PAKR47 is 350 and 1200 RLU/OD, respectively. The calculated K_{ind} was 5 μM and 0.5 μM 3-oxo-C12-HSL, respectively.

6.3.2.2 Determination of K_{ind} for lasI-lux reporter

In order to characterize the antagonistic potential of the 3-oxo-C12-HSL analogues, we analysed the effect of the most promising molecules on another promoter, which is also regulated by LasR. The *lasI-lux* fusion was inserted into the *P. aeruginosa* PAKR38 strain (Δ*lasI*::Gm^R *lasI-lux*) background. This fusion was first tested for its activation by 3-oxo-C12-HSL (Fig. 2). Unexpectedly, although the *lasI* promoter fusion *lasI-lux* (amplified using the primers PlasIFW1 and PlasIRV1) included the known LasR binding site (*lux*-box) previously determined (Fig. 3), the promoter was not induced by the addition of increasing amounts of 3-oxo-C12-HSL (Fig. 2). However, when constructing the fusion PAKR46 (Δ*lasI*::Gm^R *lasI2-lux*) comprising a larger fragment of *lasI* promoter (amplified using the primers PlasIFW2 and PlasIRV2) and including part of *rsaL* and *lasR*, the fusion was properly induced by 3-oxo-C12-HSL (Fig. 3). Hence, the existence of a previously unidentified region of the *lasI* promoter activated by the LasR-3-oxo-C12-HSL complex seemed possible.

In order to investigate this hypothesis, different *lasI-lux* fusions were constructed to identify the minimum promoter region sequence necessary for the activation by 3-oxo-C12-HSL (Fig. 3). Both the PAKR39 (Δ*lasI*::Gm^R *lasI3-lux*) and the PAKR47 (Δ*lasI*::Gm^R *lasI4-lux*) strains were induced by 3-oxo-C12-HSL as much as PAKR46 (Δ*lasI*::Gm^R *lasI2-lux*), (Fig. 2). These results suggest that there is a specific sequence between -153 bp and -119 bp (between PlasIFW1 and PlasIFW4) or between +15 bp and +54 bp (between PlasIRV1 and PlasIRV2) responsible for *lasI* activation by LasR and 3-oxo-C12-HSL. To investigate this further, two additional fusions were constructed. The PAKR66 (Δ*lasI*::Gm^R *lasI5-lux*) and PAKR67 (Δ*lasI*::Gm^R *lasI6-lux*) fusions were tested for their response to 3-oxo-C12-HSL. While *lasI6-lux* and *lasI1-lux* showed a similar low response to 3-oxo-C12-HSL addition, *lasI5-lux* were highly induced (Fig. 2). However, the levels of *lasI5-lux* activation when adding 3-oxo-C12-HSL were not restored to the ones observed when using *lasI4-lux*.

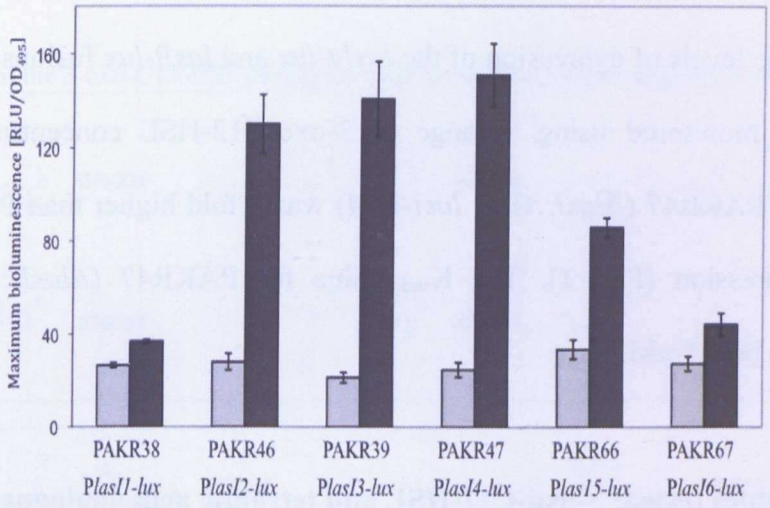


Figure 6-2. Induction of the *lasI* fusion by 3-oxo-C12-HSL.

The *lasI-lux* fusions amplified with different primers respond differently to the addition of the 3-oxo-C12-HSL (20 μM) signal molecule (dark) compared to the addition of the solvent as a control (light gray). Bioluminescence intensity was divided by OD_{495} of cell culture. The highest activity obtained with *lasI4-lux* was set to 100% and all other values are relative. The standard deviation was derived from 3 independent measurements.

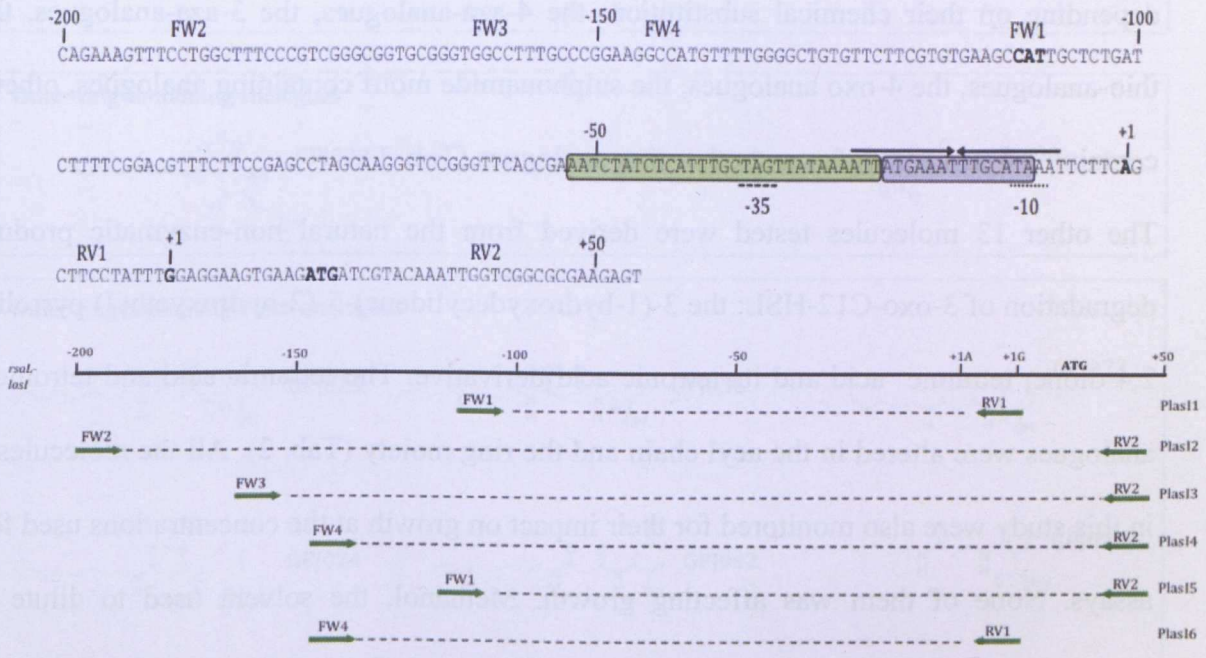


Figure 6-3. The *rsaL-lasI* intergenic region and primers used to amlify different fragments of the *lasI* promoter.

The *lasI* ATG starting codon and the nucleotides complementary to the starting codon of *rsaL* (CAT) are in boldface. The principal *lasI* transcriptional starting site is marked as +1, the G in bold indicates the secondary transcriptional starting site [78]. The 5'-AAnTTATGnAA-3' inverted repeats are indicated by arrows. The potential σ^{70}-dependent -35 and -10 consensus sequences are indicated by dashed lines. The sequence bound by RsaL is purple shaded, while the sequence bound by LasR is green [304]. Schematic representation of the way the different promoter *lasI* fusions were made is presented.

In addition, the levels of expression of the *lasI4-lux* and *lasB-lux* fusions were compared. The fusions were monitored using a range of 3-oxo-C12-HSL concentrations. The maximal expression of PAKR47 (Δ*lasI*::Gm^R *lasI-lux4*) was 4 fold higher than PAKR02 (Δ*lasI*::Gm^R *lasB-lux*) expression (Fig. 1). The K_{ind} value for PAKR47 (Δ*lasI*::Gm^R *lasI-lux4*) was determined to be 0.5 μM.

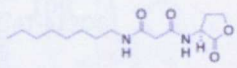
6.3.3 Molecules tested: 3-oxo-C12-HSL and tetramic acid analogues

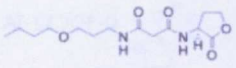
A total of 33 analogues of 3-oxo-C12-HSL were tested at a range of concentrations. From these, 20 were molecules derived from 3-oxo-C12-HSL with alterations on the alkyl chain, the 3-oxo substituent or the heterocyclic ring. These molecules were divided into 7 groups depending on their chemical substitution: the 4-aza-analogues, the 3-aza-analogues, the 3-thio-analogues, the 4-oxo analogues, the sulphonamide motif containing analogues, other ring containing 4-aza analogues and other ring analogues (Tab. 1).

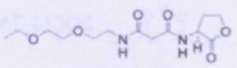
The other 13 molecules tested were derived from the natural non-enzymatic product of degradation of 3-oxo-C12-HSL: the 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl) pyrrolidine-2,4-dione, tetramic acid and its tetronic acid derivative. The tetramic acid and tetronic acid analogues were altered in the acyl chain and the ring moiety (Tab. 2). All the molecules used in this study were also monitored for their impact on growth at the concentrations used for the assays. None of them was affecting growth. Methanol, the solvent used to dilute these molecules (MeOH), showed no inhibitory effect on cell growth or on the *lasB* or *lasI* promoter activity.

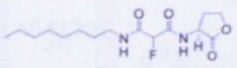
Table 1. Structure of the 3-oxo-C12-HSL analogues used in the agonist and antagonist studies.

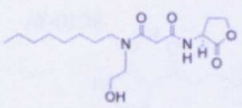
4-Aza analogues


GPJ001

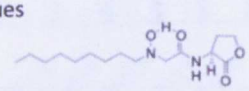

GPJ034

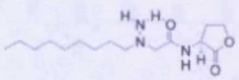

GPJ021


GPJ005

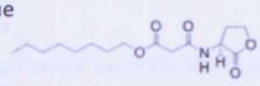

GPJ033

3-Aza analogues

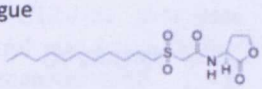

GPJ009


GPJ017

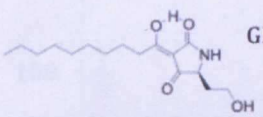
4-Oxo analogue

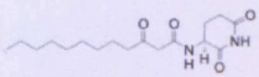

GPJ007

3-Thio analogue

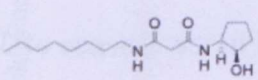

GPJ016

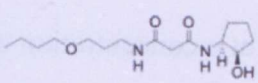
Other ring containing analogues

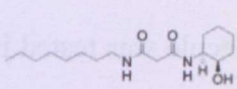

GPJ030

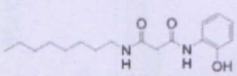

GPJ0004

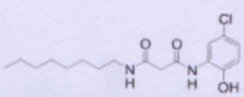
Other ring containing 4-Aza analogues

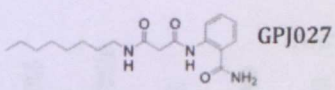

GPJ025

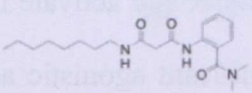

GPJ036

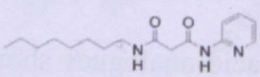

GPJ023


GPJ024


GPJ042


GPJ027


GPJ022


GPJ028

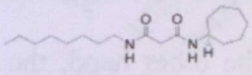
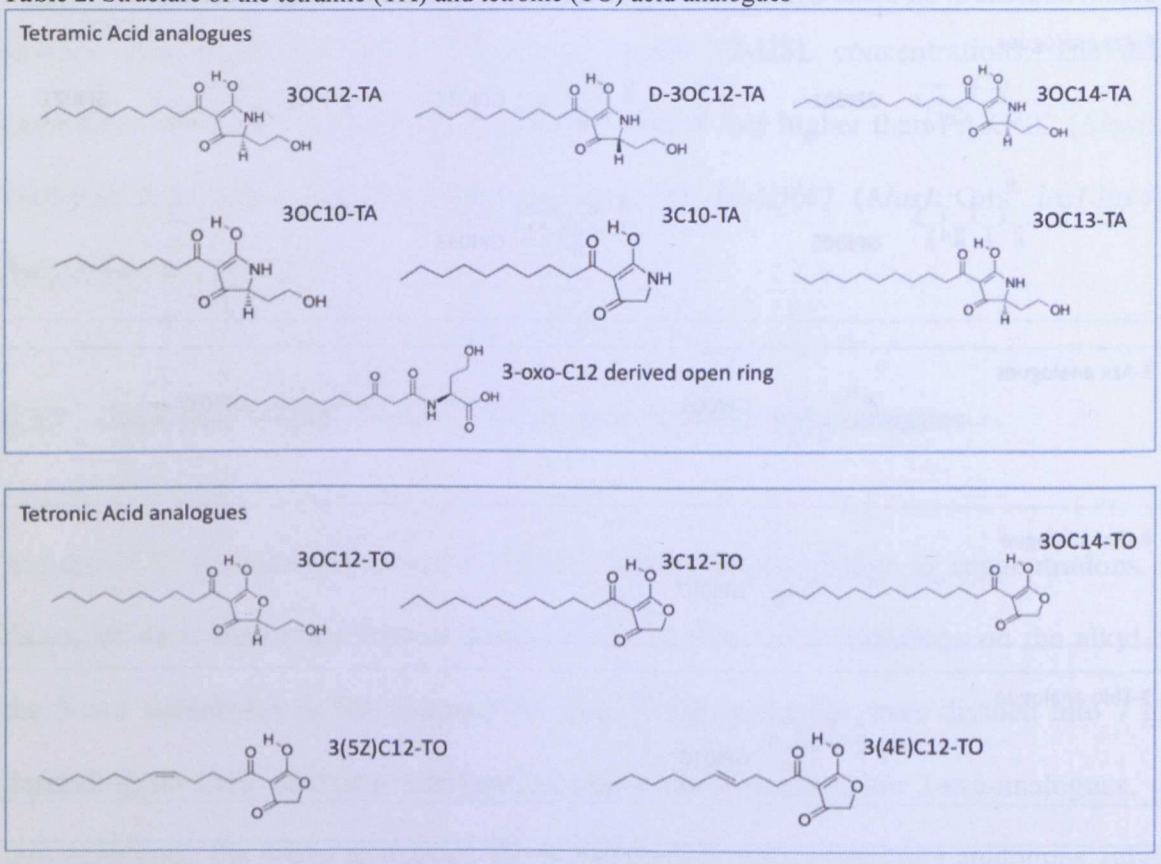

GPJ029

Table 2. Structure of the tetramic (TA) and tetronic (TO) acid analogues



6.3.4 Screening of AHL analogues for agonistic activity on the *lasB-lux* reporter

Each molecule was tested individually at a concentration of 100 µM in the absence of 3-oxo-C12-HSL for its ability to activate *lasB* expression by inducing luminescence in the reporter strain.

Out of 20 of the 3-oxo-C12-HSL analogues tested, only GPJ005 and GPJ007 displayed partial inducing activity, suggesting that these molecules possibly bind to LasR and activate it (Fig. 4). On the other hand, the tetramic acid analogues showed no significant agonistic activity (Fig. 5).

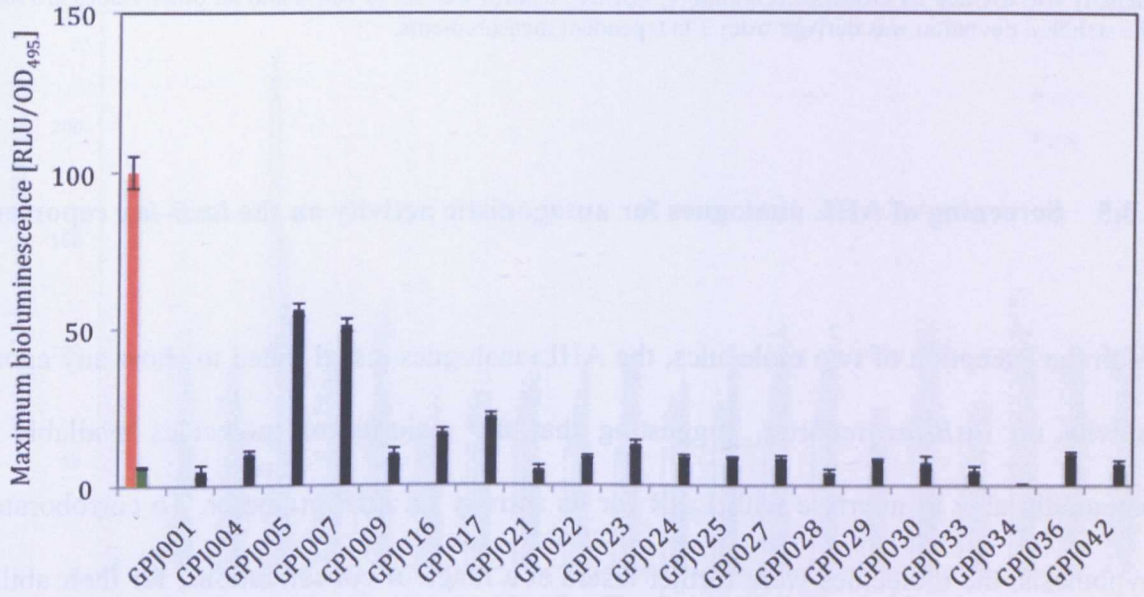


Figure 6-4. Agonistic activity of 3-oxo-C12-HSL analogues on *lasB-lux* reporter fusion.

