‘New insights into the repressor role of PqsE over *pqsA*’

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COVID-19 STATEMENT:

During the progress of this PhD, several experiments were affected by the pandemic COVID-19, which ultimately had an impact on the final outcomes of the present work. Among the general factors, there was restrictive access to BDI, with 8 hours available to do laboratory work without including the cleaning times prior and after every shift. There was no weekend access for several months and some weeks were allocated to work from home only. There were long waiting times for the obtention of reagents and supplies, along with shortage of essential laboratory tools.

The experiments listed below were performed under COVID-19 restrictions. All of them had adjustments, delays and therefore led to important hypothesis remain inconclusive in this work

- **Relative expression of *pqsA* under anaerobic conditions**: Unfortunately, this experiment was severely delayed due to the lack of access to an anaerobic cabinet placed in other areas of BDI. After more than 6 months of requests, it was not possible to gain access to it, therefore, this experiment was adjusted as to described in **Chapter 2, section 2.3**, and carried out under the best conditions available in our laboratory C75-C81.
- **Gene expression assays for pqsX**: It could not be concluded whether a mutation on the sequence of the empty plasmid was causing some changes in phenotype. It was not possible to repeat these experiments with an alternative vector due to limited time and other essential experiments ongoing in parallel. To date, the whole sequencing of the plasmid is still ongoing.
- **Protein purification of PA2705 and NirQ** : Protein purification was performed by the supervision of Philip Bardelang as he was the only post-doctorate authorized to book the ÄKTA pure machine placed in a separate room from our lab, in this scenario, all the procedures involving AKTA purification were carried out depending on the availability of the machine and Phil.
- **EMSA**: This experiment was the last to be performed as it required the purification of both proteins mentioned above. This experiment was carried out as pilot only due to more tests are required for the proteins under study. This is to confirm whether they are properly folded and present in an active form. EMSA assay also requires extensive optimizations therefore no conclusion could be obtained at this point

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.

Natalia Romo

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen able to thrive in a wide variety of environments. Its highly diverse and adaptable behaviour is an increasing threat in chronic and hospital acquired infections (HAI). The exceptional ecological success of *P. aeruginosa* can be attributed to its vast metabolic versatility and its sophisticated cell- to- cell communication system quorum sensing (QS). QS network enables *P. aeruginosa* control the expression of diverse virulence factors such as pyocyanin, elastase, exotoxins, rhamnolipids, and biofilm formation. QS in *P. aeruginosa* is closely regulated by at least three different but highly interconnected systems namely, the Las, Rhl, and quinolone-based QS system, PQS. The PQS system acts mainly via 2-heptyl-3-hydroxy-4-quinolone, named the *Pseudomonas* quinolone signal (PQS), which connects the LysR-type transcriptional regulator PqsR to stimulate their own synthesis along with the expression of several virulence factors. The synthesis of PQS is driven by the *pqsABCDE* operon*, phnAB* and *pqsH,* located further in the *P. aeruginosa* genome. PqsE, encoded by the last gene of the *pqs* operon, functions as a pathway-specific thioesterase involved in the synthesis of HHQ and PQS, however its role in this pathway can be replaced by the broad-specificity thioesterase TesB, which reveals why *pqsE* deletion mutants maintains the synthesis of PQS. PqsE also adds independent roles onto the control of bacterial virulence through an unidentified mechanism. Among others, it balances the levels of QS signal molecules and secondary metabolites deriving from the PQS pathway by repressing the *pqsA* promoter, however, since PqsE does not possess DNA binding domain, its molecular mechanism remains elusive. Previously at the University of Nottingham, two DNA promoter pull down analysis were performed in a PAO1 *pqsE* Ind strain. In this setup, the overexpression of *pqsE* at the early and late stage of growth of *P. aeruginosa* showed a diverse protein profile binding the *pqsA* promoter, suggesting that

they could potentially act as intermediate of the action of this effector in this regulation.

Chapter 3 shows PA2705 bound the *pqsA* promoter in high abundance and only when *pqsE* was overexpressed, hence becoming a potential candidate to mediate the action of this effector in early growth. Due to significant changes in the *pqsA* expression using two pMiniCTX-*lux* reporters in a *PA2705* mutant, in depth analysis of the promoter regions included in these constructs revealed that the -311 *rhlR-box* was interrupted in one of reporters and that has a pivotal role in regulation of the P_{pqsA}. This analysis contributed to elucidate that (i) PA2705 is an inductor of the *pqsA* expression and that (ii) PqsE is a repressor of *pqsA* at the post-transcriptional level. Furthermore, it was evidenced that *PA2705* is under the regulation of PqsE, but it is not essential for the PqsE-mediated repression of *pqsA*.

At the late stage of growth of *P. aeruginosa*, fewer proteins were found binding the *pqsA* promoter. In **Chapter 4**, mutation of the main candidates genes showed that the denitrification regulatory protein *nirQ* caused the major disruption towards the expression of *pqsA,* becoming the main candidate to analyse in further studies. qRT-PCR in *P. aeruginosa* grown anaerobically indicated that *nirQ* is a repressor of *pqsA.* Moreover, *g*ene expression analysis unveiled that *nirQ* is under the regulation of PqsE, but like *PA2705*, it is dispensable for the PqsE-mediated repression of *pqsA*.

The analysis of *PA2705* and *nirQ* in the PqsE-mediated repression of *pqsA* evidenced that this regulation is especially complex and seems to involve other regulatory elements. In the search for the mediator of PqsE and to better understand the regulation of *pqsA* at the post-transcriptional level, further work in this direction was addressed in **Chapter 5**. The mutation of the main QS regulators *lasR, rhlR* and *pqsR* within the wild-type and the *pqsE* Ind genetic background revealed that the PqsE-mediated repression of *pqsA* is dependent on PqsR. In addition, it is suggested that a ncRNA expressed at the -339 transcriptional start site of the *pqsA* and that is under the control of RhlR participates in the repression of *pqsA*. Nevertheless, this finding differs to that

indicated in another study and conflicts with the mechanism proposed by the authors. Last, a broader view of the *pqsABCDE* operon led to investigate whether *pqsE* is independently regulated. A CRP like box found upstream the *pqsE* coding sequence elucidated the regulatory role of Vfr towards *pqsE*.

In summary, various regulatory venues were explored in the *P. aeruginosa pqsA* regulation that reveals new perspectives to understand the cell- to- cell communication in this bacterium and exhibits the dynamics and yet obscure events occurring within this regulation, knowledge of which could pave the path towards new studies in this pathogen.

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First of all, Miguel and Stephan, I met two amazing people, thank you for all your support, consistent guidance, for always having a smile of comfort whenever I felt lost. You made me feel much welcomed since the very first day. I am very lucky to have both of you as my supervisors. I really enjoyed working on this project, it was eye opening to see a whole world of fascinating mysteries in science that I never imagined they existed.

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This is especially for you Carol, grandpa Chai and Caco.