The Δ*lasI lasB-lux* reporter was grown in the presence of methanol, the solvent used to dilute the compounds as a negative control (green), and in the presence of 5 μM of 3-oxo-C12-HSL as a positive control (orange) or 100 μM of 3-oxo-C12-HSL analogues. Bioluminescence intensity was divided by the OD_{495} of the cell culture. Positive control was set to 100% and all other values are relative. The standard deviation was derived from 3 independent measurements.

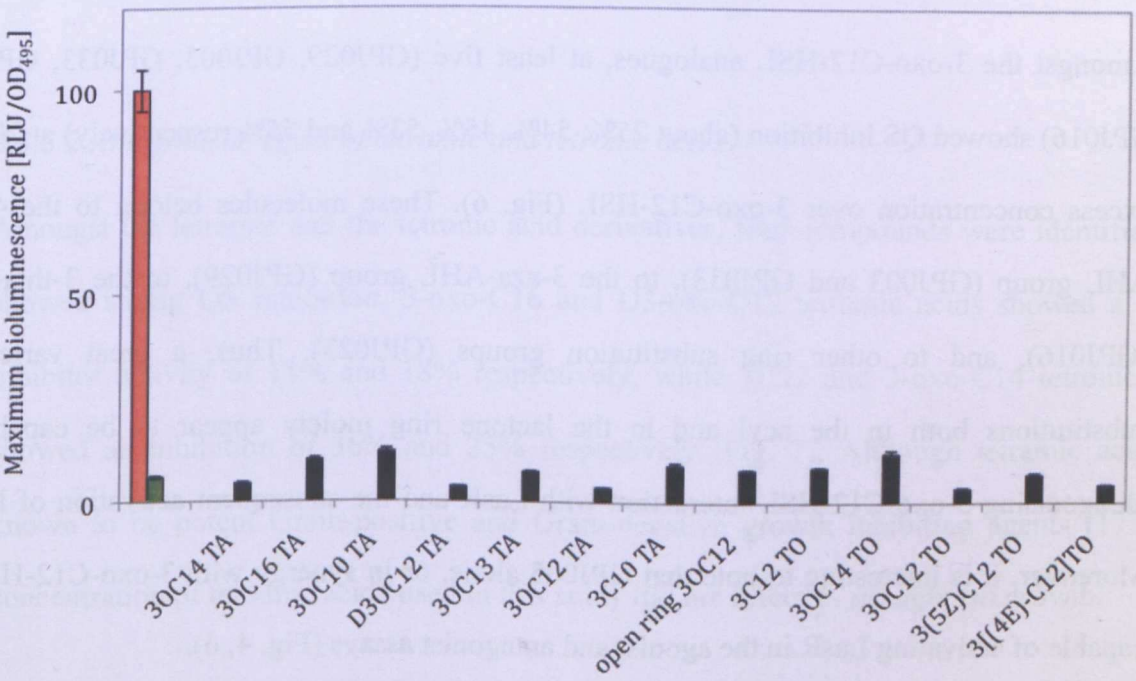


Figure 6-5. Agonistic activity of tetramic and tetronic acid analogues on *lasB-lux* reporter fusion.

The Δ*lasI lasB-lux* reporter was grown in the presence of MeOH, the solvent used to dilute the compounds as a negative control (green), and in the presence of 5 μM of 3-oxo-C12-HSL (orange) as a positive control. Tetramic (TA) or tetronic (TO) analogues at a concentration of 100 μM were added for the study. Bioluminescence

intensity was divided by OD_{495} of cell culture. Positive control was set to 100% and all other values are relative. The standard deviation was derived from 3 independent measurements.

6.3.5 Screening of AHL analogues for antagonistic activity on the *lasB-lux* reporter

With the exception of two molecules, the AHL analogues tested failed to show any agonistic activity on *lasB-lux* reporter, suggesting that the majority of molecules available were potentially able to interfere with LasR for its activity on *lasB* promoter. To corroborate this hypothesis, the molecules were further tested at a range of concentrations, for their ability to compete with the natural signal molecule for the activation of the the *lasB* promoter. The results were calculated as percentage of inhibition of relative bioluminescence by an analogue compared to 3-oxo-C12-HSL (100% positive control).

6.3.5.1 Antagonistic effect of 3-oxo-C12-HSL analogues

Amongst the 3-oxo-C12-HSL analogues, at least five (GPJ029, GPJ003, GPJ033, GPJ023, GPJ016) showed QS inhibition (about 25%, 54%, 45%, 53% and 55% respectively) at 20-fold excess concentration over 3-oxo-C12-HSL (Fig. 6). These molecules belong to the 4-aza-AHL group (GPJ003 and GPJ033), to the 3-aza-AHL group (GPJ029), to the 3-thia-AHL (GPJ016), and to other ring substitution groups (GPJ023). Thus, a great variety of substitutions both in the acyl and in the lactone ring moiety appear to be capable of antagonising 3-oxo-C12-HSL interaction with LasR and the subsequent activation of PlasB. Moreover, it is interesting to note that GPJ005 alone, or in synergy with 3-oxo-C12-HSL, is capable of activating LasR in the agonist and antagonist assays (Fig. 4, 6).

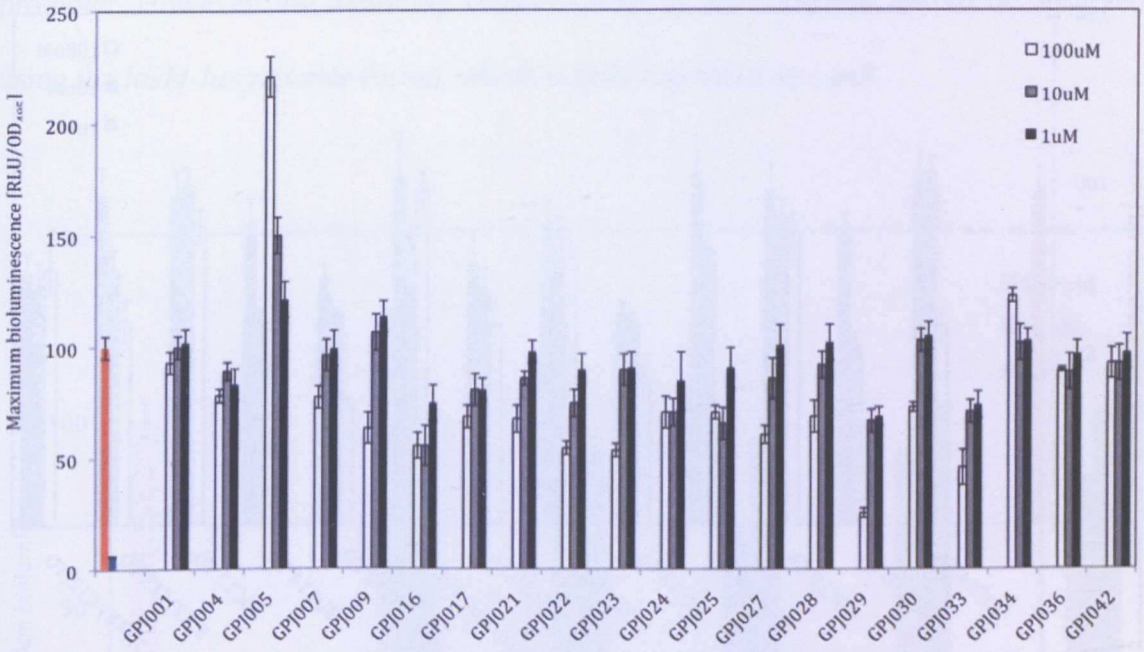


Figure 6-6. Antagonistic activity of 3-oxo-C12-HSL analogues on *lasB-lux* reporter fusion.

PAKR02 (Δ*lasI lasB-lux)* reporter was grown in the presence of MeOH, the solvent used to dilute the compounds (green), and in the presence of 5 μM of 3OC12-HSL (orange). The gray bars represent *lasB* promoter activation in the presence of 5 μM of 3-oxo-C12-HSL and in the presence of 100 μM, 10 μM and 1 μM of tetramic (TA) or tetronic (TO) analogues. Bioluminescence intensity was divided by OD_{495} of cell culture. Positive control was set to 100% and all other values are relative. The standard deviation was derived from 3 independent measures.

6.3.5.2 Antagonistic effect of tetramic and tetronic acids

Amongst the tetramic and the tetronic acid derivatives, four compounds were identified that showed strong QS inhibition. 3-oxo-C16 and D3-oxo-C12 tetramic acids showed a strong inhibitor activity of 11% and 18% respectively, while 3C12 and 3-oxo-C14 tetronic acids showed an inhibition of 36% and 25% respectively (Fig. 7). Although tetramic acids are known to be potent Gram-positive and Gram-negative growth inhibiting agents [173], the concentration of tetramic acids used in this study did not affect *P. aeruginosa* growth.

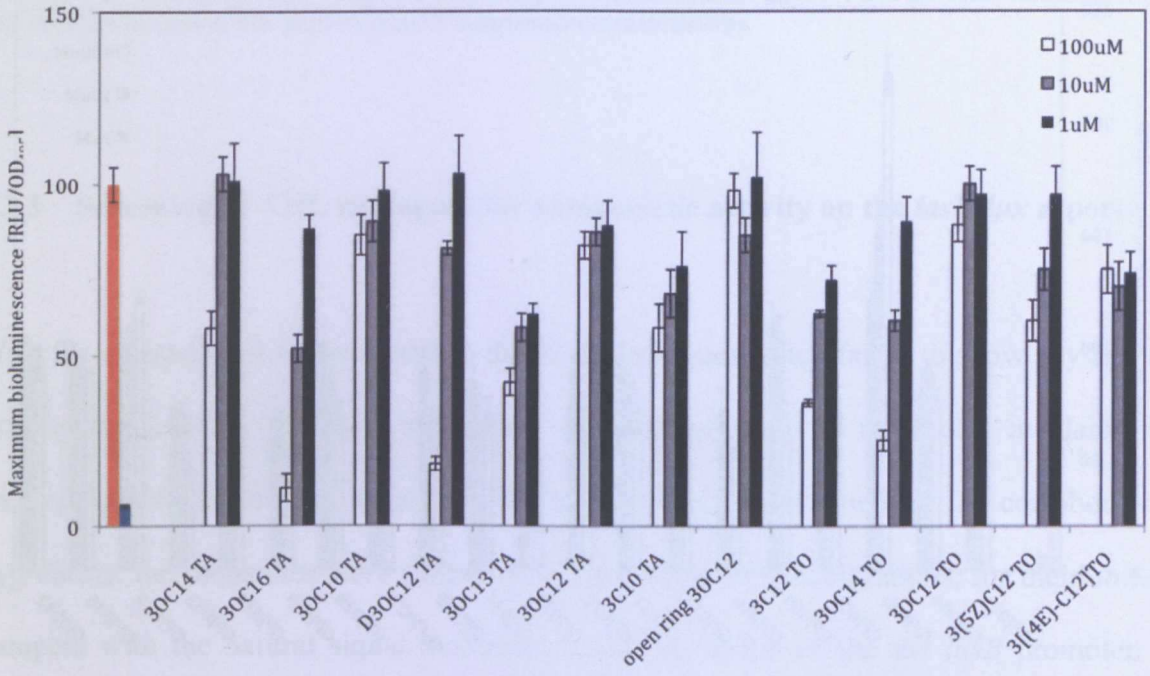


Figure 6-7. Antagonistic activity of tetronic and tetramic acid analogues on *lasB-lux* reporter fusion.

Activity of the PAKR02 (Δ*lasI lasB-lux*) biosensor is measured in the presence of 5 μM of 3-oxo-C12-HSL as a positive control (orange). Negative control represents background expression in the absence of 3-oxo-C12-HSL or any analogues with the presence of 5 % (v/v) MeOH (green). The gray bars represent the *lasB* promoter activation in the presence of 5 μM of 3-oxo-C12-HSL and in the presence of 100 μM, 10 μM and 1 μM of analogues. Bioluminescence intensity was divided by OD_{495} of cell culture. The positive control was set to 100% and all other values are relative. The standard deviation was derived from 3 independent measurements.

6.3.6 Screening of analogues for antagonistic activity on the *lasI-lux* reporter

The 3-oxo-C12-HSL analogues GPJ029, GPJ022, GPJ033, GPJ023 and GPJ016, previously identified as antagonists of LasR in the induction of *lasB* transcription, were also tested on the strain PAKR47 (Δ*lasI*::Gm^R *lasI4-lux*). The majority of the molecules tested on this reporter fusion inhibited the activity of the promoter (GPJ029 33%, GPJ022 47%, GPJ033 35%, GPJ023 30%), confirming that these molecules were acting as antagonists of LasR (Fig. 8). On the other hand, GPJ016 does not exert such a strong inhibitory activity on the PAKR47 reporter (Δ*lasI*::Gm^R *lasI4-lux*) (97%) as it does on the PAKR02 (Δ*lasI*::Gm^R *lasB-lux*) reporter fusion (55%). This suggests that GPJ016 might act as antagonist of RhlR on the *lasB*

promoter. However, the inhibitory effect exerted by this molecule cannot be observed when using the *lasI4-lux* reporter fusion, which is only regulated by LasR.

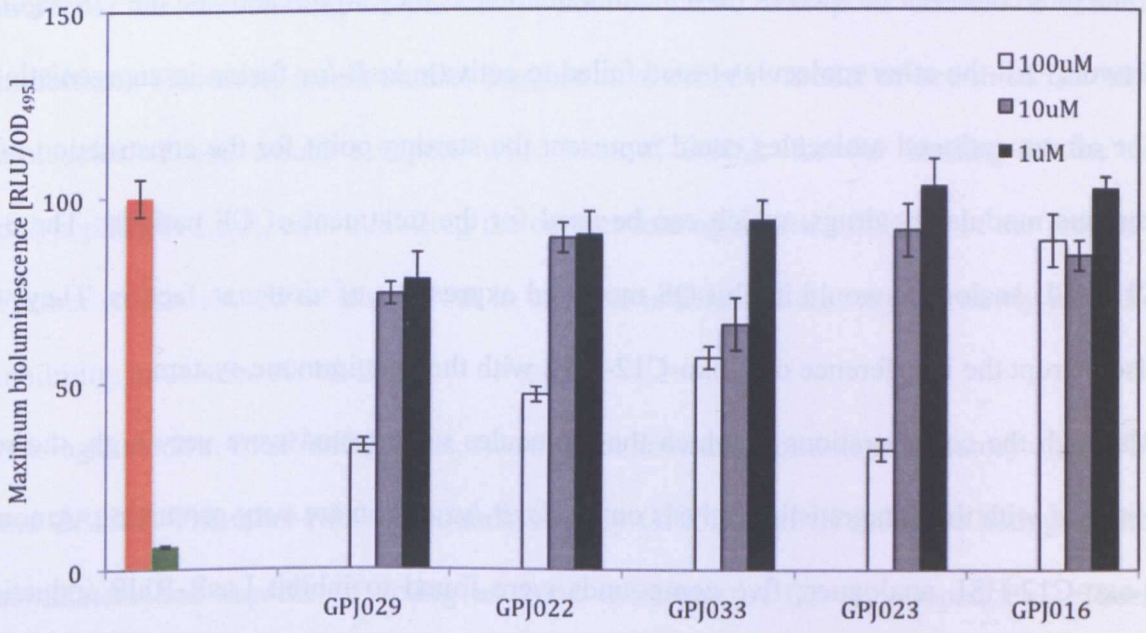


Figure 6-8. Antagonistic activity of 3-oxo-C12-HSL analogues on *lasI4-lux* reporter fusion.

Activity of the PAKR47 (Δ*lasI::Gm*^{R} *lasI4-lux*) biosensor measured in the presence of 0.5 µM of 3OC12-HSL (orange). Negative control represents background expression in the absence of 3OC12-HSL or any analogues (green). Gray bars represent *lasI* promoter activation in the presence of 1 µM of the 3OC12-HSL and in the presence of 100 µM, 10 µM and 1 µM of analogues. Bioluminescence intensity was divided by OD_{495} of cell culture. Positive control was set to 100% and all other values are relative. The standard deviation was derived from 3 independent experiments.

6.4 DISCUSSION

Following previous work on the optimisation of the immune modulatory activity of 3-oxo-C12-HSL (R. Gopal, PhD Thesis), 33 compounds were tested. These molecules were derived from the *P. aeruginosa* signal 3-oxo-C12-HSL and from its tetramic acid derivative, and were screened for their agonistic and antagonistic activity towards the *las* QS system.

The purpose of this study was to find immune modulatory molecules able to inhibit LasR activity on *P. aeruginosa* QS mediated virulence phenotype (*lasB* expression was chosen as

way of measuring LasR activity). Amongst the compounds previously found to have immune modulatory activity, only GPJ005 and GPJ009 were found to have agonistic activity on the *las* QS system by activating the transcription of the *lasB* target gene. For this reason, these molecules could not be used as immune modulators, as they would activate the QS regulatory network. All the other molecules tested failed to activate *lasB-lux* fusion in an agonistic way. The aforementioned molecules could represent the starting point for the construction of new immune modulatory drugs, which can be used for the treatment of CF patients. The 3-oxo-C12-HSL analogues would inhibit QS mediated expression of virulence factors. They would also disrupt the interference of 3-oxo-C12-HSL with the host immune system.

Although the concentrations at which the molecules were tested were very high, the results obtained with the antagonistic analysis on the *lasB-lux* fusion are very promising. Among the 3-oxo-C12-HSL analogues, five compounds were found to inhibit LasR-RhlR induction of the *lasB* promoter. This preliminary analysis was conducted using a bio-reporter system based on the *lasB* promoter. However, it is well known that the transcription of this gene is both regulated by LasR and RhlR [240]. DNaseI protection assays conducted on the *lasB* promoter allowed for the identification of two operators containing *lux-box* sites: OP1 (located -42 bp from the +1 transcription starting site), controlled by RhlR, and OP2 (located -101 bp from the +1 transcription starting site), LasR regulated [77].