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List of abbreviations

ºC	Celsius degrees
3OC12-HSL	N-(3-oxododecanoyl)-L-homoserine lactone
AAA	ATPases associated with various cellular activities
AHL	N-acyl-L-homoserine lactone
Amp	Ampicillin
AQs	4-hydroxy-2-alkylquinolines
AraC	Arabinose operon regulatory protein
bp	Base pair
C4-HSL	N-butanoyl-L-homoserine lactone
cAMP	Cyclic adenosine monophosphate
Cb	Carbenicillin
CF	Cystic fibrosis
Cam	Chloramphenicol
CGN	CbbQ/GvpN/NorQ
CMC	Critical micelle concentration
COG	NCBI Clusters of Orthologous Groups
CRP	cAMP regulator protein
Ct	Cycle threshold
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Ds-F	Downstream Forward
Ds-R	Downstream Reverse
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
FT	Flow trough
g	Grams

GFP	Green fluorescent protein
Gm	Gentamicin
HHQ	2-heptyl-4-quinolone
His	Histidine
HRP	HorseradishPeroxidase
IPTG	Isopropyl-1-thio-(β-D-galactopyranoside)
Kb	Kilobase
kDa	Kilodalton
LB	Luria Bertani
LC-MS	Liquid chromatography-tandem mass spectrometry
M	Molar
MVs	Membrane vesicles
mPQS	2-Methyl-3-Hydroxy-4-Quinolone
MetOH	Methanol
Millisec	Milliseconds
min	Minute
mL	Millilitre
mM	Millimolar concentration
mRNA	Messenger Ribonucleic Acid
MRP	MoxR proper
ncRNA	Non-coding RNA
ng	Nanograms
OMVs	Outer membrane vesicles
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered Saline
PCR	Polymerase chain reaction
PIA	Pseudomonas isolation agar
PQS quinolone	Pseudomonas quinolone signal, 2-heptyl-3-hydroxy4-
P_{nirQ}	*nirQ* promoter

P_{pqsA}	*pqsA* promoter
P_{pqsE}	*pqsE* promoter
P_{PA2705}	*PA2705* promoter
QS	Quorum sensing
RLU	Relative Luminescent Units
RBS	Ribosome Binding Site
rcf	Relative Centrifugal Force
RLU	Relative luminescent units
RNA	Ribonucleic acid
rpm	Revolutions per minute
RRs	Response regulators
RT	Room Temperature
SDS	Sodium dodecyl Sulphate
SDS-PAGE	Sodium dodecyl Sulphate Polyacrylamide Gel
sec	Second
SEC	Size exclusion chromatography
Sm	Streptomycin
TAE	Tris Acetic Acid EDTA Buffer
TBE	Tris borate EDTA
Tet	Tetracycline
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
TCSs	Two component systems
Us-F	Upstream Forward
Us-R	Upstream Reverse
V	Volt
v/v	Volume by volume
w/v	Weight by volume
µg	Microgram
µL	Microliter
µM	Micromolar
µM	Micromolar concentration

1 Chapter One: General Introduction

1.1 *Pseudomonas aeruginosa*: an overview

P. aeruginosa, is a ubiquitous gram negative bacterium belonging to the bacterial family Pseudomonadaceae. With a substantial metabolic diversity. *P. aeruginosa* thrives in a wide variety of environments with a wide range of nutrient sources. It is normally present in terrestrial and freshwater environments (Silby et al., 2011) and can also cause root infection diseases or be lethal to plants such as Arabidopsis and sweet basil (Soc et al., 1995; Walker et al., 2004). More predominantly, *P. aeruginosa* has become an emerging opportunistic pathogen of clinical relevance. It produces a vast arsenal of virulence factors, including extracellular proteases and small toxic molecules like pyocyanin to overcome host cellular defenses. This γ-proteobacterium has a high capacity to adapt to environmental changes. In humans, it causes severe infections, especially in patients with compromised host defenses such as in neutropenia, severe burns, or cystic fibrosis (CF) (Govan & Deretic, 1996).

1.2 *P. aeruginosa* and nosocomial infections:

P. aeruginosa infections are often nosocomial and usually related to compromised host defenses, being nowadays, responsible for the 10-15% infections affecting patients worldwide. (Blanc et al., 1998; Pachori et al., 2019; Spagnolo et al., 2021). Inaccurate antibiotic therapy and/or incomplete treatments have led to adverse outcomes, promoting the emergence of resistance of this pathogen to antimicrobials (C. Kang et al., 2003). In addition, treatment of chronic and acute *P. aeruginosa* infections has become challenging due to the high levels of intrinsic and acquired resistance mechanisms of this organism against antibiotics. These include the cell envelope barrier, efflux pumps and high mutation rate (Henrichfreise et al., 2007; Mandsberg et al., 2009; Azam & Khan, 2019). Therefore, new prevention and treatment approaches are urgently required to improve the outcome of patients with *P. aeruginosa* infections. In the human host, *P. aeruginosa* can

cause infections of the respiratory and urinary tracks as well as surgical wound, amongst others, in some cases leading to bacteremia (Inamatsu, 1991) **(Table 1.1)**, being pneumonia and sepsis directly linked to the high rates of morbidity and lethally.

Table 1.1. Infection caused by *P. aeruginosa*

Infection	Major risk factors
Soft tissue	Burns, open wounds, post-surgery,
Urinary tract	Urinary catheter
Bacteremia	Immunocompromised
Diabetic foot	Diabetes, impaired microvascular circulation
Respiratory pneumonia	Elderly, Chronic obstructive pulmonary disease (COPD), cystic fibrosis, mechanical ventilation
Otitis externa	Tissue injury, water blockage in ear canal
Otitis media	Improperly cleaned hot tubs
Keratitis	Extended contact lens wears, contaminated contact lens solution

Regarding the infections affecting the respiratory track, these can be classified as acute or chronic. Acute infections are commonly associated with epithelium damage due to physical stress such as an intubation process or smoke inhalation. If *P. aeruginosa* is not eradicated during the acute infection phase, it can adapt to the lung environment through biofilm growth leading to chronic infection (Gellatly & Hancock, 2013). Chronic lower airway infections present a considerable co-morbidity in CF patients (Emerson et al., 2002). CF results from an autosomal recessive genetic mutation of the CF transmembrane conductance regulator (CFTR) protein. CFRT is a chloride channel regulated by adenosine 3',5'-monophosphate (cAMP) that requires binding of adenosine triphosphate (ATP) for channel opening (Rommens et al., 1989). In the lung, the

transepithelial ion fluxes of chloride and sodium are combined with water flux to maintain adequate hydration of the epithelium surface, thus, dysfunction of CFTR results in impaired chloride transport at the apical surface of epithelial cells, resulting in a thick dehydrated sputum that obstructs the respiratory track preventing the expulsion of dust, bacteria and other impurities from the lungs (Boucher., 2007; Button & Boucher, 2008). The subsequent decreased mucociliary clearance and increased mucus viscosity creates a suitable niche for *P. aeruginosa* lung colonization, in which the mucoid phenotype of this pathogen in children becomes the predominant form found in culture (Li et al., 2005) recognized as one of the most important pulmonary pathogens and the predominant cause of morbidity associated with CF.

1.3 *P. aeruginosa* and cell-to-cell communication: Quorum sensing

Bacteria can communicate each other through small diffusible molecules that are secreted to the external milieu. These low molecular weight molecules are known as autoinducers as they typically induce their own biosynthesis. Once they reach threshold concentrations, these enable bacteria to act in a coordinated manner in the regulation of the expression of virulence factor genes such as those for elastase, protease, LecA lectin, siderophores, as well as biofilm differentiation (Kievit et al., 2001; Nadal Jimenez et al., 2012). This communication system is named quorum sensing (QS) and contributes to the pathogenesis of diverse bacteria (Bassler & Losick, 2006), becoming therefore, an attractive target for novel antimicrobial drugs. The first reported QS system was in *Vibrio fisheri*, which at high population density, induces the production of bioluminescence (Meighen, 1993).

In *P. aeruginosa*, approximately over 10% of its genome is under the transcriptional control of QS (Martin Schuster et al., 2003a; Wagner et al., 2003), evidencing the complexity of this gene regulatory network. To date, three main QS systems have been extensively described in this organism, the *las*, *rhl* and *pqs* systems. These systems and their interconnections are reviewed trough this study.

1.4 The *las/rhl* systems: A sophisticated QS network

The main molecular components of an *N*-acylhomoserine lactone (AHL)-based QS system include a LuxI homologue, gene which directs the synthesis of the AHL signal and a LuxR homologue gene, which is an AHL-dependent transcriptional regulator that regulates QS genes (Meighen ,1993).

Two hierarchically related QS systems exist in *P. aeruginosa* using AHLs as signal molecules, the *las* and *rhl* systems (Pesci et al., 1997: Glessner et al., 1999). The *las* system consists of the LuxR homologue, LasR transcriptional regulator and the LuxI homologue, LasI signal synthase protein. LasI directs the biosynthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), the first autoinducer, that binds the transcriptional factor LasR inducing the transcription of virulence factor genes such as those for the extracellular protease (*lasA*), elastase (*lasB*), alkaline protease (*apr*), and exotoxins (*toxA*) **(Figure 1.1)** (Gambello et al., 1993); moreover, LasR is able to promote *lasI* expression, therefore generating a positive feedback resulting in a rapid increase in the levels of its own autoinducer 3OC12-HSL (Pesci et al., 1997). Subsequently, LasI/LasR induce the activation of the second system *rhlI*/*rhlR*. The RhlI synthase directs the biosynthesis of the second autoinducer, *N*-butyryl-L-homoserine lactone (C4-HSL) that once bound to RhlR induces the expression of genes responsible for the production of pyocyanin (*phz*), rhamnolipids (*rhlAB*) and elastase (*lasB*), as well as of *rhlI*, generating the second positive feedback loop (Winson et al., 1995; Nadal Jimenez et al., 2012). Notably, RhlR has also been shown to impact the *las* system, this is due to the ability of the RhlR/C4-HSL complex to induce *lasI* expression and therefore restore the production of C12-HSL in the absence of LasR (Dekimpe & Déziel, 2009).

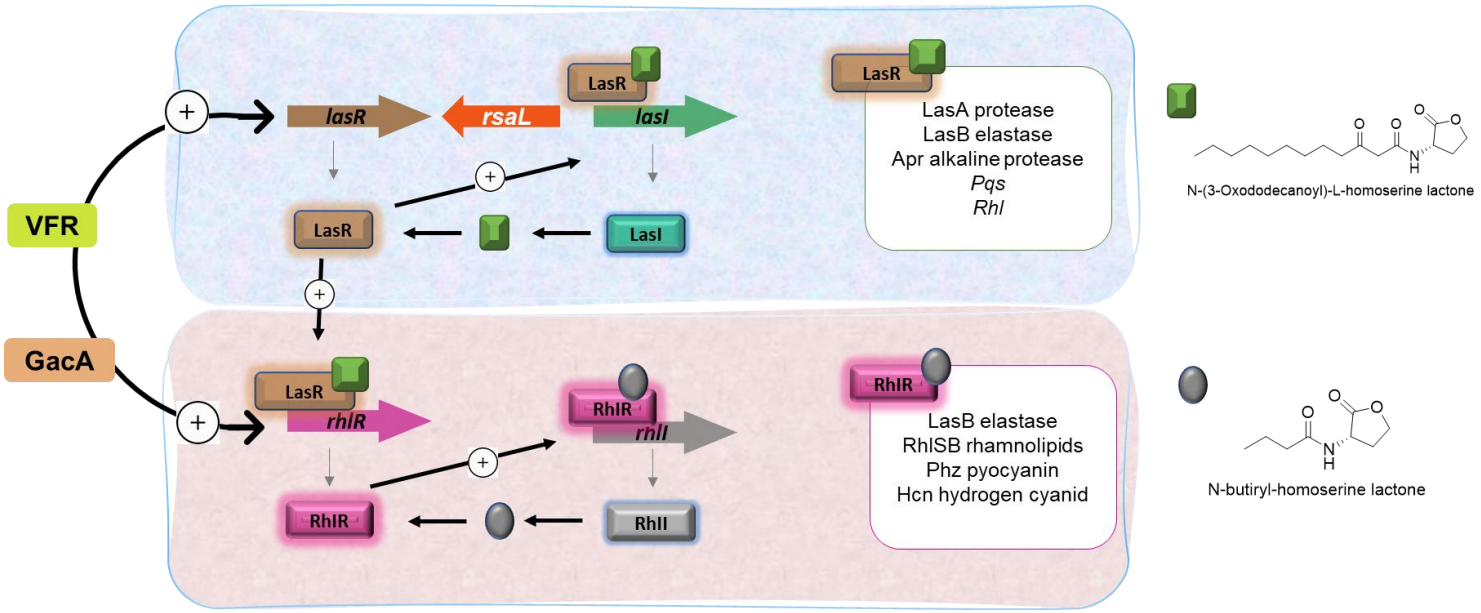


Figure 1.1. Representation of the two Homoserine-lactone based quorum-sensing systems in *P. aeruginosa*.

The QS autoinducer signal 3OC12-HSL is produced by *lasI,* and together with LasR activate the expression of *rhlR*. The second QS autoinducer C4-HSL is encoded by *rhl,* which binds its response regulator RhlR, activating the second QS pathway. Both systems upregulate their own expression, thus creating a positive feedback loop. LasR induces the expression of *rsaL*, a transcriptional repressor of *lasI* and *lasR*. Vfr and GacA positively regulate *lasR* and *rhlR*. Virulence factors regulated by each respective receptor-ligand complex are detailed on the right. Modified from Nadal Jimenez et al., (2012)).

The (3OC12-HSL)-LasR complex also induces the transcription of *rsaL*, a gene integrated within the *las* QS system coding for the transcriptional regulator RsaL (de Kievit et al., 1999). This negative regulator, genetically placed in the intergenic region between *lasR* and *lasI*, disrupts the activation of QS by repressing the transcription of *lasI*, responsible of the production of 3OC12-HSL. On the other hand, *rsaL* expression requires LasR, but does not require RhlR (de Kievit et al., 1999).