Since the *lasB* promoter fragment used in this study comprised 480 bp from the +1 transcriptional starting site, the molecules tested could exert their agonistic and antagonist effect by modulating RhlR activity on the *lasB* promoter. For this reason, a second *lasI* reporter fusion was constructed using the promoter region known to be uniquely activated by the LasR-3-oxo-C12-HSL complex [77]. The five molecules which showed an effect on the *lasB-lux* fusion were thus tested on *lasI-lux4*. From this analysis, it appears that four out the five molecules tested were acting as LasR antagonists. Although GPJ016 shows a strong inhibitory activity on the *lasB* promoter, this effect is abolished when testing the compound

on the *lasI* fusion. This could be due to the fact that GPJ016 acts as an antagonist of RhlR (modulates *lasB* expression) rather than on LasR (modulates both *lasB* and *lasI* expression).

The hypothesis is that GPJ029, GPJ033, GPJ022 and GPJ023 compete with the signal molecule for the binding to the active site of LasR, failing to activate it (competitive inhibition). However, recent studies suggest an alternative model of action of QS inhibiting molecules. These would compete with 3-oxo-C12-HSL for the binding to the nascent polypeptide of LasR, thus inhibiting the correct folding and/or solubility of the protein [305]. Further studies should be carried out in order to understand the way the newly identified QS inhibiting molecules act on LasR.

Amongst the four LasR antagonists, two did not have any immune modulatory effect: GPJ022 and GPJ023. The other two compounds, namely GPJ029 and GPJ033, are both good immune modulators and capable of acting as good inhibitors of QS (R. Gopal, PhD Thesis). In particular, GPJ029 appears to be the most interesting one since it is a stronger immune modulator compared with the *P. aeruginosa* natural signal molecule. Moreover, GPJ029 displays the strongest QS inhibitor effect (25%) compared with the 3-oxo-C12-HSL analogues tested.

In the lungs of CF patients infected with *P. aeruginosa*, the main cause of tissue damage is not due to the bacteria *per se*, but rather due to the activation of a prolonged immune inflammatory response. This inflammatory response is ultimately responsible for the necrosis of the lung tissue and the loss of the pulmonary function [306]. For this reason, the molecules GPJ029 and GPJ033 could represent a good starting point for the development of drugs used in the treatment of *P. aeruginosa* disease, in which both the immune repression and the QS inhibition activity is required (for example in cystic fibrosis chronic infections).

The 3-oxo-C12-HSL analogues tested were constructed docking the potential analogues into the active pocket of two LasR homologues. The known crystal structures of TraR, from the plant pathogen *Agrobacterium tumefaciens*, and SdiA, from *E. coli*, were used for this

purpose [307, 308]. The identification of 3-oxo-C12-HSL analogues competing for LasR binding represents a significant contribution to the understanding of the active site pocket of the LasR regulator.

The recent determination of the LasR crystal structure provides a new approach for the screening of LasR antagonists [305]. It is thus possible to create QS inhibitors modelled to fit ad hoc the active site of LasR.

The molecules derived from 3-oxo-C12-HSL non enzymatic degradation, namely tetramic acids (TA), were previously tested for immune modulation (R. Gopal, PhD Thesis). Although these molecules were shown to act as good immune inhibitors, they also displayed a strong antibacterial activity against many Gram-positive strains [173]. Hence, TA (and any other molecules derived from tetramic acids) can interfere with the commensally natural bacteria present in the human system. Therefore, these molecules are not good candidates for the development of immune modulatory therapies.

Finally, the analysis conducted on the *lasI* promoter allowed for the identification of two important regions required for the *lasI* promoter activation by 3-oxo-C12-HSL. These are located between the -153 and -119 bp region and between the +15 and +54 bp region. In particular the region downstream the second transcriptional starting point (G) is essential for the capability of the promoter to respond to the addition of the signal molecule, while the region from -153 to -119 is important for the amplitude of the response. These results suggest that there is a region between +15 bp and +54 bp, which is involved in the promoter's response to the addition of the signal molecule 3-oxo-C12-HSL. In addition, a second ATG translational starting site was found a few bp downstream of the first one, suggesting that there is a third potential +1 transcriptional starting site and an ATG translational start further downstream. These findings suggest the existence of additional regulators acting on *lasI* transcription. However, a second *lux-box* or another visible palindromic repeat was not found on the promoter of *lasI*. It is possible that the regulation occurs at the post-transcriptional

level. The mRNA resulting when using RV2P*lasI* could be more stable than the one resulting when amplifying with RV1P*lasI*. To investigate this, an in silico analysis was performed on the sequence comprised between the two reverse primers, RV2PlasI and RV1PlasI. In order to find secondary structures, the RNA*fold* WebServer was used (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). A secondary RNA stem loop structure forming between the +1 (A) and +21 bp was found.

The hypothesis suggested is the following: LasR could bind with the same affinity to the promoters tested, however the mRNA derived when including the +21 region is more stable than the one derived when amplifying the *lasI* promoter until the +1 (A). Since two transcriptional starting sites were identified, [78] this hypothesis seems to be feasible.

At lower 3-oxo-C12-HSL concentrations, the transcription of *lasI* might start from the second transcriptional starting site (G), giving a basal level of transcription, and the mRNA generated could be degraded (possible post-transcriptional regulation). On the other hand, in the presence of high 3-oxo-C12-HSL the transcription could start from the first transcriptional starting site (A), giving rise to the more stable mRNA. This hypothesis would fit with our observations. When using the PlasIRV2 primer in combination with the PlasIFW1 primer to amplify the *lasI* promoter, the resulting mRNA would include the stem loop that stabilizes the transcript. On the other hand, when the PlasIRV1 is used, the stem loop could not be formed, promoting the mRNA degradation (Fig. 9).

To test this hypothesis, it would be necessary to study the degradation (e.g. Real Time PCR) of a target gene cloned downstream of the two different promoter fragments.

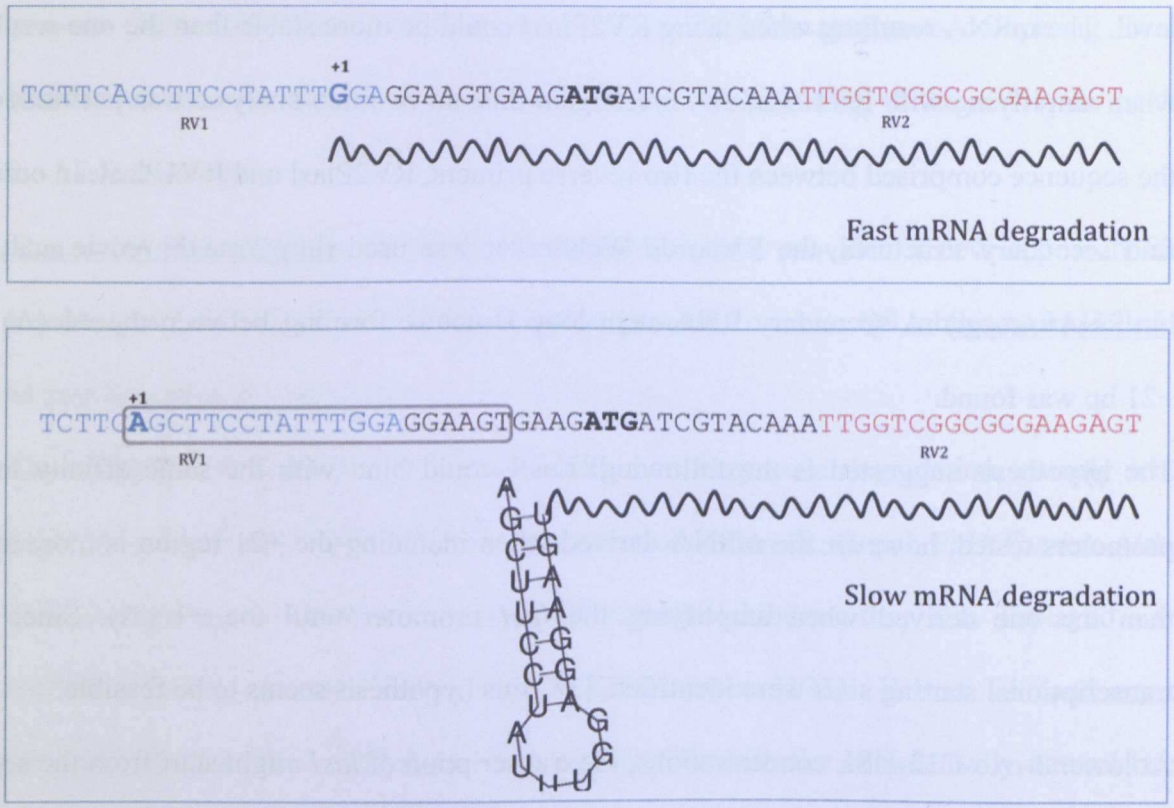


Figure 6-9. Schematic representation of the region between the RV1lasI and the RV2lasI of the *lasI* promoter and the resulting transcript.

The formation of the secondary RNA structure depends on the transcriptional starting site. This influences the stability of the resulting mRNA. When using the PlasIRV1 (blue) to amplify the *lasI* promoter, the secondary structure is disrupted and the resulting mRNA is less stable. When using PlasIRV2 (red) to amplify the *lasI* promoter, the secondary structure is included in the transcript, promoting mRNA stability.

6.5 Conclusion

The structural homology of the LuxR-proteins family (SdiA and TraR) allows for the use of these homologues as a reference for docking studies of AHLs derived compounds. A range of 3-oxo-C12-HSL derived molecules with an altered acyl-chain or lactone ring was analysed for its agonistic and antagonistic activity toward the LasR QS regulator. From this study, it is apparent that by analyzing these molecules' indirect effect on the transcription of *lasB* and *lasI*, a preliminary selection of the molecules with the strongest QSI activity can be obtained (Fig. 10). The reporters constructed and tested here are available for future screenings of new potential QS modulator molecules designed to interfere with the *las* QS system.

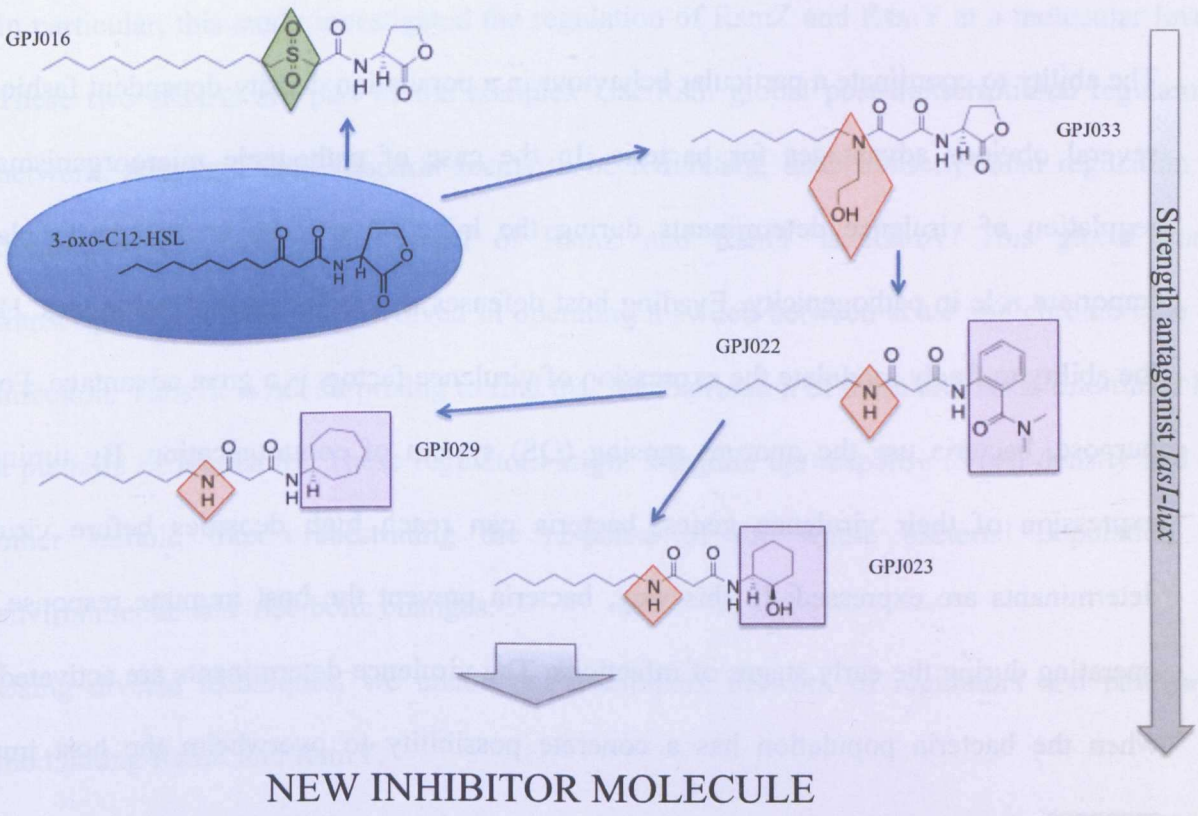


Figure 6-10. Compounds derived from 3-oxo-C12-HSL potentially able to have an antagonistic effect on LasR.

Through the analysis of the ability of these compounds to inhibit *lasI*, new inhibitor molecules altered in their acyl-chain or lactone ring, can be produced.

7 General conclusions and future directions

The ability to coordinate a particular behaviour in a population density-dependent fashion has several obvious advantages for bacteria. In the case of pathogenic microorganisms, the regulation of virulence determinants during the infection process is thought to play an important role in pathogenicity. Evading host defenses is a major goal of pathogens. Hence, the ability to finely modulate the expression of virulence factors is a great advantage. For this purpose, bacteria use the quorum sensing (QS) system of communication. By timing the expression of their virulence genes, bacteria can reach high densities before virulence determinants are expressed. In this way, bacteria prevent the host immune response from operating during the early stages of infections. The virulence determinants are activated only when the bacteria population has a concrete possibility to overwhelm the host immune response.

Bacteria have also evolved ways to efficiently respond to extracellular signals. By this means, bacteria induce the expression of a series of genes, which allow them to survive in a constantly changing environment. For this purpose, they have developed a wide range of two component systems (TCS), which sense external stimuli and activate responses.

The importance of both QS and TCS systems for the control of *P. aeruginosa* virulence determinants implies that the study of these networks is essential to develop new antimicrobial strategies. Understanding the modulation of these networks will contribute to the discovery and development of new antibacterial targets.

In Chapters 3, 4 and 5, we concentrated on the study of RsmZ and RsmY expression, which is modulated by regulators (AguR, RhlR), extracellular (PQS, CDA), and intracellular (cyclic-di-GMP) signal molecules. In Chapter 6, we were interested in testing the potential of QS inhibition *in vitro* using synthetic molecules. A clinical pilot study was also conducted to test

the potential of natural occurring QS inhibitor molecules to promote the clearance of *P. aeruginosa* infections (see Appendix).

In particular, this study investigated the regulation of RsmZ and RsmY at a molecular level. These two sRNAs are part of the complex Gac/Rsm global post-transcriptional regulatory network. Although their function seems to be redundant, their transcriptional regulation is dissimilar [156, 160]. The target of RsmZ and RsmY is RsmA. This global post-transcriptional regulator is involved in operating a switch between acute and chronic type of infection. Thus, it is not surprising to find that the expression of these sRNAs is fine-tuned by a plethora of regulators. These regulators might integrate the response to cell density and to other stimuli, thus orchestrating the response of the whole bacterial population to environmental and metabolic changes.

Using diverse techniques, we uncovered a complex network of regulators and pathways modulating RsmZ and RsmY.

Using the promoter pull down technique, we identified a new regulator, AguR, which affects the timing of the *rsmZ* transcription. This regulator is responsible for the modulation of the polyamine synthetic pathway. Recent studies show that polyamines are important for biofilm formation and modulate type III secretion system, suggesting a link between polyamines and the RsmA global regulatory network [287]. AguR could exert its role on biofilm and type III secretion through the modulation of *rsmZ* and thus RsmA. In the future, it will be interesting to use the purified AguR protein to conduct band-shift analysis on the *rsmZ* promoter.

Using the transposon mutagenesis approach, we were able to link the Gac/Rsm pathway to cyclic-di-GMP, a key secondary messenger molecule. The involvement of this molecule in biofilm formation, adhesion and virulence has been extensively studied [309-311]. In particular, high levels of cyclic-di-GMP are usually associated with biofilm formation. On other hand, RsmA negatively regulates this phenotype. The discovery that *rsmZ* is positively

regulated by cyclic-di-GMP suggests that this second messenger molecule can act on the biofilm phenotype through the modulation of RsmA.

In 2001, Pessi *et al.* discovered the link between QS and the global post-transcriptional regulator RsmA [152]. Using translational fusions, they found that RsmA negatively regulates the expression of *lasI* and *rhlI* encoding for the two QS HSL synthase enzymes. Using a genetic bank approach, we found that both RhlR and LasR are capable of activating *rsmZ* transcription. Supporting this result, we discovered a *lux* box on the *rsmZ* promoter region, which is not present in the *rsmY* promoter. These findings, together with the discovery of the PQS molecule in one of the fractions activating *rsmZ* transcription, prompted us to analyse the role of QS on the Gac TCS.

Until now, QS system signal molecules were known to have an effect on the transcription of target genes binding to a regulator protein belonging to the LuxR family. This protein would then act at a transcriptional level on the expression of target genes. The discovery of PQS activation of *rsmZ* transcription suggests that this QS signal potentially modulates the expression of target genes also at the post-transcriptional level. This hypothesis was verified by constructing a translational fusion based on the *lecA* gene, which is negatively regulated by RsmA [94].