The expression of the *las/rhl* systems is modulated by Vfr and GacA. Vfr is a global regulator of virulence factor expression in *P. aeruginosa*. It belongs to the CRP/FNR superfamily of transcription factors (West et al., 1994). Its action is dependent on cyclic adenosine monophosphate (cAMP) albeit cAMP independent mechanisms have also been reported (Fuchs et al., 2010). Vfr positively regulates the expression of *lasR* in a cAMP-independent manner whereas *rhlR* is positively or negatively regulated due to its multiple Vfr binding sites found in its promoter (Albus et al., 1997; Medina et al., 2003; Fuchs et al., 2010; Croda-García et al., 2011). GacA, in contrast, is a response regulator part of the GacS/GacA two component systems and positively controls the quorum-sensing machinery and the expression of extracellular products via two small regulatory RNAs, RsmY and RsmZ. GacA is conserved in Gram-negative bacteria and positively regulates the transcription of *lasR and rhlR* **(Figure 1.1)** (Reimmann et al., 1997)

1.5 QscR, the third LasR/RhlR homologue in *P. aeruginosa*

QscR (QS control repressor, PA1898), which was known as an orphan AHL sensor (Chugani et al., 2001) corresponds to a third *P. aeruginosa* AHL-responsive transcription factor. Initial experiments demonstrated that deletion of *qscR* results in an acceleration of LasR activation as measured by earlier synthesis of the signals 3OC12-HSL and C4-HSL. Further analysis performed by Lequette et al., (2006) reported that a *qscR* null mutant grown to different stages differentially expressed 424 different genes compared to the wild type PAO1. Most of these genes were downregulated by QscR suggesting an important counterregulatory role in *P. aeruginosa*. The three QS regulators and two signal synthases comprise a hierarchical cascade, where QscR phase-differentially represses both LasR and RhlR. Here, *las* regulates *rhl* and *qscR,* explaining why QscR is activated by the LasR cognate signal 3OC12-HSL (Lequette et al., 2006). QscR in turn represses *lasI* at early stages and *rhlI* at late stages of growth (Chugani et al., 2001) as well as *lasR* and *rhlR* probably through the formation of inactive heterodimers (Ledgham et al., 2003), impacting the timing of the activation of the *las/rhl* system**.** QscR regulates pyocyanin production (Mavrodi et al., 2001) by negatively controlling *phz1* (Chugani et al., 2001) and more strongly *phz2* expression (Ledgham et al., 2003) likely trough the *las/rhl* system. QscR is regulated by GacA (Ledgham et al., 2003) , which is strictly required for the production of several virulence factors (Laville et al., 1992). Recent studies using chromatin immunoprecipitation analysis indicated that QscR regulates QS gene expression by activating one single operon PA1895-1897 (Ding et al., 2018). The latter finding gained validation when the deletion of PA1897 resulted in an early QS activation phenotype like a QscR-null mutant, and that the latter mutation was only complemented by full restoration of the PA1895-1897 operon, suggesting that the QS antiactivation caused by QscR, is mediated trough the action of the genes PA1895-1897 in this pathogen (**Figure 1.6**).

1.6 The Pseudomonas quinolone signal: PQS system

Quinolones are broad spectrum antibiotics based on the 4-quinolone-structure. There is a second class of quinolone-based molecules with natural antimicrobial properties that are produced by *P. aeruginosa*. These molecules named 4-hydroxy-2-alkylquinolines(AQs) (Heeb et al., 2011) belong to a family of more than 50 compounds (Deziel et al., 2004) , where 2-heptyl-3-hydroxy-4(1H)-quinolone, termed Pseudomonas quinolone signal (PQS) and 2-heptyl-4-hydroxyquinoline (HHQ) have been largely studied given their role as QS signal molecules. PQS is a poorly soluble AQs that regulates the expression of numerous virulence genes including those coding for elastase, rhamnolipid biosynthesis enzymes, galactophilic lectin LecA and pyocyanin. Moreover, it also plays an important role in biofilm development. PQS biosynthesis is driven by the *pqsABCDE* operon (PA0996-PA1000), *phnAB* (PA1001-PA1002) and *pqsH* (PA2687), being direct regulated by the LysR-type transcriptional regulator PqsR (PA1003) (also known as MfvR) **(Figure 1.2).** PqsR when bound to PQS positively regulates the expression of the *pqsABCDE* AQ biosynthetic operon, generating a positive feedback loop (Xiao et al., 2006). Maximum PQS production occurs at the end of the exponential growth phase of *P. aeruginosa* (Bala et al., 2013) and mutation of *pqsR* was shown to abrogate AQs synthesis, suggesting to be necessary for PQS transduction (Cao et al., 2001; Gallagher et al., 2002).

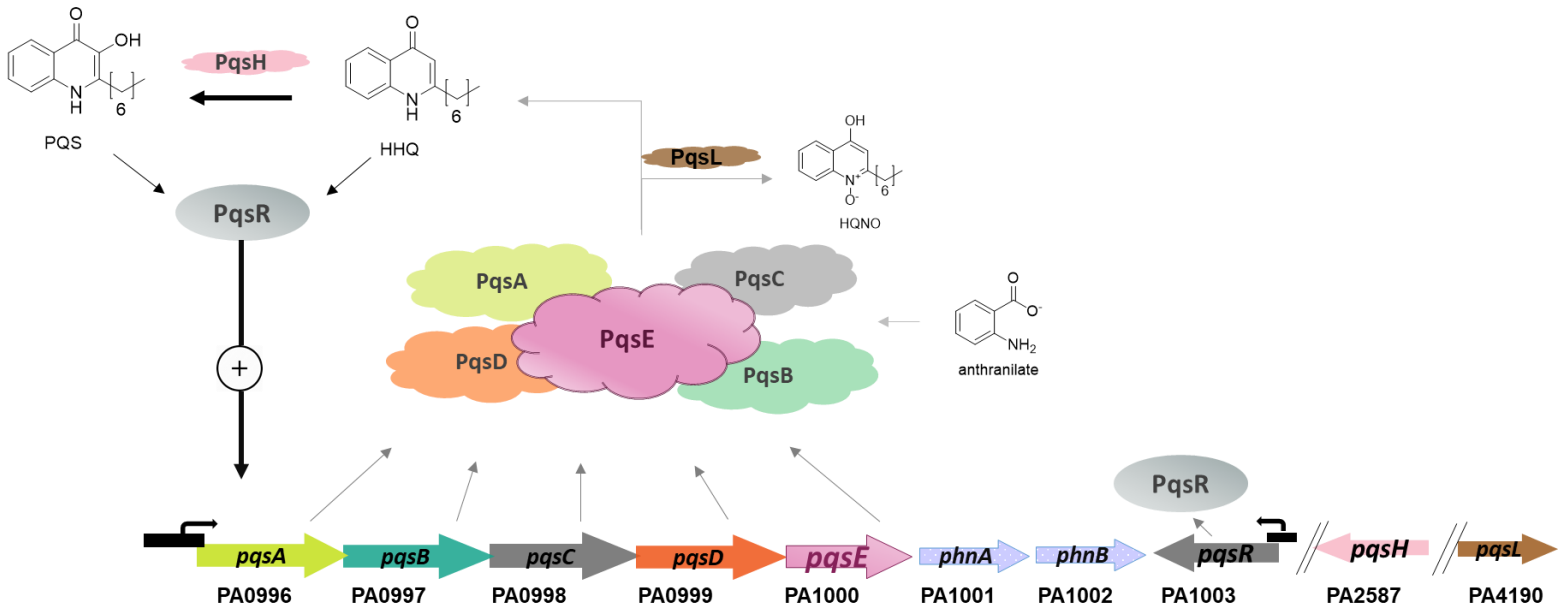


Figure 1.2.Overall view of the PQS system in *P. aeruginosa*.