We also conducted epistasis analysis to prove that PQS action on *lecA* was actually mediated by inducing *rsmZ* transcription. The presented data demonstrate that *rsmZ* is involved in the PQS-mediated post-transcriptional regulation, acting in antagonism with RsmA-mediated repression of *lecA*. In contrast with what was initially thought, we showed that PQS acts weakly on the *lecA* promoter. This suggests that QS regulation on this important virulence factor is mainly exerted post-transcriptionally.

Finally, although PQS activation of *rsmZ* transcription was found to act through the *lux* box, its action appears to be independent from PqsR, PqsE, LasR and RhlR. It would be interesting

to investigate if PQS action goes through the "orphans" LuxR proteins QscR or VqsR [90, 312].

In conclusion, the finding that PQS acts on post-transcriptional regulation of gene expression increases the complexity of the *P. aeruginosa* QS regulon. Furthermore, it is clear from our work that *rsmZ* and *rsmY* are differently regulated by PQS, MvaT, MvaU, and possibly by other regulators, in agreement with what has been previously observed [156, 160].

Investigating the regulation of target genes by QS and the Gac/Rsm pathway could lead to a deeper understanding of the regulation of virulence by *P. aeruginosa*. In this study, we gave evidence for the existence of a bidirectional link connecting QS and the Gac/Rsm pathway in a complex network. Infection studies (using *in vivo* models) must be carried out to appreciate the biological relevance of PQS post-transcriptional modulation of target virulence genes.

In the course of this study, new vectors and a whole range of chromosomal mutants have been generated. Of particular interest, the double transcriptional-translational reporter fusion (pMW02), which will be used in future studies aimed to the identification of the extracellular signal(s) modulating the Gac/Rsm system. We also produced a wide range of mutants both in the QS and the Gac/Rsm system, as well as a range of transcriptional and translational fusions that represent an excellent starting point to reveal PQS (and in general QS) involvement in post-transcriptional regulation of target genes.

8 Appendix: Garlic against *Pseudomonas aeruginosa* infections - a clinical study

8.1 Introduction

Eukaryotes and bacteria have coexisted for millions of years, it is then not surprising that many plants and fungi have evolved ways to interfere with the bacterial cell-to-cell communication system. This allows them to inhibit virulence, or to promote the growth of beneficial bacteria. The search for quorum sensing inhibiting (QSI) compounds has been extended to the natural environment. In particular much effort has been dedicated in the study of plants such as carrot, soybean, water lily, tomato, pea seedlings, habanero and garlic, which have been found to produce molecules capable of interfering with QS in bacteria [180, 303]. Garlic is known to be an antimicrobial agent since the ancient times. Garlic extracts have been reported to exhibit a broad antibacterial spectrum against both Gram-positive and Gram-negative bacteria [313-315]. This bactericidal activity has been recently coupled with QS inhibition properties. Recent *in vivo* studies, which were conducted on a pulmonary mouse, on a *Caenorhabditis elegans* and on nematode infection models, showed that treatment with garlic extracts lowers *P. aeruginosa* infection by improving the clearance of the infection [299, 316]. The hypothesis that garlic inhibits QS was further corroborated by results obtained *in vitro*. Microarray data indicates that garlic extracts specifically inhibit QS-regulated genes, probably by interacting with the signal sensor/response regulator proteins [316]. Moreover, it was demonstrated that addition of garlic extracts to *P. aeruginosa* biofilms reduces the tolerance of the bacteria to tobramycin treatment [180]. Finally, it has been found that

P. aeruginosa culture treated with garlic extract produces lower amounts of signal molecules and, consequently, of virulence factors [317].

Raw garlic extracts contain the compounds responsible for QS inhibition activity (still unknown) and the molecules mediating its antimicrobial activity. *In vitro* studies showed that when the concentration of garlic extracts is too high, the antimicrobial effect prevails affecting bacterial survival [318]. Thus, in order to use garlic extracts in the future treatment of *P. aeruginosa* infections, we need to identify the QSI molecule(s). It is also essential to understand the real potential of garlic extract to act as QS inhibitor in human treatments.

8.2 AIM OF THE STUDY

The aim of this study was to conduct an analysis on the impact of macerated garlic oil on *P aeruginosa* infection clearance in CF patients.

A group of CF paediatric and adult patients with chronic *P. aeruginosa* lung infection was given garlic (or placebo) capsules for a period of two months. The levels of QS signal molecules, both in the sputum and in the blood, were analysed before and after the treatment to monitor *P. aeruginosa* infection. The general clinical improvement of the patients was also monitored.

Our goal was to demonstrate the QS inhibitor potential of garlic extracts *in vivo,* and to determine whether it was associated with clinical improvement. This pilot study was the first study of a QS inhibitor to be conducted in humans.

The study was conducted in collaboration with Alan Smyth (Division of Child Health, University of Nottingham, UK), who conducted the clinical trial, and Catherine Ortori (Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham, UK), who conducted the analysis of the extracted sputum and plasma samples.

8.3 RESULTS

8.3.1 Garlic extracts effect on the *lasB-lux* biosensor

Prior to the clinical study, we performed some *in vitro* experiments in order to test the potential of the crude garlic extracts for QSI activity. For this purpose, we used the strain PAKR02 (Δ*lasI*::Gm^R *lasB-lux*) and tested the effect of increasing amounts of garlic extracts on the activation of *lasB-lux* fusion (Fig. 1). At a concentration of 5% (v/v), the garlic extract significantly reduced the activation of the *lasB-lux* reporter by lowering the induction by about two folds. However, when lower concentrations (1%) were added, we again observed a significant reduction on *lasB-lux* activation. The garlic concentration did not influence growth when used at concentrations below 2%. These results confirm previous observations [180], which showed that crude garlic extracts have an effect on QS regulated genes such as *lasB*. The observed effect is caused by a still unknown molecule, which may act as an antagonist of the LasR regulator.

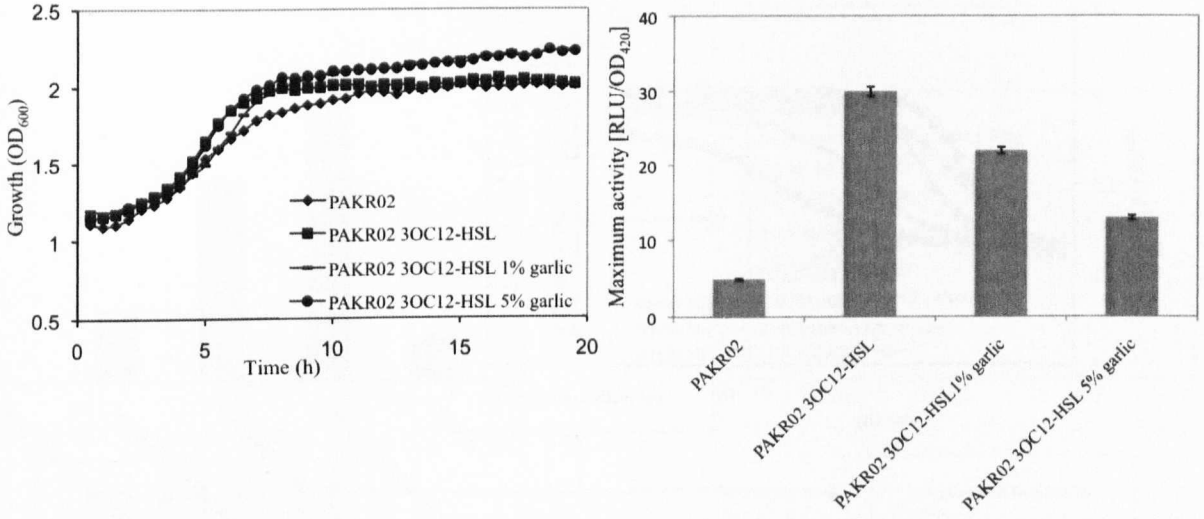


Figure 1. Influence of garlic extracts on growth and on the activation of the *lasB-lux* fusion.
On the left, growth curve of the strain PAKR02 (Δ*lasI lasB-lux*) tested in the presence of the 3-oxo-C12-HSL signal molecule and of different concentrations of garlic extracts.
On the right, the maximum activity of the PAKR02 sensor strain in the absence and in the presence of 3-oxo-C12-HSL (5 µM) or in the presence of 3-oxo-C12-HSL (5 µM) and a range of garlic extract concentrations (1% or 5% v/v).

8.3.2 Ajoene effect on the *lasB-lux* biosensor

The compound(s) acting as QSI in garlic extracts are still unknown. However, previous studies found that the antimicrobial activity of garlic is related to the presence of growth inhibitory sulfur-containing compounds such as allicin and ajoene [319, 320]. Since allicin is not the QSI compound [321], we decided to analyse the effect of ajoene on the PAKR02 sensor strain. Ajoene was found to have an effect inhibiting *P. aeruginosa* growth at a concentration of 5µM, in discordance with previous publications [320]. Therefore, the observed effect of ajoene on *lasB* transcription appears to be due to its growth inhibition potential (Fig.2).

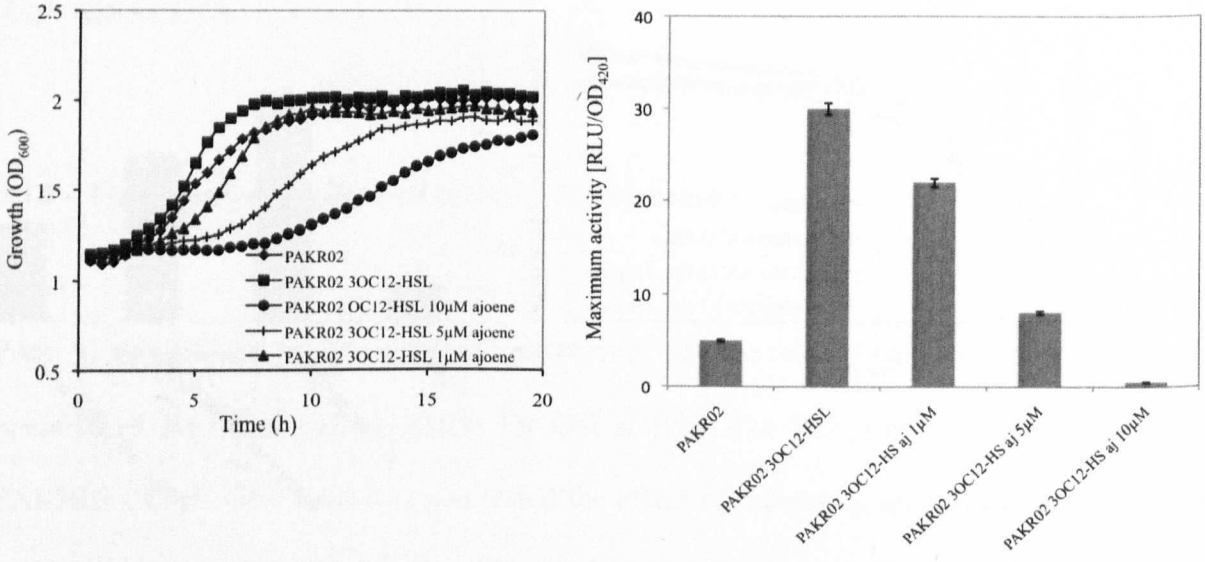


Figure 2. Influence of ajoene on growth and activation of the *lasB-lux* fusion.
On the left, growth curve of the strain PAKR02 (Δ*lasI lasB-lux*) tested in the presence of the 3-oxo-C12-HSL signal molecule and of different concentrations of ajoene.
On the right, the maximum activity of the PAKR02 sensor strain in the absence and in the presence of 3-oxo-C12-HSL (5 µM) or in the presence of 3-oxo-C12-HSL (5 µM) and a range of ajoene concentrations.

8.3.3 Patient enrolment

We tested the hypothesis that garlic treatment would reduce *P. aeruginosa* QS signal molecule levels in the sputum and plasma of 97 cystic fibrosis patients, colonized by *P. aeruginosa.* We randomised 34 patients, 8 patients withdrew, leaving 26 patients for the analysis (Fig. 3). Enrolment took place between May and September 2007 at a single centre (the Cystic Fibrosis Centre at Nottingham University Hospital's NHS Trust).

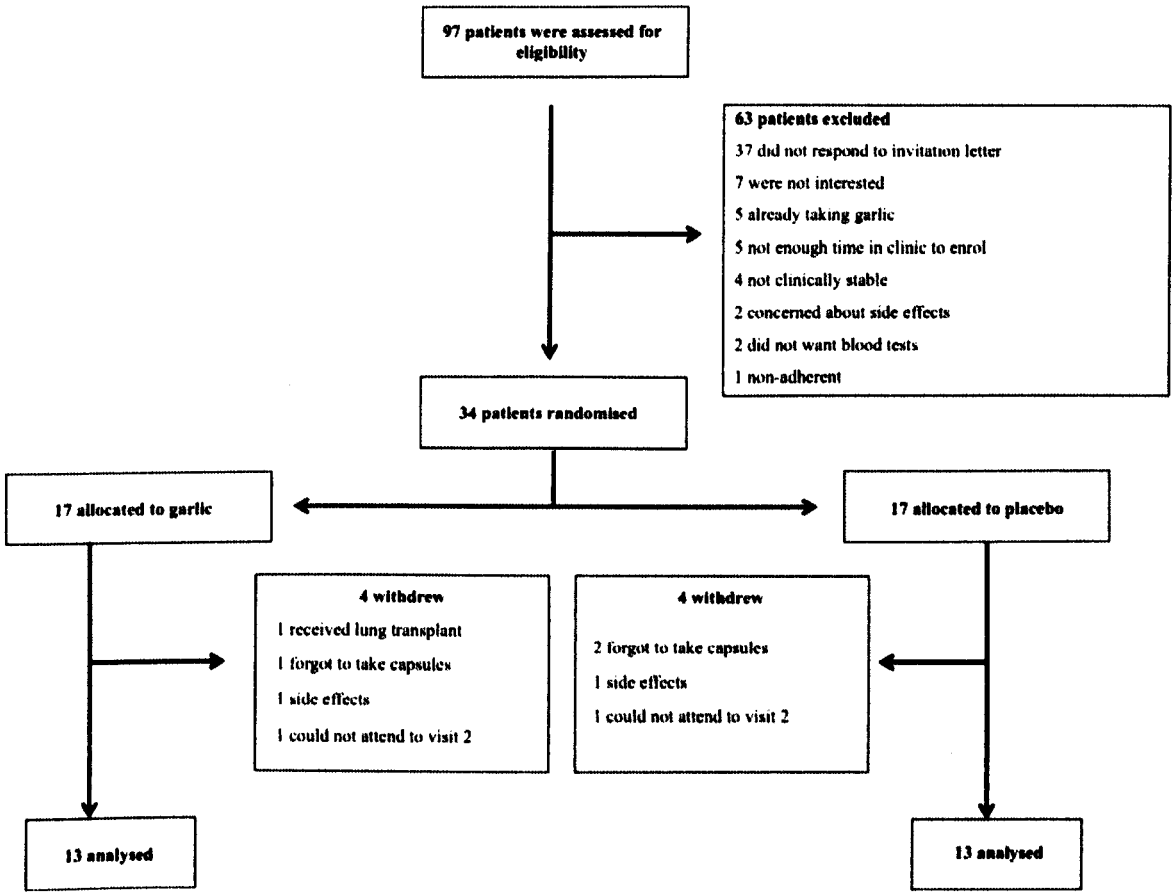


Figure 3. Patient enrolment and follow-up.

8.3.4 Clinical outcome

Thirteen patients received garlic treatment and thirteen received placebo treatment.

The garlic capsules were 656 mg of garlic oil macerate and 10 mg of cardamom oil, the placebo capsules were 656 mg of olive oil and 10 mg of cardamom oil. The cardamom oil was used as an odour control agent. The dose of garlic was chosen following the recommended dose of garlic as a nutritional supplement.

At entry into the study, the two groups were comparable regarding sex, age distribution, weight, FEV1 (forced expiratory volume in 1 second) and clinical score (Tab. 1). At the end

of the treatment, five garlic patients reported minor adverse effects such as diarrhoea, halitosis, abdominal pain, dysuria. In the placebo group, one patient reported a minor haemoptysis. Otherwise, the treatment was well tolerated and no other clinical of haematological adverse reactions occurred.

	Placebo	Garlic	Total
Total	13	13	26
Adults	9 (63%)	8 (62%)	17
Children	4 (31%)	5 (38%)	9
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
FEV1	1.91 (1.05)	1.84 (0.79)	1.87 (0.91)
Weight (Kg)	54.2 (14.1)	46.7 (14.6)	50.4 (14.5)
	Median (Range)	**Median (Range)**	**Median (Range)**
Clinical score	4 (0,6)	2 (0,6)	3 (0,6)

Table 1. Baseline characteristics of study participants

The clinical scores at the start of the treatment did not differ significantly between the two groups of patients completing the study (Tab. 2), even though the FEV1 values showed a greater decline in the placebo group (-3.6%) than in the garlic group (-2.0%). Both groups showed an increase in weight. The mean increase was greater in the garlic treated group than in the placebo. Concerning the Jensen clinical score (Material and Methods section 2.18.6), the values obtained at the beginning of the study were subtracted to the ones measured at the end of the study. Therefore, a negative score means decreased symptoms. In the garlic group the median change in clinical score was -1, while in the placebo group was calculated as +1. Moreover, the amount of antibiotic received during the 8 weeks of treatment was compared.

In the garlic group seven patients had the antibiotic treatment, while in the placebo group only five patients took the antibiotic treatment.