PQS, the most active signal, correspond to 2-heptyl-3-hydroxi-4-quinolone, and it is product of the action of de FAD-dependent monooxygenase *pqsH* towards its precursor 2-heptyl-4-quinolone, also known as HHQ. Both can activate the expression of *pqsA* by binding the LysR transcriptional regulator PqsR, however HHQ is less potent. The synthesis of PQS is driven by the operon *pqsABCDE* (PA0996-PA1000), and *phnAB* (PA1001-PA1002), which provide the initial substrate, anthranilate. The PA number is indicated below the genes according to the Pseudomonas Genome Database. Modified from Rampioni et al., (2016).

The *PQS* biosynthetic pathway requires a series of enzymatic reactions that begins from anthranilate to finally generate HHQ and PQS **(Figure 1.3)**. To begin with, *phnAB* encodes the anthranilate synthase that converts chorismate to anthranilate which upon the action of the anthranilate-coenzyme A ligase PqsA leads to the formation of anthraniloyl-CoA **(Figure 1.3)** (Farrow et al., 2008). Next PqsD converts anthraniloyl-CoA into 2-aminobenzoylacetyl-CoA through a condensation reaction with manoyil. Subsequently, PqsE hydrolysates 2-aminobenzoylacetyl-CoA to form 2-aminobenzoylacetate which is then condensed with octanoyl-CoA by PqsBC to form HHQ. PQS is finally synthesized from the hydroxylation of HHQ at the 3 position by the NADH-dependent flavin mono-oxygenase PqsH (Bredenbruch et al., 2005). Two active secondary metabolites 2,4-dihydroxyquinoline (DHQ) and 2-aminoacetophenone (2-AA) are produced during AQs biosynthesis. DHQ correspond to a by-product of the reaction catalyzed by *pqsAD* (Zhang et al., 2008) whereas 2-AA is originated from decarboxylative decomposition of 2-ABA (Drees & Fetzner, 2015). PqsE has shown to be dispensable for AQs biosynthesis, largely due to the substitution role from the broad-specificity thioesterase enzyme TesB distantly located in the chromosome (Drees & Fetzner, 2015). The latter, explains why a *pqsE* deletion mutant still produces wild-type levels of AQs (Deziel et al., 2004; Gallagher et al., 2002) and that differs to the impact caused by the specific mutations in the *pqsA, pqsB, pqsC* or *pqsD* biosynthetic genes or *pqsR,* that abolish the AQs production (Gallagher et al., 2002; Diggle et al., 2003). Like *pqsH*, *pqsL* is also found distant from the *pqsABCDE* operon. The structure of PqsL resembles a class A flavoprotein monooxygenases and is the key enzyme responsible for the generation of 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) in the PQS biosynthetic pathway. More specifically, PqsL uses as a substrate 2-aminobenzoylacetate, molecule formed after the action of PqsE, resulting in the formation of an unstable product 2-hydroxylaminobenzoylacetate(2-HABA), molecule that is a preferred substrate for the complex PqsBC and that results in the final formation of HQNO (Drees et al., 2018), evidencing why a mutation of *pqsH* accumulates HHQ and HQNO, whereas mutation of *pqsL* accumulates HHQ and PQS (Deziel et al., 2004).

The formation of PQS depends on the presence of the initiator molecule anthranilate **(Figure 1.3)**. The latter, is also a precursor of tryptophan biosynthesis (Essar et al., 1990; Calfee et al., 2001; Oglesby et al., 2008) and when accumulated, it can be degraded into TCA cycle intermediates by the functions of *antABC* and *catA*, trough the action of AntR (Oglesby et al., 2008), placing anthranilate as a pivotal branch point during the PQS biosynthesis **(Figure 1.3)**. To date, three sources of anthranilate maintain the cellular pool of this metabolite in *P. aeruginosa.* The anthranilate synthases TrpEG and PhnAB, supply anthranilate for AQ biosynthesis from chorismite (Essar et al., 1990), however, PhnAB appears to supply anthranilate only under nutrient-limiting conditions (Knoten et al., 2014). The kynurenine pathway on the contrary, is considered the main source of anthranilate formation when tryptophan is present (J. M. Farrow & Pesci, 2007; Oglesby et al., 2008) which degradation occurs trough action of the enzymes tryptophan-2,3-dioxygenase (encoded by *kynA)*, kynurenine formamidase (encoded by *kynB*), and kynureninase (encoded by *kynU*), conducing the production of formylkynurenine, kynurenine and anthranilate, respectively (Kurnasov et al., 2003) **(Figure 1.3)**.

HHQ, the precursor of PQS, can also activate the *pqs* system, however it has been demonstrated to be less potent in strain PA14 (Xiao et al., 2006). In addition, a different study carried out in PAO1, revealed that the promoter activity of *pqsA* (P_{pqsA}) was most sensitively activated by HHQ (Fletcher et al., 2007) , possibly because HHQ exclusively regulates the *pqsABCDE-phnAB* transcriptional unit, contrary to PQS that regulates the mRNA levels of another 182 genes (Rampioni et al., 2016). Among these, the majority of the up-regulated genes were related to iron starvation, including *pvdS, pchR* and the genes involved in siderophore biosynthesis (Rampioni et al., 2016). The latter finding, supported the role of PQS in iron entrapment, that was first observed by Bredenbruch et al., (2006), when the addition of exogenous PQS to PAO1 cultures resulted in depleted iron from the medium comparable to that when using the chelating agent 2'2-dipyridyl. This observation was further validated when Diggle et al., (2007) evidenced the formation of complexes between PQS but not HHQ, with iron(III) at physiological pH. In the same work, the authors

also reported the capability of PQS in promoting the production of the siderophore pyoverdine, probably due to its chelation properties that contribute with an iron starvation response. This QS signal, however, is not likely to be a siderophore, because it has shown to be unable to deliver iron into the bacterial cell without a functional pyoverdine or pyochelin, hence propounding that PQS, serves a reservoir of iron that may be then captured by pyoverdine and/or pyochelin and delivered back into the cell trough the respective siderophore receptor (Diggle et al., 2007).