	Placebo Mean (SD)	Garlic Mean (SD)	Mean difference Garlic-Placebo	P value
% Change from baseline in FEV_1	-3.60 (11.31)	-2.01 (12.30)	1.59	0.8
% Change from baseline in weight (Kg)	0.61 (2.00)	0.99 (2.03)	0.38	0.6
	Median (Range)	Median (Range)	Median difference, Hodges-Lehmann	
Change in symptom score	1 (-1,4)	-1 (-3,5)	-1 (-3,0)	0.16

Table 2. Results for clinical outcome measures

8.3.5 Sputum and Plasma analysis

Before and after the treatment, both sputum and plasma samples were collected and analysed for the levels of AHLs, as described in Material and Methods (section 2.18.11). The levels of 3-oxo-C12-HSL and C4-HSL were determined using LC-MS/MS analysis, and their relative concentration was obtained by calculating the peak area produced after the LC-MS/MS run. The concentrations of C4-HSL and 3-oxo-C12-HSL collected before the treatment in sputum were much higher than the ones detected in plasma (Fig.4 and 5).

In sputum, with the exception of patient number 2, 34 and 40, 3-oxo-C12-HSL was detectable in all the samples collected. On the other hand, C4-HSL was detected in lower amounts and only in few patients (Fig. 4).

In plasma, 3-oxo-C12-HSL was detectable in all the patients samples, with the exception of patient number 34 and 53. C4-HSL was detected only in patient number 33 (Fig. 5).

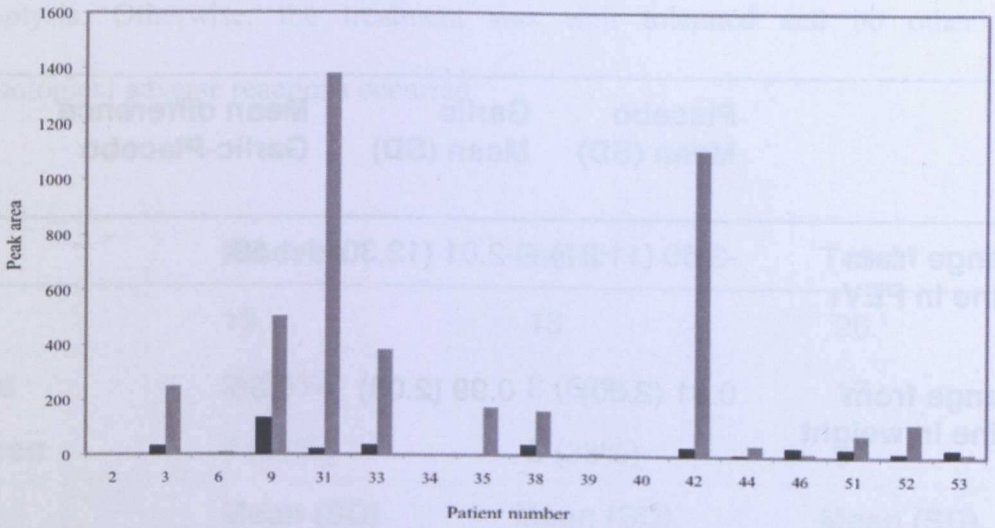


Figure 4. Presence of AHL molecules in sputum samples collected from CF patients.
Peak area C4-HSL (in black) and 3-oxo-C12-HSL (in gray) in sputum samples collected at the beginning of the treatment.

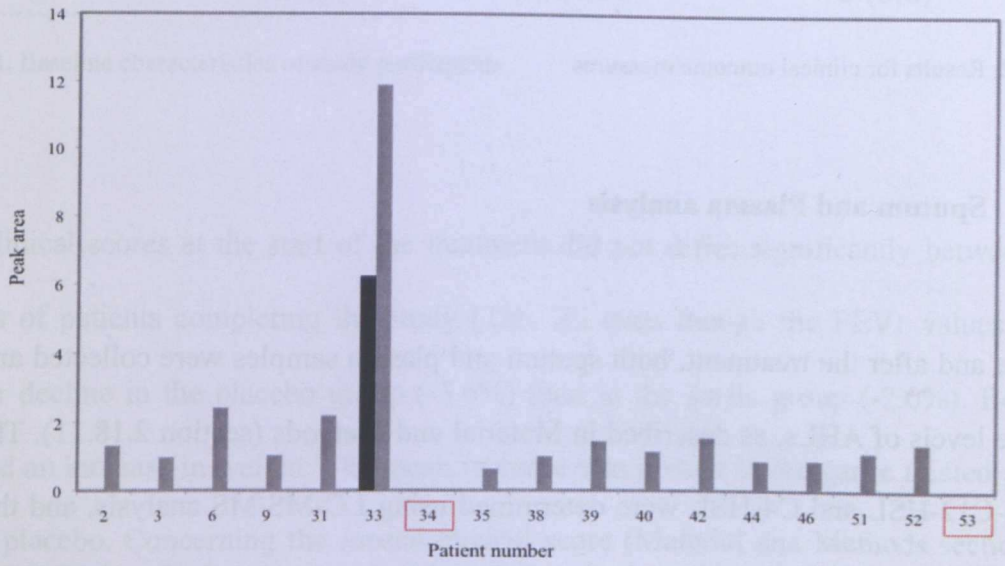


Figure 5. Presence of AHL molecules in plasma samples collected from CF patients.
Peak area C4-HSL (in black) and 3-oxo-C12-HSL (in gray) in plasma samples collected at the beginning of the treatment. Squared is the patient number in which the levels of 3-oxo-C12-HSL and C4-HSL was below the detectable levels.

Although this analysis represents a starting point for future studies on garlic extracts efficacy on *P. aeruginosa* infection clearance in CF patients, there were insufficient patients completing the treatment to evaluate the effect of garlic.

However, it was interesting to compare the levels of 3-oxo-C12-HSL in the sputum and plasma samples of the patients receiving the placebo. Measurable concentrations of 3-oxo-C12-HSL were obtained for both plasma and sputum samples from seven patients. There is a highly significant correlation between plasma and sputum measurements of 3-oxo-C12-HSL (Pearson correlation coefficient = 0.914, p = 0.004) (Fig. 6). This result although promising, derives from a very small group of patients, thus a larger study is needed to corroborate this findings. It is worth mentioning that when considering the plasma and sputum samples from all the patients entered into the study, the correlation between 3-oxo-C12-HSL in sputum vs. plasma is not evident (data not shown).

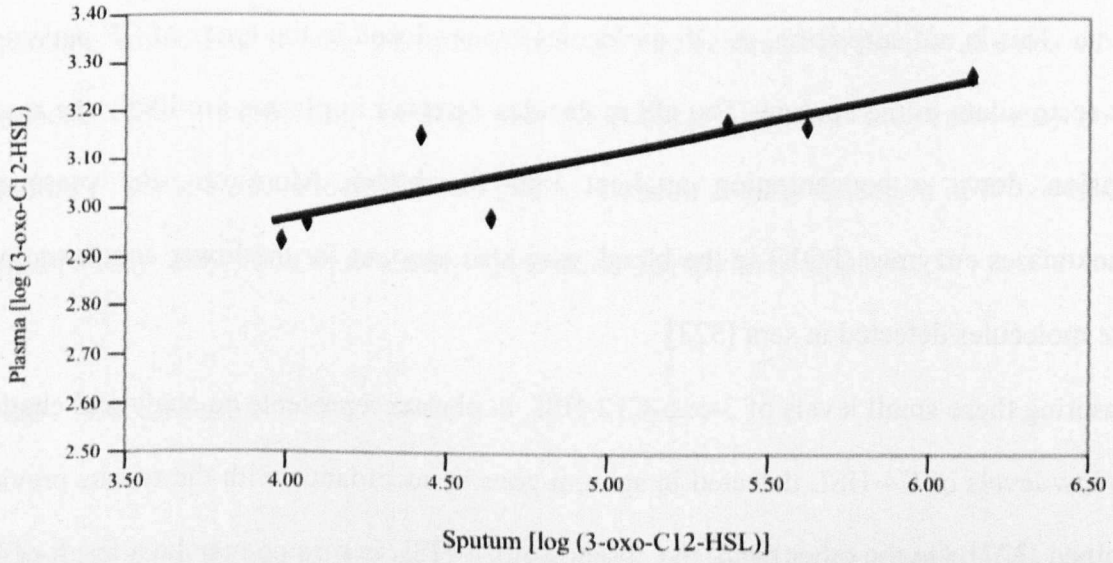


Figure 6. Correlation between concentration of 3-oxo-C12-HSL found in plasma vs. concentration of 3-oxo-C12-HSL found in sputum.
Samples derive from seven placebo patients collected at the beginning of the treatment.

8.4 DISCUSSION

This is the first study of QS inhibition conducted in human. Although no significant results were obtained comparing the placebo with the garlic treatment therapy, this study provides the basis for a bigger study conducted on a larger number of patients. Garlic capsules do not seem to affect the general clinical state of the patients, although there were some adverse effects reported, many of these were mild or intrinsic features of CF.

Up to now the QS signal molecules from CF patients colonised by *P. aeruginosa* have been detected only in sputa and lung tissue. The molecule 3-oxo-C12-HSL has been found at nanomolar concentrations [322].

In this study, by analysing the 3-oxo-C12-HSL content in blood and sputum with a LC-MS/MS, it was possible for the first time to show that plasma levels correlate closely with the levels found in sputum. The mean level of 3-oxo-C12-HSL in plasma present in the seven patients (Fig. 5) was measured to be 1.380 (arbitrary unit). This compares to the 89.817 for sputum, indicating that the levels in sputum are more than fifty fold greater than those in plasma. This is not surprising, as QS molecules are produced in the lungs of CF patients and thus accumulate in the sputum. The QS molecules detected in plasma are likely the result of diffusion down a concentration gradient into the blood. Moreover, the presence of paraoxonases enzymes (PON) in the blood, may also account for the lower concentrations of these molecules detected in sera [323].

Measuring these small levels of 3-oxo-C12-HSL in plasma represents an analytical challenge. The low levels of C4-HSL detected in sputum goes in accordance with the results previously obtained [322]. On the other hand, the absence of C4-HSL in plasma may be a result of lower levels of this molecule produced and differences in the rate of metabolism or transfer from the lungs to the blood. However there were not sufficient patients that ended the study to compare the difference levels of 3-oxo-C12-HSL between the beginning and the end of the study in placebo and garlic treated patients.

The 3-oxo-C12-HSL molecule was previously detected using a biosensor system, in blood from sepsis patients and its presence has been correlated with the severity of the disease in these patients [324]. Hence the correlation between 3-oxo-C12-HSL detected in blood and sputum from CF patients could be used in the future to establish a connection between the presence of these molecules in sputum and their clinical status. Moreover the detection of 3-oxo-C12-HSL in plasma could be used as a biomarker for *P. aeruginosa* in CF patients. Further studies must be conducted to improve the sensitivity of the detection and to determine if the 3-oxo-C12-HSL levels correlate with the amount of *P. aeruginosa* in the lungs of CF patients. One of the main problems after a lung transplantation event is that often the transplanted lungs are re-colonised by *P. aeruginosa* [325]. For this reason it is important to monitor the presence of 3-oxo-C12-HSL signal molecule in order to proceed with specific antibiotic treatments. However once the signal molecules are detected in sputum, the infection has reached the chronic state [326]. It was recently shown that bronchoalveolar lavages from patients which had a lung transplant, had QS molecules even in the absence of apparent infection [327], supporting the idea that the detection of these molecules may be possible before *P. aeruginosa* is identified in sputum. Hence the early detection of 3-oxo-C12-HSL in plasma may allow for a more timely eradication therapy to be applied.

8.5 CONCLUSION

Although there are many studies conducted in animal models demonstrating the efficiency of natural and synthetic compounds for the treatment of *P. aeruginosa* infections by down regulating QS dependent virulence genes, up to now there have been no previous trial designed to evaluate the potential of a drug as QS inhibitor in humans. This trial was however too small to demonstrate statistically significant improvements in clinical outcomes and/or decrease in the signal molecule present in plasma and sputum. A much larger study would

therefore be needed to determine if garlic treatment is beneficial. Moreover, since garlic has been proven to have a strong anti bactericidal potential, further studies must be carried out for the identification of the active QS inhibiting component (or components) among the heterogeneous mixture of sulphur containing compounds found in macerated garlic oil [328]. This would allow for a more specific and probably more powerful treatment.

9 Bibliography

1. Finch, R.G., et al., *Quorum sensing: a novel target for anti-infective therapy.* J Antimicrob Chemother, 1998. **42**(5): p. 569-71.
2. Palleroni, N.J., *Pseudomonas classification. A new case history in the taxonomy of gram-negative bacteria.* Antonie Van Leeuwenhoek, 1993. **64**(3-4): p. 231-51.
3. Palleroni, N., *Human and animal-pathogenic pseudomonasds.* 2nd ed. The prokaryotes, a handbook on the biology of bacteria, ecophysiology, isolation, identification and applications, , ed. H.G.T. In a A. Belows, M. Dworkin, E. Harder, and K.H Schleifer Vol. III. 1992, New York. 3086-3103.
4. Van Delden, C., *Virulence factors in Pseudomonas aeruginosa.* In J.-L. Ramos (ed), Pseudomonas. Vol. II. 2004, New York: Kluwer Academic/Plenum Pubishers. 3-45.
5. Stover, C.K., et al., *Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen.* Nature, 2000. **406**(6799): p. 959-64.
6. Van Delden, C. and B.H. Iglewski, *Cell-to-cell signaling and Pseudomonas aeruginosa infections.* Emerg Infect Dis, 1998. **4**(4): p. 551-60.
7. Goodman, A.L. and S. Lory, *Analysis of regulatory networks in Pseudomonas aeruginosa by genomewide transcriptional profiling.* Curr Opin Microbiol, 2004. **7**(1): p. 39-44.
8. Sadikot, R.T., et al., *Pathogen-host interactions in Pseudomonas aeruginosa pneumonia.* Am J Respir Crit Care Med, 2005. **171**(11): p. 1209-23.
9. Gjodsbol, K., et al., *Multiple bacterial species reside in chronic wounds: a longitudinal study.* Int Wound J, 2006. **3**(3): p. 225-31.
10. Govan, J.R. and V. Deretic, *Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia.* Microbiol Rev, 1996. **60**(3): p. 539-74.
11. Middleton, B., et al., *Direct detection of N-acylhomoserine lactones in cystic fibrosis sputum.* FEMS Microbiol Lett, 2002. **207**(1): p. 1-7.
12. Lyczak, J.B., C.L. Cannon, and G.B. Pier, *Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist.* Microbes Infect, 2000. **2**(9): p. 1051-60.
13. Murray, T.S., M. Egan, and B.I. Kazmierczak, *Pseudomonas aeruginosa chronic colonization in cystic fibrosis patients.* Curr Opin Pediatr, 2007. **19**(1): p. 83-8.
14. Diekema, D.J., et al., *A cluster of serious Escherichia coli infections in a neonatal intensive-care unit.* Infect Control Hosp Epidemiol, 1997. **18**(11): p. 774-6.
15. Rello, J. and E. Diaz, *Pneumonia in the intensive care unit.* Crit Care Med, 2003. **31**(10): p. 2544-51.
16. Furukawa, K., *[Importance of appropriate carbapenem use to reduce carbapenem--resistant Pseudomonas aeruginosa].* Nippon Rinsho, 2007. **65 Suppl 2 Pt. 1**: p. 258-64.
17. Singh, P.K., et al., *Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms.* Nature, 2000. **407**(6805): p. 762-4.
18. Feldman, M., et al., *Role of flagella in pathogenesis of Pseudomonas aeruginosa pulmonary infection.* Infect Immun, 1998. **66**(1): p. 43-51.
19. DiMango, E., et al., *Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8.* J Clin Invest, 1995. **96**(5): p. 2204-10.

20. Adamo, R., et al., *Pseudomonas aeruginosa flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5.* Am J Respir Cell Mol Biol, 2004. **30**(5): p. 627-34.
21. Mahenthiralingam, E., M.E. Campbell, and D.P. Speert, *Nonmotility and phagocytic resistance of Pseudomonas aeruginosa isolates from chronically colonized patients with cystic fibrosis.* Infect Immun, 1994. **62**(2): p. 596-605.
22. Mattick, J.S., *Type IV pili and twitching motility.* Annu Rev Microbiol, 2002. **56**: p. 289-314.
23. Gupta, S.K., et al., *Pili and lipopolysaccharide of Pseudomonas aeruginosa bind to the glycolipid asialo GM1.* Infect Immun, 1994. **62**(10): p. 4572-9.
24. Hahn, H.P., *The type-4 pilus is the major virulence-associated adhesin of Pseudomonas aeruginosa--a review.* Gene, 1997. **192**(1): p. 99-108.
25. Farinha, M.A., et al., *Alteration of the pilin adhesin of Pseudomonas aeruginosa PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte cells and decreased virulence in mice.* Infect Immun, 1994. **62**(10): p. 4118-23.
26. Comolli, J.C., et al., *Pseudomonas aeruginosa gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia.* Infect Immun, 1999. **67**(7): p. 3625-30.
27. Hajjar, A.M., et al., *Human Toll-like receptor 4 recognizes host-specific LPS modifications.* Nat Immunol, 2002. **3**(4): p. 354-9.
28. Davies, J.C., *New therapeutic approaches for cystic fibrosis lung disease.* J R Soc Med, 2002. **95 Suppl 41**: p. 58-67.
29. Hentzer, M., et al., *Alginate overproduction affects Pseudomonas aeruginosa biofilm structure and function.* J Bacteriol, 2001. **183**(18): p. 5395-401.
30. Nicas, T.I. and B.H. Iglewski, *The contribution of exoproducts to virulence of Pseudomonas aeruginosa.* Can J Microbiol, 1985. **31**(4): p. 387-92.
31. Denning, G.M., et al., *Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells.* Infect Immun, 1998. **66**(12): p. 5777-84.
32. Leidal, K.G., K.L. Munson, and G.M. Denning, *Small molecular weight secretory factors from Pseudomonas aeruginosa have opposite effects on IL-8 and RANTES expression by human airway epithelial cells.* Am J Respir Cell Mol Biol, 2001. **25**(2): p. 186-95.
33. Allen, L., et al., *Pyocyanin production by Pseudomonas aeruginosa induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo.* J Immunol, 2005. **174**(6): p. 3643-9.
34. Lau, G.W., et al., *The role of pyocyanin in Pseudomonas aeruginosa infection.* Trends Mol Med, 2004. **10**(12): p. 599-606.
35. O'Malley, Y.Q., et al., *Pseudomonas aeruginosa pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells.* Am J Physiol Lung Cell Mol Physiol, 2004. **287**(1): p. L94-103.
36. Gallagher, L.A. and C. Manoil, *Pseudomonas aeruginosa PAO1 kills Caenorhabditis elegans by cyanide poisoning.* J Bacteriol, 2001. **183**(21): p. 6207-14.
37. Goldfarb, W.B. and H. Margraf, *Cyanide production by pseudomonas aeruginosa.* Ann Surg, 1967. **165**(1): p. 104-10.
38. Visca, P., F. Imperi, and I.L. Lamont, *Pyoverdine siderophores: from biogenesis to biosignificance.* Trends Microbiol, 2007. **15**(1): p. 22-30.
39. Wick, M.J., et al., *Structure, function, and regulation of Pseudomonas aeruginosa exotoxin A.* Annu Rev Microbiol, 1990. **44**: p. 335-63.
40. Schultz, M.J., et al., *Impairment of host defence by exotoxin A in Pseudomonas aeruginosa pneumonia in mice.* J Med Microbiol, 2001. **50**(9): p. 822-7.

41. Parmely, M., et al., *Proteolytic inactivation of cytokines by Pseudomonas aeruginosa.* Infect Immun, 1990. **58**(9): p. 3009-14.
42. Galloway, D.R., *Pseudomonas aeruginosa elastase and elastolysis revisited: recent developments.* Mol Microbiol, 1991. **5**(10): p. 2315-21.
43. Azghani, A.O., T. Bedinghaus, and R. Klein, *Detection of elastase from Pseudomonas aeruginosa in sputum and its potential role in epithelial cell permeability.* Lung, 2000. **178**(3): p. 181-9.
44. Kon, Y., et al., *The role of Pseudomonas aeruginosa elastase as a potent inflammatory factor in a rat air pouch inflammation model.* FEMS IMMUNOL MED MICROBIOL, 1999. **25**(3): p. 313-21.
45. Read, R.C., et al., *Effect of Pseudomonas aeruginosa rhamnolipids on mucociliary transport and ciliary beating.* J Appl Physiol, 1992. **72**(6): p. 2271-7.
46. Sawa, T. and J.P. Wiener-Kronish, *A therapeutic strategy against the shared virulence mechanism utilized by both Yersinia pestis and Pseudomonas aeruginosa.* Anesthesiol Clin North America, 2004. **22**(3): p. 591-606, viii-ix.
47. Lee, V.T., et al., *Activities of Pseudomonas aeruginosa effectors secreted by the Type III secretion system in vitro and during infection.* Infect Immun, 2005. **73**(3): p. 1695-705.
48. Parsek, M.R. and E.P. Greenberg, *Quorum sensing signals in development of Pseudomonas aeruginosa biofilms.* Methods Enzymol, 1999. **310**: p. 43-55.
49. kolter, R., *Microbial sciences: the superficial life of micorbes.* Nature, 2006. **441**: p. 300-302.
50. Greenberg, E.P., *Bacterial communication: tiny teamwork.* Nature, 2003. **424**(6945): p. 134.
51. Hentzer, M., et al., *Quorum sensing : a novel target for the treatment of biofilm infections.* BioDrugs, 2003. **17**(4): p. 241-50.
52. O'Toole, G.A., et al., *The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa.* J Bacteriol, 2000. **182**(2): p. 425-31.
53. Latifi, A., et al., *Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in Pseudomonas aeruginosa PAO1.* Mol Microbiol, 1995. **17**(2): p. 333-43.
54. Aeschlimann, J.R., *The role of multidrug efflux pumps in the antibiotic resistance of Pseudomonas aeruginosa and other gram-negative bacteria. Insights from the Society of Infectious Diseases Pharmacists.* Pharmacotherapy, 2003. **23**(7): p. 916-24.
55. Bonomo, R.A. and D. Szabo, *Mechanisms of multidrug resistance in Acinetobacter species and Pseudomonas aeruginosa.* Clin Infect Dis, 2006. **43 Suppl 2**: p. S49-56.
56. Gottesman, S., *Bacterial regulation: global regulatory networks.* Annu Rev Genet, 1984. **18**: p. 415-41.
57. Gruber, T.M. and C.A. Gross, *Multiple sigma subunits and the partitioning of bacterial transcription space.* Annu Rev Microbiol, 2003. **57**: p. 441-66.
58. Potvin, E., F. Sanschagrin, and R.C. Levesque, *Sigma factors in Pseudomonas aeruginosa.* FEMS Microbiol Rev, 2008. **32**(1): p. 38-55.
59. Atlung, T. and H. Ingmer, *H-NS: a modulator of environmentally regulated gene expression.* Mol Microbiol, 1997. **24**(1): p. 7-17.
60. Bruckner, R. and F. Titgemeyer, *Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization.* FEMS Microbiol Lett, 2002. **209**(2): p. 141-8.
61. Pruss, B.M., et al., *A complex transcription network controls the early stages of biofilm development by Escherichia coli.* J Bacteriol, 2006. **188**(11): p. 3731-9.

62. Nealson, K.H. and J.W. Hastings, *Bacterial bioluminescence: its control and ecological significance.* Microbiol Rev, 1979. **43**(4): p. 496-518.
63. Waters, C.M. and B.L. Bassler, *Quorum sensing: cell-to-cell communication in bacteria.* Annu Rev Cell Dev Biol, 2005. **21**: p. 319-46.
64. Bassler, B.L., *Small talk. Cell-to-cell communication in bacteria.* Cell, 2002. **109**(4): p. 421-4.
65. Fuqua, C., M.R. Parsek, and E.P. Greenberg, *Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing.* Annu Rev Genet, 2001. **35**: p. 439-68.
66. Sturme, M.H., et al., *Cell to cell communication by autoinducing peptides in gram-positive bacteria.* Antonie Van Leeuwenhoek, 2002. **81**(1-4): p. 233-43.
67. Schaefer, A.L., et al., *Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified Vibrio fischeri LuxI protein.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9505-9.
68. Stevens, E.J., et al., *A quantitative model of invasive Pseudomonas infection in burn injury.* J Burn Care Rehabil, 1994. **15**(3): p. 232-5.
69. Slock, J., et al., *Critical regions of the Vibrio fischeri luxR protein defined by mutational analysis.* J Bacteriol, 1990. **172**(7): p. 3974-9.
70. Gambello, M.J. and B.H. Iglewski, *Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression.* J Bacteriol, 1991. **173**(9): p. 3000-9.
71. Pearson, J.P., et al., *Structure of the autoinducer required for expression of Pseudomonas aeruginosa virulence genes.* Proc Natl Acad Sci U S A, 1994. **91**(1): p. 197-201.
72. Duan, K., et al., *Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication.* Mol Microbiol, 2003. **50**(5): p. 1477-91.
73. Ben Jacob, E., et al., *Bacterial linguistic communication and social intelligence.* Trends Microbiol, 2004. **12**(8): p. 366-72.
74. Eberl, L. and B. Tummler, *Pseudomonas aeruginosa and Burkholderia cepacia in cystic fibrosis: genome evolution, interactions and adaptation.* Int J Med Microbiol, 2004. **294**(2-3): p. 123-31.
75. Smith, R.S. and B.H. Iglewski, *Pseudomonas aeruginosa quorum sensing as a potential antimicrobial target.* J Clin Invest, 2003. **112**(10): p. 1460-5.
76. Whitehead, N.A., et al., *Quorum-sensing in Gram-negative bacteria.* FEMS Microbiol Rev, 2001. **25**(4): p. 365-404.
77. Schuster, M., M.L. Urbanowski, and E.P. Greenberg, *Promoter specificity in Pseudomonas aeruginosa quorum sensing revealed by DNA binding of purified LasR.* Proc Natl Acad Sci U S A, 2004. **101**(45): p. 15833-9.
78. Seed, P.C., L. Passador, and B.H. Iglewski, *Activation of the Pseudomonas aeruginosa lasI gene by LasR and the Pseudomonas autoinducer PAI: an autoinduction regulatory hierarchy.* J Bacteriol, 1995. **177**(3): p. 654-9.
79. de Kievit, T.R. and B.H. Iglewski, *Bacterial quorum sensing in pathogenic relationships.* Infect Immun, 2000. **68**(9): p. 4839-49.
80. Latifi, A., et al., *A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS.* Mol Microbiol, 1996. **21**(6): p. 1137-46.
81. Pessi, G. and D. Haas, *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): p. 6940-9.

82. Pearson, J.P., et al., *A second N-acylhomoserine lactone signal produced by Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 1995. **92**(5): p. 1490-4.
83. Pesci, E.C., et al., *Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa.* J Bacteriol, 1997. **179**(10): p. 3127-32.
84. Pesci, E.C. and B.H. Iglewski, *The chain of command in Pseudomonas quorum sensing.* Trends Microbiol, 1997. **5**(4): p. 132-4; discussion 134-5.
85. Chugani, S.A., et al., *QscR, a modulator of quorum-sensing signal synthesis and virulence in Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 2001. **98**(5): p. 2752-7.
86. Ledgham, F., et al., *Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of Pseudomonas aeruginosa LasR and RhlR.* Mol Microbiol, 2003. **48**(1): p. 199-210.
87. Lee, J.H., Y. Lequette, and E.P. Greenberg, *Activity of purified QscR, a Pseudomonas aeruginosa orphan quorum-sensing transcription factor.* Mol Microbiol, 2006. **59**(2): p. 602-9.
88. Lequette, Y., et al., *A distinct QscR regulon in the Pseudomonas aeruginosa quorum-sensing circuit.* J Bacteriol, 2006. **188**(9): p. 3365-70.
89. Juhas, M., et al., *Global regulation of quorum sensing and virulence by VqsR in Pseudomonas aeruginosa.* Microbiology, 2004. **150**(Pt 4): p. 831-41.
90. Li, L.L., J.E. Malone, and B.H. Iglewski, *Regulation of the Pseudomonas aeruginosa quorum-sensing regulator VqsR.* J Bacteriol, 2007. **189**(12): p. 4367-74.
91. Williams, P. and M. Camara, *Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules.* Curr Opin Microbiol, 2009. **12**(2): p. 182-91.
92. Pesci, E.C., et al., *Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 11229-34.
93. McKnight, S.L., B.H. Iglewski, and E.C. Pesci, *The Pseudomonas quinolone signal regulates rhl quorum sensing in Pseudomonas aeruginosa.* J Bacteriol, 2000. **182**(10): p. 2702-8.
94. Diggle, S.P., et al., *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.* Mol Microbiol, 2003. **50**(1): p. 29-43.
95. Wade, D.S., et al., *Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa.* J Bacteriol, 2005. **187**(13): p. 4372-80.
96. McGrath, S., D.S. Wade, and E.C. Pesci, *Dueling quorum sensing systems in Pseudomonas aeruginosa control the production of the Pseudomonas quinolone signal (PQS).* FEMS Microbiol Lett, 2004. **230**(1): p. 27-34.
97. Xiao, G., J. He, and L.G. Rahme, *Mutation analysis of the Pseudomonas aeruginosa mvfR and pqsABCDE gene promoters demonstrates complex quorum-sensing circuitry.* Microbiology, 2006. **152**(Pt 6): p. 1679-86.
98. Diggle, S.P., et al., *The Pseudomonas aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment.* Chem Biol, 2007. **14**(1): p. 87-96.
99. Haussler, S. and T. Becker, *The pseudomonas quinolone signal (PQS) balances life and death in Pseudomonas aeruginosa populations.* PLoS Pathog, 2008. **4**(9): p. e1000166.
100. Mashburn, L.M., et al., *Staphylococcus aureus serves as an iron source for Pseudomonas aeruginosa during in vivo coculture.* J Bacteriol, 2005. **187**(2): p. 554-66.

101. D'Argenio, D.A., et al., *Autolysis and autoaggregation in Pseudomonas aeruginosa colony morphology mutants.* J Bacteriol, 2002. **184**(23): p. 6481-9.
102. Allesen-Holm, M., et al., *A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms.* Mol Microbiol, 2006. **59**(4): p. 1114-28.
103. Collier, D.N., et al., *A bacterial cell to cell signal in the lungs of cystic fibrosis patients.* FEMS Microbiol Lett, 2002. **215**(1): p. 41-6.
104. Gallagher, L.A., et al., *Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa.* J Bacteriol, 2002. **184**(23): p. 6472-80.
105. Mahajan-Miklos, S., et al., *Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis model.* Cell, 1999. **96**(1): p. 47-56.
106. Hooi, D.S., et al., *Differential immune modulatory activity of Pseudomonas aeruginosa quorum-sensing signal molecules.* Infect Immun, 2004. **72**(11): p. 6463-70.
107. Skindersoe, M.E., et al., *Pseudomonas aeruginosa quorum-sensing signal molecules interfere with dendritic cell-induced T-cell proliferation.* FEMS IMMUNOL MED MICROBIOL, 2009. **55**(3): p. 335-45.
108. Kim, K., et al., *HHQ and PQS, two Pseudomonas aeruginosa quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway.* Immunology. **129**(4): p. 578-88.
109. Deziel, E., et al., *Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1339-44.
110. Lepine, F., et al., *Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by Pseudomonas aeruginosa.* J Am Soc Mass Spectrom, 2004. **15**(6): p. 862-9.
111. Lepine, F., et al., *A stable isotope dilution assay for the quantification of the Pseudomonas quinolone signal in Pseudomonas aeruginosa cultures.* Biochim Biophys Acta, 2003. **1622**(1): p. 36-41.
112. Yu, S., et al., *Structure elucidation and preliminary assessment of hydrolase activity of PqsE, the Pseudomonas quinolone signal (PQS) response protein.* Biochemistry, 2009. **48**(43): p. 10298-307.
113. Rampioni, G., et al., *Transcriptomic analysis reveals a global alkyl-quinolone-independent regulatory role for PqsE in facilitating the environmental adaptation of Pseudomonas aeruginosa to plant and animal hosts.* Environ Microbiol. **12**(6): p. 1659-73.
114. Diggle, S.P., et al., *Functional genetic analysis reveals a 2-Alkyl-4-quinolone signaling system in the human pathogen Burkholderia pseudomallei and related bacteria.* Chem Biol, 2006. **13**(7): p. 701-10.
115. Calfee, M.W., et al., *Solubility and bioactivity of the Pseudomonas quinolone signal are increased by a Pseudomonas aeruginosa-produced surfactant.* Infect Immun, 2005. **73**(2): p. 878-82.
116. Wagner, V.E., et al., *Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment.* J Bacteriol, 2003. **185**(7): p. 2080-95.
117. Schuster, M., et al., *Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis.* J Bacteriol, 2003. **185**(7): p. 2066-79.
118. Hentzer, M., et al., *Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors.* EMBO J, 2003. **22**(15): p. 3803-15.

119. Whiteley, M., K.M. Lee, and E.P. Greenberg, *Identification of genes controlled by quorum sensing in Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 13904-9.
120. Venturi, V., *Regulation of quorum sensing in Pseudomonas.* FEMS Microbiol Rev, 2006. **30**(2): p. 274-91.
121. Wadhams, G.H. and J.P. Armitage, *Making sense of it all: bacterial chemotaxis.* Nat Rev Mol Cell Biol, 2004. **5**(12): p. 1024-37.
122. Galperin, M.Y., *Sampling of microbial diversity by complete genomes.* Environ Microbiol, 2006. **8**(8): p. 1313-7.
123. Stock, J. and S. Da Re, *Signal transduction: response regulators on and off.* Curr Biol, 2000. **10**(11): p. R420-4.
124. Mascher, T., et al., *The CiaRH system of Streptococcus pneumoniae prevents lysis during stress induced by treatment with cell wall inhibitors and by mutations in pbp2x involved in beta-lactam resistance.* J Bacteriol, 2006. **188**(5): p. 1959-68.
125. Rodrigue, A., et al., *Two-component systems in Pseudomonas aeruginosa: why so many?* Trends Microbiol, 2000. **8**(11): p. 498-504.
126. Romby, P., F. Vandenesch, and E.G. Wagner, *The role of RNAs in the regulation of virulence-gene expression.* Curr Opin Microbiol, 2006. **9**(2): p. 229-36.
127. Toledo-Arana, A., F. Repoila, and P. Cossart, *Small noncoding RNAs controlling pathogenesis.* Curr Opin Microbiol, 2007. **10**(2): p. 182-8.
128. Valverde, C. and D. Haas, *Small RNAs controlled by two-component systems.* Adv Exp Med Biol, 2008. **631**: p. 54-79.
129. Majdalani, N., et al., *Role of RcsF in signaling to the Rcs phosphorelay pathway in Escherichia coli.* J Bacteriol, 2005. **187**(19): p. 6770-8.
130. Heidrich, N., et al., *The small untranslated RNA SR1 from the Bacillus subtilis genome is involved in the regulation of arginine catabolism.* Mol Microbiol, 2006. **62**(2): p. 520-36.
131. Babitzke, P. and T. Romeo, *CsrB sRNA family: sequestration of RNA-binding regulatory proteins.* Curr Opin Microbiol, 2007. **10**(2): p. 156-63.
132. Storz, G., S. Altuvia, and K.M. Wassarman, *An abundance of RNA regulators.* Annu Rev Biochem, 2005. **74**: p. 199-217.
133. Romeo, T., et al., *Identification and molecular characterization of csrA, a pleiotropic gene from Escherichia coli that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties.* J Bacteriol, 1993. **175**(15): p. 4744-55.
134. Romeo, T., *Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB.* Mol Microbiol, 1998. **29**(6): p. 1321-30.
135. Wei, B.L., et al., *Positive regulation of motility and flhDC expression by the RNA-binding protein CsrA of Escherichia coli.* Mol Microbiol, 2001. **40**(1): p. 245-56.
136. Weilbacher, T., et al., *A novel sRNA component of the carbon storage regulatory system of Escherichia coli.* Mol Microbiol, 2003. **48**(3): p. 657-70.
137. Burrowes, E., et al., *Characterisation of the regulatory RNA RsmB from Pseudomonas aeruginosa PAO1.* Res Microbiol, 2005. **156**(1): p. 7-16.
138. White, D., M.E. Hart, and T. Romeo, *Phylogenetic distribution of the global regulatory gene csrA among eubacteria.* Gene, 1996. **182**(1-2): p. 221-3.
139. Chen, Y.T., et al., *Evolutionary analysis of the two-component systems in Pseudomonas aeruginosa PAO1.* J Mol Evol, 2004. **59**(6): p. 725-37.
140. Hsu, J.L., et al., *Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in Pseudomonas aeruginosa PAO1.* J Biol Chem, 2008. **283**(15): p. 9933-44.