In addition to its role in QS, PQS has also shown to be important for stimulating membrane vesicles (MVs) formation, signal traffic strategy that *P. aeruginosa* utilizes to deliver toxins, the antibiotic resistance protein β-lactamase, or virulence factors to target cells, among others (Kuehn & Kesty, 2005; Mashburn & Whiteley, 2005). PQS is among the molecules mobilized by MVs to the external milieu, due to its hydrophobic nature that prevents it to freely diffuse out of bacterial cells. About 86% of the PQS produced by this pathogen are present in MVs, in contrast to the *las/rhl* AHL signals, which presence is nearly null (1%) (Mashburn & Whiteley, 2005). Further studies indicated that PQS strongly interacts with constituents of the LPS structure 4'-phosphate and acyl chains of lipid A, and more specifically, the third position hydroxyl, as well as the alkyl chain of PQS are the components required for this interaction (Mashburn-Warren et al., 2008). Further, Mashburn-Warren et al., (2009) evidenced that the third position hydroxyl of PQS is critical for stimulating MV formation as well its association with MVs. The five-carbon alkyl chain is the minimum requirement for notable association with MVs and for integration into LPS, indicative that these components are important for inducing the MV LPS aggregates. Interestingly, a recent report indicated that the formation of outer membrane vesicles (OMVs) is strain dependent and it does not correlate to the total production of PQS, but rather to exported amount to the external milieu. This was evidenced when a poor OMVs producer PAO1 presented less amount of exported PQS than the strong OMVs producer PA14, which exported a large majority of its PQS to the supernatant (Florez et al., 2017). Besides, PQS also participate in balancing life and death of *P. aeruginosa* when this is

exposed to non-favourable conditions. In Häussler & Becker, (2008), *P. aeruginosa* deficient in PQS production was more tolerant to antibiotics and resistant to oxidative stress. Due to its role in inducing cytotoxicity (Vrla et al., 2020) PQS has a protective role when the external conditions are not favorable by increasing its susceptibility towards these stressors. Interestingly, this role was only attributed to PQS but not to HHQ, evidencing a predominant role of the former in *Pseudomonas*.

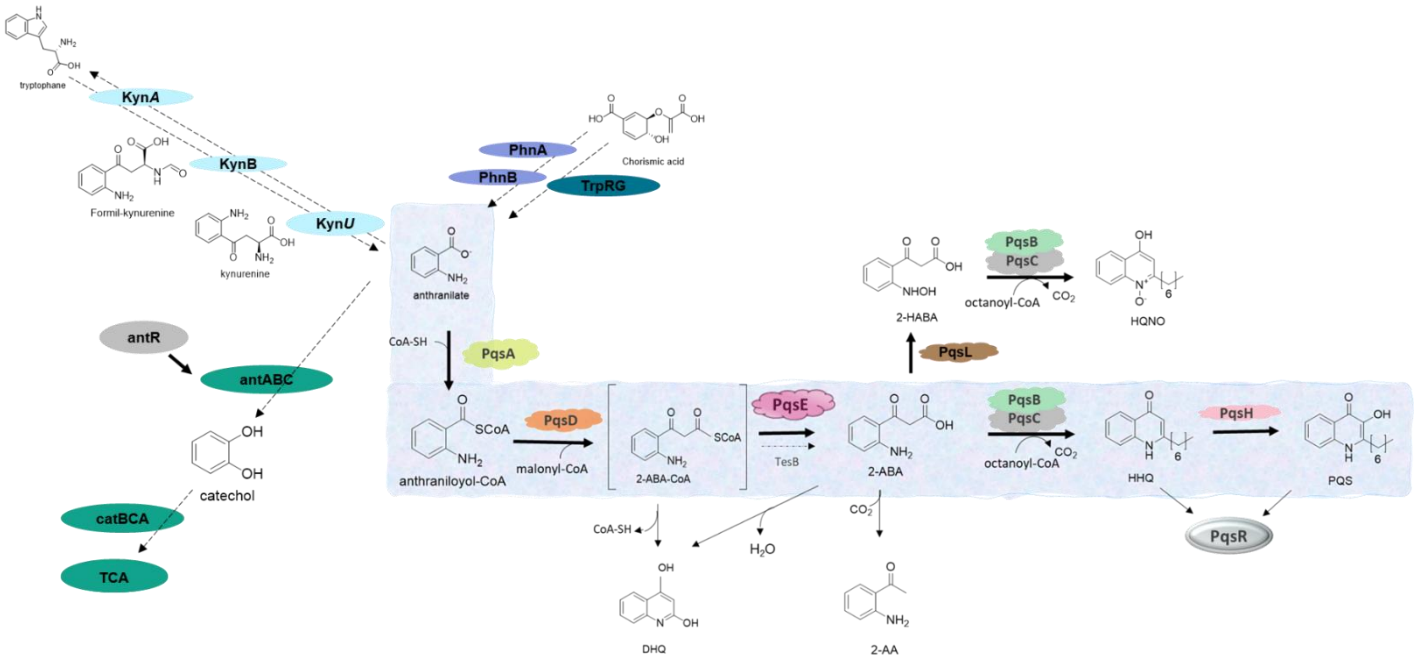


Figure 1.3.PQS biosynthesis pathway in *P. aeruginosa*.

Anthranilate for PQS synthesis is provided through tryptophan or chorismite degradation. The accumulation of anthranilate activates its degradation towards the TCA cycle. Anthranilate is converted into PQS by the action of the *pqsABCDE* operon, encoding for the enzymes PqsA, PqsB , PqsC, PqsD and PqsE (Refer to text for more details). Abbreviations: CoA, coenzyme A; 2-ABA-CoA, 2'-aminobenzoylacetyl-CoA; 2-ABA, 2'-aminobenzoylacetate; DHQ, dihydroxyquinoline; 2-AA, 2'-aminoacetophenone; 2-HABA, 2'-hydroxylaminobenzoylacetate; HHQ, 2-heptyl-4-(1H)-quinolone; HQNO, 4-hydroxy-2-heptylquinoline-N-oxide; PQS, Pseudomonas quinolone signal. Modified from Schütz & Empting, (2018).

1.7 Regulation of the *pqs* system and relationships with the *las/rhl* systems.

P. aeruginosa controls the production of many virulence factors through the *las/rhl* system. Together, these QS systems control 6% to 11% of the *P. aeruginosa* genome (Whiteley, Lee, and Greenberg., 1999; Schuster et al., 2003; Wagner et al., 2003). In 1999, Pesci et al. reported the discovery of the third QS signal, PQS. The latter showed to regulate the expression of the elastase gene *lasB* even in the absence of LasR, however, this regulator was required for the production of PQS (Pesci et al., 1999; McGrath et al., 2004). This suggested that the gene or genes in charge of the biosynthesis of this QS signal were under the positive control of the *las* system (**Figure 1.4**). Wade et al., (2005) demonstrated that the induction of PQS by the *las* system was indirect trough the activation of *pqsR* (**Figure 1.6**). Moreover, Xiao et al., (2006) demonstrated that LasR binds the *las/rhl box* CTAACAAAAGACATAG, centred at -513 bp upstream of the *pqsR* translational start site. Recent studies have identified four transcriptional start sites (TSS) within the *pqsR* promoter (Wade et al., 2005; Eckweiler et al., 2012; Farrow et al., 2015; Farrow & Pesci, 2017). A deeper analysis of the regulations taking part within this promoter indicated that LasR regulates transcription at the *pqsR* TSS3/4 **(Figure 1.5)**, generating not only a mRNA that carries the *pqsR* coding region, but also small transcripts that terminated prematurely between the TSS1/2, suggesting an additional regulatory point in the expression of *pqsR* (Farrow & Pesci, 2017). The '*pqsR*-mediated PQS regulator' PmpR, belonging to the YebC protein family, showed to negatively impact the regulation of *pqsR*, nevertheless, its precise binding site within the *pqsR* promoter has not been elucidated yet (Liang et al., 2008). The LysR-type transcriptional regulator CysB, competes with LasR for binding to the *pqsR* promoter at the *las-box*, which in contrast to LasR, acts a repressor of the expression of *pqsR* (Farrow et al., 2015). The functionally redundant H-NS family members MvaT and MvaU, interact with the *pqsR-nadA* intergenic region and silence transcription (Castang et al., 2008) repressing *pqsR* expression by binding at its proximal TSS (Farrow & Pesci, 2017) **(Figure 1.5).**