141. Pessi, G. and D. Haas, *Dual control of hydrogen cyanide biosynthesis by the global activator GacA in Pseudomonas aeruginosa PAO1.* FEMS Microbiol Lett, 2001. **200**(1): p. 73-8.
142. Rahme, L.G., et al., *Use of model plant hosts to identify Pseudomonas aeruginosa virulence factors.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 13245-50.
143. Tan, M.W., S. Mahajan-Miklos, and F.M. Ausubel, *Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 715-20.
144. Jander, G., L.G. Rahme, and F.M. Ausubel, *Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects.* J Bacteriol, 2000. **182**(13): p. 3843-5.
145. Brinkman, F.S., et al., *Evolutionary relationships among virulence-associated histidine kinases.* Infect Immun, 2001. **69**(8): p. 5207-11.
146. Chieda, Y., et al., *Pathogenicity of gacA mutant of Pseudomonas aeruginosa PA01 in the silkworm, Bombyx mori.* FEMS Microbiol Lett, 2005. **244**(1): p. 181-6.
147. Reimmann, C., et al., *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.* Mol Microbiol, 1997. **24**(2): p. 309-19.
148. Parkins, M.D., H. Ceri, and D.G. Storey, *Pseudomonas aeruginosa GacA, a factor in multihost virulence, is also essential for biofilm formation.* Mol Microbiol, 2001. **40**(5): p. 1215-26.
149. Chatterjee, A., et al., *Inactivation of rsmA leads to overproduction of extracellular pectinases, cellulases, and proteases in Erwinia carotovora subsp. carotovora in the absence of the starvation/cell density-sensing signal, N-(3-oxohexanoyl)-L-homoserine lactone.* Appl Environ Microbiol, 1995. **61**(5): p. 1959-67.
150. Brencic, A. and S. Lory, *Determination of the regulon and identification of novel mRNA targets of Pseudomonas aeruginosa RsmA.* Mol Microbiol, 2009. **72**(3): p. 612-32.
151. Heurlier, K., et al., *Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in Pseudomonas aeruginosa PAO1.* J Bacteriol, 2004. **186**(10): p. 2936-45.
152. Pessi, G., et al., *The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in Pseudomonas aeruginosa.* J Bacteriol, 2001. **183**(22): p. 6676-83.
153. Cui, Y., et al., *Identification of a global repressor gene, rsmA, of Erwinia carotovora subsp. carotovora that controls extracellular enzymes, N-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting Erwinia spp.* J Bacteriol, 1995. **177**(17): p. 5108-15.
154. Goodman, A.L., et al., *A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in Pseudomonas aeruginosa.* Dev Cell, 2004. **7**(5): p. 745-54.
155. Ventre, I., et al., *Multiple sensors control reciprocal expression of Pseudomonas aeruginosa regulatory RNA and virulence genes.* Proc Natl Acad Sci U S A, 2006. **103**(1): p. 171-6.
156. Goodman, A.L., et al., *Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen.* Genes Dev, 2009. **23**(2): p. 249-59.
157. Laskowski, M.A., E. Osborn, and B.I. Kazmierczak, *A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in Pseudomonas aeruginosa.* Mol Microbiol, 2004. **54**(4): p. 1090-103.

158. Laskowski, M.A. and B.I. Kazmierczak, *Mutational analysis of RetS, an unusual sensor kinase-response regulator hybrid required for Pseudomonas aeruginosa virulence.* Infect Immun, 2006. **74**(8): p. 4462-73.
159. Petrova, O.E. and K. Sauer, *The novel Two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA rsmZ through CafA.* J. Bac, 2010. **192**(20): p. e1000668.
160. Bordi, C., et al., *Regulatory RNAs and the HptB/RetS signalling pathways fine-tune Pseudomonas aeruginosa pathogenesis.* Mol Microbiol. **76**(6): p. 1427-43.
161. Tang, H.B., et al., *Contribution of specific Pseudomonas aeruginosa virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection.* Infect Immun, 1996. **64**(1): p. 37-43.
162. Telford, G., et al., *The Pseudomonas aeruginosa quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity.* Infect Immun, 1998. **66**(1): p. 36-42.
163. Pritchard, D.I., et al., *Alleviation of insulitis and moderation of diabetes in NOD mice following treatment with a synthetic Pseudomonas aeruginosa signal molecule, N-(3-oxododecanoyl)-L-homoserine lactone.* Acta Diabetol, 2005. **42**(3): p. 119-22.
164. Smith, R.S., et al., *IL-8 production in human lung fibroblasts and epithelial cells activated by the Pseudomonas autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2.* J Immunol, 2001. **167**(1): p. 366-74.
165. Smith, R.S., et al., *The Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)homoserine lactone contributes to virulence and induces inflammation in vivo.* J Bacteriol, 2002. **184**(4): p. 1132-9.
166. Hassett, D.J., et al., *Pseudomonas aeruginosa biofilm sensitivity to biocides: use of hydrogen peroxide as model antimicrobial agent for examining resistance mechanisms.* Methods Enzymol, 1999. **310**: p. 599-608.
167. Smith, R.S. and B.H. Iglewski, *P. aeruginosa quorum-sensing systems and virulence.* Curr Opin Microbiol, 2003. **6**(1): p. 56-60.
168. Lawrence, R.N., et al., *The Pseudomonas aeruginosa quorum-sensing signal molecule, N-(3-oxododecanoyl)-L-homoserine lactone, inhibits porcine arterial smooth muscle contraction.* Br J Pharmacol, 1999. **128**(4): p. 845-8.
169. Hentzer, M. and M. Givskov, *Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections.* J Clin Invest, 2003. **112**(9): p. 1300-7.
170. Parsek, M.R., et al., *Acyl homoserine-lactone quorum-sensing signal generation.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4360-5.
171. Byers, J.T., et al., *Nonenzymatic turnover of an Erwinia carotovora quorum-sensing signaling molecule.* J Bacteriol, 2002. **184**(4): p. 1163-71.
172. Yates, E.A., et al., *N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of Yersinia pseudotuberculosis and Pseudomonas aeruginosa.* Infect Immun, 2002. **70**(10): p. 5635-46.
173. Kaufmann, G.F., et al., *Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones.* Proc Natl Acad Sci U S A, 2005. **102**(2): p. 309-14.
174. Dong, Y.H., et al., *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): p. 3526-31.
175. Leadbetter, J.R. and E.P. Greenberg, *Metabolism of acyl-homoserine lactone quorum-sensing signals by Variovorax paradoxus.* J Bacteriol, 2000. **182**(24): p. 6921-6.

176. Chun, C.K., et al., *Inactivation of a Pseudomonas aeruginosa quorum-sensing signal by human airway epithelia.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3587-90.
177. Ozer, E.A., et al., *Human and murine paraoxonase 1 are host modulators of Pseudomonas aeruginosa quorum-sensing.* FEMS Microbiol Lett, 2005. **253**(1): p. 29-37.
178. Cha, C., et al., *Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria.* Mol Plant Microbe Interact, 1998. **11**(11): p. 1119-29.
179. Gao, M., et al., *Production of substances by Medicago truncatula that affect bacterial quorum sensing.* Mol Plant Microbe Interact, 2003. **16**(9): p. 827-34.
180. Rasmussen, T.B., et al., *Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector.* J Bacteriol, 2005. **187**(5): p. 1799-814.
181. de Nys, R., et al., *Furanones.* Prog Mol Subcell Biol, 2006. **42**: p. 55-86.
182. Hentzer, M., et al., *Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound.* Microbiology, 2002. **148**(Pt 1): p. 87-102.
183. Manefield, M., et al., *Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover.* Microbiology, 2002. **148**(Pt 4): p. 1119-27.
184. Chhabra, S.R., et al., *Synthetic analogues of the bacterial signal (quorum sensing) molecule N-(3-oxododecanoyl)-L-homoserine lactone as immune modulators.* J Med Chem, 2003. **46**(1): p. 97-104.
185. Frank, L.H. and R.D. Demoss, *On the biosynthesis of pyocyanine.* J Bacteriol, 1959. **77**(6): p. 776-82.
186. Rashid, M.H. and A. Kornberg, *Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 2000. **97**(9): p. 4885-90.
187. Grant, S.G., et al., *Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4645-9.
188. Simon, M. and J.S. Hong, *Direct homocysteine biosynthesis from O-succinylhomoserine in Escherichia coli: an alternate pathway that bypasses cystathionine.* J Bacteriol, 1983. **153**(1): p. 558-61.
189. Studier, F.W. and B.A. Moffatt, *Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes.* J Mol Biol, 1986. **189**(1): p. 113-30.
190. Van Houdt, R., et al., *N-acyl-L-homoserine lactone signal interception by Escherichia coli.* FEMS Microbiol Lett, 2006. **256**(1): p. 83-9.
191. Voisard, C., et al., *Cyanide production by Pseudomonas fluorescens helps suppress black root rot of tobacco under gnotobiotic conditions.* EMBO J, 1989. **8**(2): p. 351-8.
192. Beatson, S.A., et al., *Differential regulation of twitching motility and elastase production by Vfr in Pseudomonas aeruginosa.* J Bacteriol, 2002. **184**(13): p. 3605-13.
193. Aendekerk, S., et al., *The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in Pseudomonas aeruginosa via 4-quinolone-dependent cell-to-cell communication.* Microbiology, 2005. **151**(Pt 4): p. 1113-25.
194. Winzer, K., et al., *The Pseudomonas aeruginosa lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS.* J Bacteriol, 2000. **182**(22): p. 6401-11.
195. Vieira, J. and J. Messing, *New pUC-derived cloning vectors with different selectable markers and DNA replication origins.* Gene, 1991. **100**: p. 189-94.
196. Larsen, R.A., et al., *Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria.* Arch Microbiol, 2002. **178**(3): p. 193-201.

197. Anand, R.D., O. Sertil, and C.V. Lowry, *Restriction digestion monitors facilitate plasmid construction and PCR cloning.* Biotechniques, 2004. **36**(6): p. 982-5.
198. Becher, A. and H.P. Schweizer, *Integration-proficient Pseudomonas aeruginosa vectors for isolation of single-copy chromosomal lacZ and lux gene fusions.* Biotechniques, 2000. **29**(5): p. 948-50, 952.
199. Hoang, T.T., et al., *A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants.* Gene, 1998. **212**(1): p. 77-86.
200. Heeb, S., et al., *Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria.* Mol Plant Microbe Interact, 2000. **13**(2): p. 232-7.
201. Schnider-Keel, U., et al., *Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent Pseudomonas fluorescens CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin.* J Bacteriol, 2000. **182**(5): p. 1215-25.
202. Heeb, S., C. Blumer, and D. Haas, *Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in Pseudomonas fluorescens CHA0.* J Bacteriol, 2002. **184**(4): p. 1046-56.
203. Del Sal G, M.G., Schneider C., *A one-tube plasmid DNA mini-preparation suitable for sequencing.* Nucleic Acids Res, 1988. **20**(1988 Oct).
204. sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning - a laboratory manual*, l. press, Editor. 1989, cold spring harbor.
205. Gamper, M., et al., *RNA processing modulates the expression of the arcDABC operon in Pseudomonas aeruginosa.* J Mol Biol, 1992. **226**(4): p. 943-57.
206. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
207. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
208. Westfall, L.W., et al., *The Pseudomonas aeruginosa global regulator MvaT specifically binds to the ptxS upstream region and enhances ptxS expression.* Microbiology, 2004. **150**(Pt 11): p. 3797-806.
209. Miller, J.H., Jr., *Experiments in molecular genetics*. 1972, N.Y.: cold spring harbor laboratory.
210. Fletcher, M.P., et al., *Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules.* Nat Protoc, 2007. **2**(5): p. 1254-62.
211. Ortori, C.A., et al., *Comprehensive profiling of N-acylhomoserine lactones produced by Yersinia pseudotuberculosis using liquid chromatography coupled to hybrid quadrupole-linear ion trap mass spectrometry.* Anal Bioanal Chem, 2007. **387**(2): p. 497-511.
212. Galperin, M.Y. and A.N. Nikolskaya, *Identification of sensory and signal-transducing domains in two-component signaling systems.* Methods Enzymol, 2007. **422**: p. 47-74.
213. Marles-Wright, J. and R.J. Lewis, *Stress responses of bacteria.* Curr Opin Struct Biol, 2007. **17**(6): p. 755-60.
214. Cegelski, L., et al., *The biology and future prospects of antivirulence therapies.* Nat Rev Microbiol, 2008. **6**(1): p. 17-27.
215. Okada, A., et al., *Walkmycin B targets WalK (YycG), a histidine kinase essential for bacterial cell growth.* J Antibiot (Tokyo). **63**(2): p. 89-94.
216. Gotoh, Y., et al., *Two-component signal transduction as potential drug targets in pathogenic bacteria.* Curr Opin Microbiol. **13**(2): p. 232-9.
217. Gotoh, Y., et al., *Novel antibacterial compounds specifically targeting the essential WalR response regulator.* J Antibiot (Tokyo). **63**(3): p. 127-34.

218. Rasko, D.A., et al., *Targeting QseC signaling and virulence for antibiotic development.* Science, 2008. **321**(5892): p. 1078-80.
219. Gooderham, W.J. and R.E. Hancock, *Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa.* FEMS Microbiol Rev, 2009. **33**(2): p. 279-94.
220. Busch, A., et al., *Bacterial sensor kinase TodS interacts with agonistic and antagonistic signals.* Proc Natl Acad Sci U S A, 2007. **104**(34): p. 13774-9.
221. Dubuis, C. and D. Haas, *Cross-species GacA-controlled induction of antibiosis in pseudomonads.* Appl Environ Microbiol, 2007. **73**(2): p. 650-4.
222. Dubuis, C., et al., *Thiamine-auxotrophic mutants of Pseudomonas fluorescens CHA0 are defective in cell-cell signaling and biocontrol factor expression.* Appl Environ Microbiol, 2006. **72**(4): p. 2606-13.
223. Kay, E., C. Dubuis, and D. Haas, *Three small RNAs jointly ensure secondary metabolism and biocontrol in Pseudomonas fluorescens CHA0.* Proc Natl Acad Sci U S A, 2005. **102**(47): p. 17136-41.
224. Zuber, S., et al., *GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of Pseudomonas fluorescens CHA0.* Mol Plant Microbe Interact, 2003. **16**(7): p. 634-44.
225. Davies, D.G. and C.N. Marques, *A fatty acid messenger is responsible for inducing dispersion in microbial biofilms.* J Bacteriol, 2009. **191**(5): p. 1393-403.
226. Brencic, A., et al., *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.* Mol Microbiol, 2009. **73**(3): p. 434-45.
227. Valverde, C., et al., *RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in Pseudomonas fluorescens CHA0.* Mol Microbiol, 2003. **50**(4): p. 1361-79.
228. Kay, E., et al., *Two GacA-dependent small RNAs modulate the quorum-sensing response in Pseudomonas aeruginosa.* J Bacteriol, 2006. **188**(16): p. 6026-33.
229. Beatson, S.A., et al., *Quorum sensing is not required for twitching motility in Pseudomonas aeruginosa.* J Bacteriol, 2002. **184**(13): p. 3598-604.
230. Blumer, C., et al., *Global GacA-steered control of cyanide and exoprotease production in Pseudomonas fluorescens involves specific ribosome binding sites.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 14073-8.
231. Dow, J.M., et al., *Biofilm dispersal in Xanthomonas campestris is controlled by cell-cell signaling and is required for full virulence to plants.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10995-1000.
232. Fouhy, Y., et al., *Diffusible signal factor-dependent cell-cell signaling and virulence in the nosocomial pathogen Stenotrophomonas maltophilia.* J Bacteriol, 2007. **189**(13): p. 4964-8.
233. He, Y.W. and L.H. Zhang, *Quorum sensing and virulence regulation in Xanthomonas campestris.* FEMS Microbiol Rev, 2008. **32**(5): p. 842-57.
234. Kulasakara, H., et al., *Analysis of Pseudomonas aeruginosa diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2839-44.
235. Kuchma, S.L., et al., *BifA, a cyclic-Di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by Pseudomonas aeruginosa PA14.* J Bacteriol, 2007. **189**(22): p. 8165-78.
236. Lee, V.T., et al., *A cyclic-di-GMP receptor required for bacterial exopolysaccharide production.* Mol Microbiol, 2007. **65**(6): p. 1474-84.

237. Merritt, J.H., et al., *SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function.* J Bacteriol, 2007. **189**(22): p. 8154-64.
238. Hickman, J.W. and C.S. Harwood, *Identification of FleQ from Pseudomonas aeruginosa as a c-di-GMP-responsive transcription factor.* Mol Microbiol, 2008. **69**(2): p. 376-89.
239. Ryan, R.P., et al., *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): p. 6712-7.
240. Pearson, J.P., E.C. Pesci, and B.H. Iglewski, *Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes.* J Bacteriol, 1997. **179**(18): p. 5756-67.
241. Diggle, S.P., et al., *The galactophilic lectin, LecA, contributes to biofilm development in Pseudomonas aeruginosa.* Environ Microbiol, 2006. **8**(6): p. 1095-104.
242. Adam, E.C., D.U. Schumacher, and U. Schumacher, *Cilia from a cystic fibrosis patient react to the ciliotoxic Pseudomonas aeruginosa II lectin in a similar manner to normal control cilia--a case report.* J Laryngol Otol, 1997. **111**(8): p. 760-2.
243. Bajolet-Laudinat, O., et al., *Cytotoxicity of Pseudomonas aeruginosa internal lectin PA-I to respiratory epithelial cells in primary culture.* Infect Immun, 1994. **62**(10): p. 4481-7.
244. Diggle, S.P., et al., *Advancing the quorum in Pseudomonas aeruginosa: MvaT and the regulation of N-acylhomoserine lactone production and virulence gene expression.* J Bacteriol, 2002. **184**(10): p. 2576-86.
245. Tendeng, C., et al., *MvaT proteins in Pseudomonas spp.: a novel class of H-NS-like proteins.* Microbiology, 2003. **149**(Pt 11): p. 3047-50.
246. Vallet, I., et al., *Biofilm formation in Pseudomonas aeruginosa: fimbrial cup gene clusters are controlled by the transcriptional regulator MvaT.* J Bacteriol, 2004. **186**(9): p. 2880-90.
247. Bazire, A., et al., *The sigma factor AlgU plays a key role in formation of robust biofilms by nonmucoid Pseudomonas aeruginosa.* J Bacteriol. **192**(12): p. 3001-10.
248. Kohler, T., et al., *Swarming of Pseudomonas aeruginosa is dependent on cell-to-cell signaling and requires flagella and pili.* J Bacteriol, 2000. **182**(21): p. 5990-6.
249. Pernestig, A.K., et al., *The Escherichia coli BarA-UvrY two-component system is needed for efficient switching between glycolytic and gluconeogenic carbon sources.* J Bacteriol, 2003. **185**(3): p. 843-53.
250. Farrow, J.M., 3rd, et al., *PqsE functions independently of PqsR-Pseudomonas quinolone signal and enhances the rhl quorum-sensing system.* J Bacteriol, 2008. **190**(21): p. 7043-51.
251. Antunes, L.C., et al., *A mutational analysis defines Vibrio fischeri LuxR binding sites.* J Bacteriol, 2008. **190**(13): p. 4392-7.
252. Lindsay, A. and B.M. Ahmer, *Effect of sdiA on biosensors of N-acylhomoserine lactones.* J Bacteriol, 2005. **187**(14): p. 5054-8.
253. Mazzola, M., et al., *Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats.* Appl Environ Microbiol, 1992. **58**(8): p. 2616-24.
254. Dietrich, L.E., et al., *The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of Pseudomonas aeruginosa.* Mol Microbiol, 2006. **61**(5): p. 1308-21.
255. Reszka, K.J., et al., *Oxidation of pyocyanin, a cytotoxic product from Pseudomonas aeruginosa, by microperoxidase 11 and hydrogen peroxide.* Free Radic Biol Med, 2004. **36**(11): p. 1448-59.

256. Price-Whelan, A., L.E. Dietrich, and D.K. Newman, *Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in Pseudomonas aeruginosa PA14.* J Bacteriol, 2007. **189**(17): p. 6372-81.
257. Mashburn, L.M. and M. Whiteley, *Membrane vesicles traffic signals and facilitate group activities in a prokaryote.* Nature, 2005. **437**(7057): p. 422-5.
258. Hogan, D.A., *Talking to themselves: autoregulation and quorum sensing in fungi.* Eukaryot Cell, 2006. **5**(4): p. 613-9.
259. Nickerson, K.W., A.L. Atkin, and J.M. Hornby, *Quorum sensing in dimorphic fungi: farnesol and beyond.* Appl Environ Microbiol, 2006. **72**(6): p. 3805-13.
260. Cugini, C., et al., *Farnesol, a common sesquiterpene, inhibits PQS production in Pseudomonas aeruginosa.* Mol Microbiol, 2007. **65**(4): p. 896-906.
261. Zaborina, O., et al., *Dynorphin activates quorum sensing quinolone signaling in Pseudomonas aeruginosa.* PLoS Pathog, 2007. **3**(3): p. e35.
262. Bredenbruch, F., et al., *The Pseudomonas aeruginosa quinolone signal (PQS) has an iron-chelating activity.* Environ Microbiol, 2006. **8**(8): p. 1318-29.
263. Rampioni, G., et al., *Transcriptomic analysis reveals a global alkyl-quinolone-independent regulatory role for PqsE in facilitating the environmental adaptation of Pseudomonas aeruginosa to plant and animal hosts.* Environ Microbiol.
264. Mondragon, V., et al., *pH-dependent activation of the BarA-UvrY two-component system in Escherichia coli.* J Bacteriol, 2006. **188**(23): p. 8303-6.
265. Lapouge, K., et al., *Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour.* Mol Microbiol, 2008. **67**(2): p. 241-53.
266. Liu, Y., et al., *Characterization of a novel RNA regulator of Erwinia carotovora ssp. carotovora that controls production of extracellular enzymes and secondary metabolites.* Mol Microbiol, 1998. **29**(1): p. 219-34.
267. Fortune, D.R., M. Suyemoto, and C. Altier, *Identification of CsrC and characterization of its role in epithelial cell invasion in Salmonella enterica serovar Typhimurium.* Infect Immun, 2006. **74**(1): p. 331-9.
268. Sahr, T., et al., *Two small ncRNAs jointly govern virulence and transmission in Legionella pneumophila.* Mol Microbiol, 2009. **72**(3): p. 741-62.
269. Kulkarni, P.R., et al., *Prediction of CsrA-regulating small RNAs in bacteria and their experimental verification in Vibrio fischeri.* Nucleic Acids Res, 2006. **34**(11): p. 3361-9.
270. Clarke, L. and J. Carbon, *Biochemical construction and selection of hybrid plasmids containing specific segments of the Escherichia coli genome.* Proc Natl Acad Sci U S A, 1975. **72**(11): p. 4361-5.
271. Liberati, N.T., et al., *An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2833-8.
272. Mathee, K., et al., *Dynamics of Pseudomonas aeruginosa genome evolution.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3100-5.
273. Canchaya, C., G. Fournous, and H. Brussow, *The impact of prophages on bacterial chromosomes.* Mol Microbiol, 2004. **53**(1): p. 9-18.
274. Hickman, J.W., D.F. Tifrea, and C.S. Harwood, *A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels.* Proc Natl Acad Sci U S A, 2005. **102**(40): p. 14422-7.
275. Goymer, P., et al., *Adaptive divergence in experimental populations of Pseudomonas fluorescens. II. Role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype.* Genetics, 2006. **173**(2): p. 515-26.

276. Nakada, Y., et al., *Molecular characterization and regulation of the aguBA operon, responsible for agmatine utilization in Pseudomonas aeruginosa PAO1.* J Bacteriol, 2001. **183**(22): p. 6517-24.
277. Igarashi, K. and K. Kashiwagi, *Polyamine transport in bacteria and yeast.* Biochem J, 1999. **344 Pt 3**: p. 633-42.
278. Reimmann, C., et al., *Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain Pseudomonas fluorescens CHA0.* J Bacteriol, 2005. **187**(1): p. 276-85.
279. Suzuki, K., et al., *Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of Escherichia coli.* J Bacteriol, 2002. **184**(18): p. 5130-40.
280. Dean, H.F., et al., *Isolates of Pseudomonas aeruginosa from Australian hospitals having R-plasmid determined antibiotic resistance.* Med J Aust, 1977. **2**(4): p. 116-9.
281. Klockgether, J., et al., *Genome diversity of Pseudomonas aeruginosa PAO1 laboratory strains.* J Bacteriol. **192**(4): p. 1113-21.
282. Williams, B.J., et al., *Discovery of an operon that participates in agmatine metabolism and regulates biofilm formation in Pseudomonas aeruginosa.* Mol Microbiol.
283. Shah, P. and E. Swiatlo, *A multifaceted role for polyamines in bacterial pathogens.* Mol Microbiol, 2008. **68**(1): p. 4-16.
284. Patel, C.N., et al., *Polyamines are essential for the formation of plague biofilm.* J Bacteriol, 2006. **188**(7): p. 2355-63.
285. Karatan, E., T.R. Duncan, and P.I. Watnick, *NspS, a predicted polyamine sensor, mediates activation of Vibrio cholerae biofilm formation by norspermidine.* J Bacteriol, 2005. **187**(21): p. 7434-43.
286. Sturgill, G. and P.N. Rather, *Evidence that putrescine acts as an extracellular signal required for swarming in Proteus mirabilis.* Mol Microbiol, 2004. **51**(2): p. 437-46.
287. Zhou, L., J. Wang, and L.H. Zhang, *Modulation of bacterial Type III secretion system by a spermidine transporter dependent signaling pathway.* PLoS One, 2007. **2**(12): p. e1291.
288. Takaya, A., et al., *Negative regulation of quorum-sensing systems in Pseudomonas aeruginosa by ATP-dependent Lon protease.* J Bacteriol, 2008. **190**(12): p. 4181-8.
289. Zhu, J. and S.C. Winans, *The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization.* Proc Natl Acad Sci U S A, 2001. **98**(4): p. 1507-12.
290. Castang, S., et al., *H-NS family members function coordinately in an opportunistic pathogen.* Proc Natl Acad Sci U S A, 2008. **105**(48): p. 18947-52.
291. Naora, H., *Involvement of ribosomal proteins in regulating cell growth and apoptosis: translational modulation or recruitment for extraribosomal activity?* Immunol Cell Biol, 1999. **77**(3): p. 197-205.
292. Zimmermann, R.A., *The double life of ribosomal proteins.* Cell, 2003. **115**(2): p. 130-2.
293. Jang, G.M., et al., *Structurally distinct elements mediate internal ribosome entry within the 5'-noncoding region of a voltage-gated potassium channel mRNA.* J Biol Chem, 2004. **279**(46): p. 47419-30.
294. Kim, S.Y. and E.A. Craig, *Broad sensitivity of Saccharomyces cerevisiae lacking ribosome-associated chaperone ssb or zuo1 to cations, including aminoglycosides.* Eukaryot Cell, 2005. **4**(1): p. 82-9.
295. Cui, K., et al., *Novel interaction between the transcription factor CHOP (GADD153) and the ribosomal protein FTE/S3a modulates erythropoiesis.* J Biol Chem, 2000. **275**(11): p. 7591-6.

296. Wan, F., et al., *Ribosomal protein S3: a KH domain subunit in NF-kappaB complexes that mediates selective gene regulation.* Cell, 2007. **131**(5): p. 927-39.
297. Pritchard, D.I., *Immune modulation by Pseudomonas aeruginosa quorum-sensing signal molecules.* Int J Med Microbiol, 2006. **296**(2-3): p. 111-6.
298. Tateda, K., et al., *The Pseudomonas aeruginosa autoinducer N-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils.* Infect Immun, 2003. **71**(10): p. 5785-93.
299. Bjarnsholt, T. and M. Givskov, *Quorum-sensing blockade as a strategy for enhancing host defences against bacterial pathogens.* Philos Trans R Soc Lond B Biol Sci, 2007. **362**(1483): p. 1213-22.
300. Smith, R.S., et al., *The Pseudomonas autoinducer N-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts: implications for inflammation.* J Immunol, 2002. **169**(5): p. 2636-42.
301. Ritchie, A.J., et al., *The Pseudomonas aeruginosa quorum-sensing molecule N-3-(oxododecanoyl)-L-homoserine lactone inhibits T-cell differentiation and cytokine production by a mechanism involving an early step in T-cell activation.* Infect Immun, 2005. **73**(3): p. 1648-55.
302. Reverchon, S., et al., *New synthetic analogues of N-acyl homoserine lactones as agonists or antagonists of transcriptional regulators involved in bacterial quorum sensing.* Bioorg Med Chem Lett, 2002. **12**(8): p. 1153-7.
303. Teplitski, M., J.B. Robinson, and W.D. Bauer, *Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria.* Mol Plant Microbe Interact, 2000. **13**(6): p. 637-48.
304. Rampioni, G., et al., *The quorum-sensing negative regulator RsaL of Pseudomonas aeruginosa binds to the lasI promoter.* J Bacteriol, 2006. **188**(2): p. 815-9.
305. Bottomley, M.J., et al., *Molecular insights into quorum sensing in the human pathogen Pseudomonas aeruginosa from the structure of the virulence regulator LasR bound to its autoinducer.* J Biol Chem, 2007. **282**(18): p. 13592-600.
306. Gibson, R.L., J.L. Burns, and B.W. Ramsey, *Pathophysiology and management of pulmonary infections in cystic fibrosis.* Am J Respir Crit Care Med, 2003. **168**(8): p. 918-51.
307. Zhang, H.B., L.H. Wang, and L.H. Zhang, *Genetic control of quorum-sensing signal turnover in Agrobacterium tumefaciens.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4638-43.
308. Yao, Y., et al., *Structure of the Escherichia coli quorum sensing protein SdiA: activation of the folding switch by acyl homoserine lactones.* J Mol Biol, 2006. **355**(2): p. 262-73.
309. Romling, U., M. Gomelsky, and M.Y. Galperin, *C-di-GMP: the dawning of a novel bacterial signalling system.* Mol Microbiol, 2005. **57**(3): p. 629-39.
310. Jenal, U., *Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria?* Curr Opin Microbiol, 2004. **7**(2): p. 185-91.
311. Romling, U. and D. Amikam, *Cyclic di-GMP as a second messenger.* Curr Opin Microbiol, 2006. **9**(2): p. 218-28.
312. Chugani, S. and E.P. Greenberg, *LuxR homolog-independent gene regulation by acyl-homoserine lactones in Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A. **107**(23): p. 10673-8.
313. Ankri, S. and D. Mirelman, *Antimicrobial properties of allicin from garlic.* Microbes Infect, 1999. **1**(2): p. 125-9.

314. O'Gara, E.A., D.J. Hill, and D.J. Maslin, *Activities of garlic oil, garlic powder, and their diallyl constituents against Helicobacter pylori.* Appl Environ Microbiol, 2000. **66**(5): p. 2269-73.
315. Tsao, S. and M. Yin, *In vitro activity of garlic oil and four diallyl sulphides against antibiotic-resistant Pseudomonas aeruginosa and Klebsiella pneumoniae.* J Antimicrob Chemother, 2001. **47**(5): p. 665-70.
316. Rasmussen, T.B., et al., *Identity and effects of quorum-sensing inhibitors produced by Penicillium species.* Microbiology, 2005. **151**(Pt 5): p. 1325-40.
317. Harjai, K., Kumar, R., Singh, Sukhuvinder, *Garlic blocks quorum sensing and attenuates the virulence of Pseudomonas aeruginosa.* FEMS Immunol. Med. Microbiol., 2009.
318. Bodini, S.F., et al., *Quorum sensing inhibition activity of garlic extract and p-coumaric acid.* Lett Appl Microbiol, 2009. **49**(5): p. 551-5.
319. Ariga, T. and T. Seki, *Antithrombotic and anticancer effects of garlic-derived sulfur compounds: a review.* Biofactors, 2006. **26**(2): p. 93-103.
320. Naganawa, R., et al., *Inhibition of microbial growth by ajoene, a sulfur-containing compound derived from garlic.* Appl Environ Microbiol, 1996. **62**(11): p. 4238-42.
321. Bjarnsholt, T., et al., *Garlic blocks quorum sensing and promotes rapid clearing of pulmonary Pseudomonas aeruginosa infections.* Microbiology, 2005. **151**(Pt 12): p. 3873-80.
322. Erickson, D.L., et al., *Pseudomonas aeruginosa quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis.* Infect Immun, 2002. **70**(4): p. 1783-90.
323. Teiber, J.F., et al., *Dominant role of paraoxonases in inactivation of the Pseudomonas aeruginosa quorum-sensing signal N-(3-oxododecanoyl)-L-homoserine lactone.* Infect Immun, 2008. **76**(6): p. 2512-9.
324. Boontham, P., et al., *Significant immunomodulatory effects of Pseudomonas aeruginosa quorum-sensing signal molecules: possible link in human sepsis.* Clin Sci (Lond), 2008. **115**(11): p. 343-51.
325. Wagener, M.M. and V.L. Yu, *Bacteremia in transplant recipients: a prospective study of demographics, etiologic agents, risk factors, and outcomes.* Am J Infect Control, 1992. **20**(5): p. 239-247.
326. Geisenberger, O., et al., *Production of N-acyl-L-homoserine lactones by P. aeruginosa isolates from chronic lung infections associated with cystic fibrosis.* FEMS Microbiol Lett, 2000. **184**(2): p. 273-8.
327. Ward, C., et al., *Preliminary findings of quorum signal molecules in clinically stable lung allograft recipients.* Thorax, 2003. **58**(5): p. 444-6.
328. Amagase, H., *Clarifying he real bioactive constituents of garlic.* Journal of Nutrition, 2006. **136**(3): p. 716S-725S.