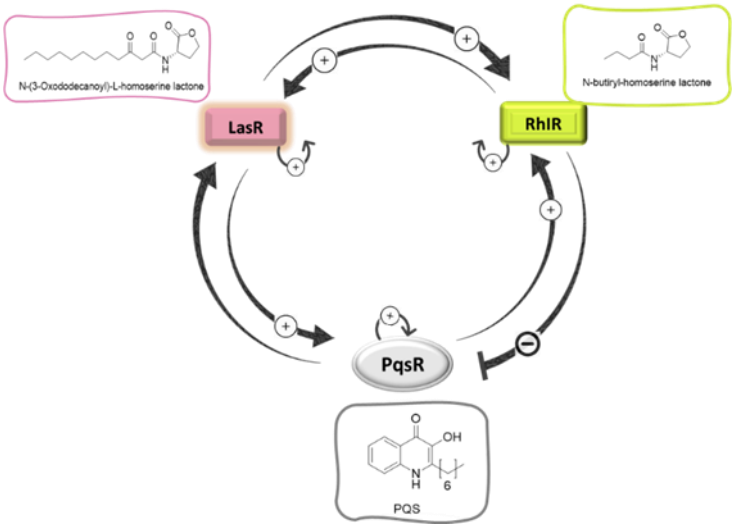


Figure 1.4. Schematic representation of the circular QS model.

The interlink regulations between the *las/rhl* and PQS systems are shown. Each system autoinducers their own expression. The interconnections between the three systems features the high complexity of this regulation. Solid black arrows indicates regulation, from which (⊕→) states for positive, whereas (⊖⊣) for negative. Modified from Allegretta et al., (2017).

PqsR in turn, has shown to be an activator of the *las* system **(Figure 1.4)**, specifically by regulating *lasR* (Allegretta et al., 2017) **(Figure 1.6)**. Surprisingly, this regulation occurs at the early stage of growth of *P. aeruginosa*, thus arising the question whether the characteristic QS hierarchical regulation model is rather circular, placing at the top of the cascade PqsR or LasR. **(Figure.1.4).**

The PQS bioactivity has also shown to depend on RhlR (Pesci et al., 1999) this was due to the inability of PQS to restore *lasB* expression in the absence of this regulator, suggesting that these systems are interlinked. McGrath et al.,(2004) reported that RhlR acts a repressor of the PQS production and Wade et al., (2005) complemented this finding by indicating that RhlR represses *pqsR* **(Figure 1.5) (Figure 1.6)**. PQS in turn, acts as an inductor of *rhlI* (McKnight et al., 2000), demonstrating that PQS is a connector of the three QS systems. PqsR

has also been shown to regulate RhlR in a positive manner **(Figure 1.4)** (Allegretta et al., 2017) however, differently from the induction at early exponential phase towards *lasR*, the *rhlR* regulation occurs at the mid-exponential phase, suggesting the idea of a probable circular regulatory model **(Figure.1.4).**

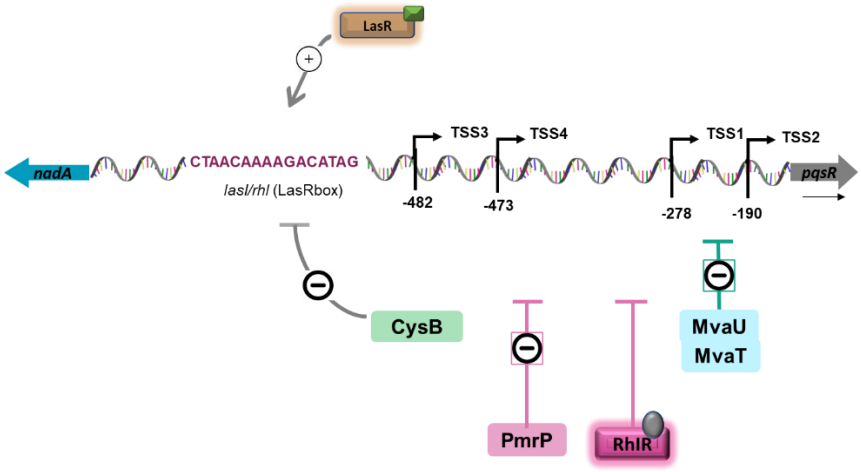


Figure 1.5. Promoter region of *pqsR*.

The *pqsR* transcriptional start sites are indicated by bent arrows showing TSS1, TSS2, TSS3 and TSS4. TSS2 and TSS1 are placed at -190 and -278 bp upstream from the *pqsR*, respectively (Wade et al., 2005). TSS3 is placed at −482 bp relative to the *pqsR* translational start site, from which TSS4 is found 19 bp downstream TSS3, at position -473 bp (J. Farrow & Pesci, 2017). As mentioned in the text CysB, PmpR, MvaT/MvaU, as well as RhlR/C4-HSL, are negative regulators on *pqsR* expression, whereas LasR is an inductor. The *pqsR* gene is represented by a grey arrow. The putative QS *las/rhl* box sequence is also shown. Modified from Farrow & Pesci, (2017) .

In addition, RhlR is believed to control the expression of *pqsA* by directly repressing this promoter (Xiao et al., 2006). The *pqsABCDE* operon holds four TSS **(Figure 1.6)**, from where two, are placed upstream and downstream of the of the start codon of *pqsA,* respectively. The first reported TSS is placed -71bp upstream of the start codon of *pqsA* (McGrath et al., 2004), which is under the positive control of PqsR, thus driving the expression of the *pqsABCDE* operon. The second TSS is placed -311 bp upstream from the -71 *pqsA* transcriptional initiation site, and that is accepted to be under the negative control of RhlR (Xiao et al., 2006), which creates a long transcript that sequesters the Ribonucleotide binding site (RBS), preventing the activation of *pqsA* by PqsR (Brouwer et al., 2014) (The in depth analysis of this regulation is covered in **Chapter 5**)**.** The third TSS is found at 31 bp upstream of the *pqsB* gene (Eckweiler et al., 2012), however, the functionality of this promoter has not been demonstrated to date, nor whether it is dependent on the *las/rhl* system. Finally, the fourth TSS is likely to be placed at least 87 bp upstream of the *pqsD* gene, however, this has only been shown to be active under nutrient-limiting conditions (Knoten et al., 2014).

On the other hand, Baker et al., (2017) evidenced the first interaction between PQS and RhlR although the level of specificity remains unknown. This is interesting as RhlR has also shown to bind other signals different than its canonical C4-HSL (Boursier et al., 2018), suggesting a high level of flexibility in terms of specificity and interactions with other regulatory pathways.

Furthermore, the FAD-dependent monooxygenase PqsH **(Figure1.3),** responsible for the conversion of HHQ to PQS, is under the positive control of the QS system LasI/R (Gallagher et al., 2002) as well as RsaL (Kang et al., 2017). Similarly PqsL, the enzyme in charge of the production of HQNO, **(Figure1.3**) has also been reported to be under the positive regulation of LasI/R **(Figure 1.6)** (Martin Schuster et al., 2003b), thus, emphasising the complexity of QS in *P. aeruginosa*.

Small RNAs (sRNAs) have also been involved in the regulation of the *pqs* system **(Figure 1.6)**. Among the major players are PhrS, ReaL and PrrF1-2. PhrS controls *pqsR* (Sonnleitner & Haas, 2011) by activating its expression and requires the

anaerobic regulation of arginine deiminase and nitrate reduction (ANR). ReaL, which is downregulated by LasR, influences the synthesis of the *pqs* quinolone signal PQS by positively impacting on the expression of *pqsC* in a post-transcriptional manner (Carloni et al., 2017). On the other hand, PrrF 1-2, which participates in iron homeostasis in conjunction with the RNA chaperone Hfq , is a repressor of *antR* (Oglesby et al., 2008). AntR is an activator of the *antABC* genes that encode for the enzymes for the degradation of anthranilate in *P. aeruginosa*, hence, favoring the accumulation of this metabolite that is subsequently used in the formation of PQS (Djapgne et al., 2018)

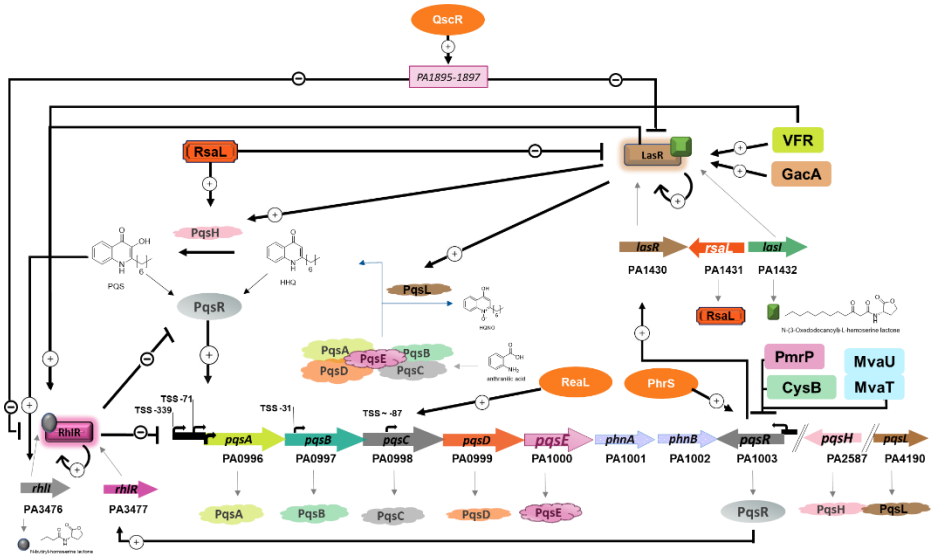


Figure 1.6. General outline of the regulation of the *pqsABCDE operon* and the interlinks with the *las/rhl* system including some of the main QS regulators in *P. aeruginosa*.

Interconnections between *pqs* and the *las/rhl* system are shown. Major regulators in *P. aeruginosa* controlling the expression of QS are also represented. QscR is a repressor of the *las* and *rhl* system. LasR, positively regulated by Vfr and GacA, activates *lasI* encoding the 3OC12-HSL synthase. The LasR/3OC12-HSL complex activates the second AHL-dependent QS system *rhlR/I* as well as the PQS system via *pqsR* and *pqsH*. The latter is negatively regulated by RsaL, which also represses *lasR*, balancing the production of QS signals in *P. aeruginosa*. The AQs biosynthesis requires PqsABCDE, in which the action of PqsE is dispensable. HHQ is transformed to PQS trough the action of PqsH. PQS and HHQ are co-inducers of PqsR, allowing the expression of the *pqsABCDE* operon. The *rhlR/I* system negatively affects PQS production by repressing *pqsA* and *pqsR* expression. The expression of *pqsR* is negatively regulated by MvaT/MvaU, PmpR and GycB. The small RNA PhrS activates *pqsR* whereas ReaL activates *pqsC.* The PA number is indicated below the genes according to the Pseudomonas Genome Database. Solid black arrows indicates regulation, from which (⊕→) states for positive, whereas (⊖⊣) for negative. Grey arrows mean to encode. Blue arrows represent enzymatic reactions.

1.8 Anthranilate as a source for the formation of PQS

The accumulation and/or degradation of anthranilate is tightly regulated by the *rhl*, *las* and *pqs* systems. QscR, which controls the expression of *rhl* and *las* in a growth dependent manner, is also a key component in the orchestrated regulation of anthranilate (**Figure 1.7**). During late exponential/early stationary phase the *pqsABCDE-phnAB* cluster and the enzymes *antA* and *catA,* are activated and repressed by LasR, respectively. This suggested that LasR favors the production of anthranilate in *P. aeruginosa*. In contrast, RhlR is a repressor of the *pqsABCDE-phnAB* cluster at the late exponential/early stationary phase, and it is an inductor of *antA* and *catA* at the late stationary phase*,* favoring the degradation of anthranilate (Schuster et al., 2003b; McGrath et al., 2004; Wade et al., 2005; Choi et al., 2011). Choi et al., (2011) using real-time PCR analysis showed that in a wild type condition, *catA* and *antA* are induced in late stationary phase, probably due to the accumulation of anthranilate like reported previously (Oglesby et al., 2008), However, the induction of *catA* and *antA* and subsequently *antR* was almost nullified in the absence of *rhlR,* suggesting that not only the degradation, but also the accumulation of anthranilate in late stationary phase is attributed to an RhlR-dependent mechanism. Under this posit, the authors discarded that the anthranilate accumulation was due to *phnAB* or the kynurenine pathway, this was because *phnAB* is repressed by RhlR in late stationary phase, whereas *kynB* and *kynU*, are positively regulated by LasR (Martin Schuster et al., 2003b). Instead, Choi et al., (2011) proposed that the accumulation of anthranilate probably occur through the induction of *phzE1,* an RhlR-dependent anthranilate synthase related to the formation of pyocyanin, and that together with *phzD*, have been suggested to generate trans-2,3-dihydro 3-hydroxyanthranilate instead of anthranilate. A mutation of *phzE1* presented lower levels of anthranilate level in the culture medium when compared to the wild-type (Choi et al., 2011) and like *antABC* and *catA*, *phzE* and *phzD* were both activated at late stationary phase (Martin Schuster et al., 2003b). These findings encourage a pivotal role of RhlR in the anthranilate metabolism that may impact the balance of the

formation of PQS in *P. aeruginosa*. PqsR, on the other hand, not only favors the production of anthranilate by the induction of *pqsABCDE-phnAB* gene cluster, but also represses its degradation by repressing *antA* expression (Oglesby et al., 2008; Choi et al., 2011), providing the initiator molecule for the production of PQS.

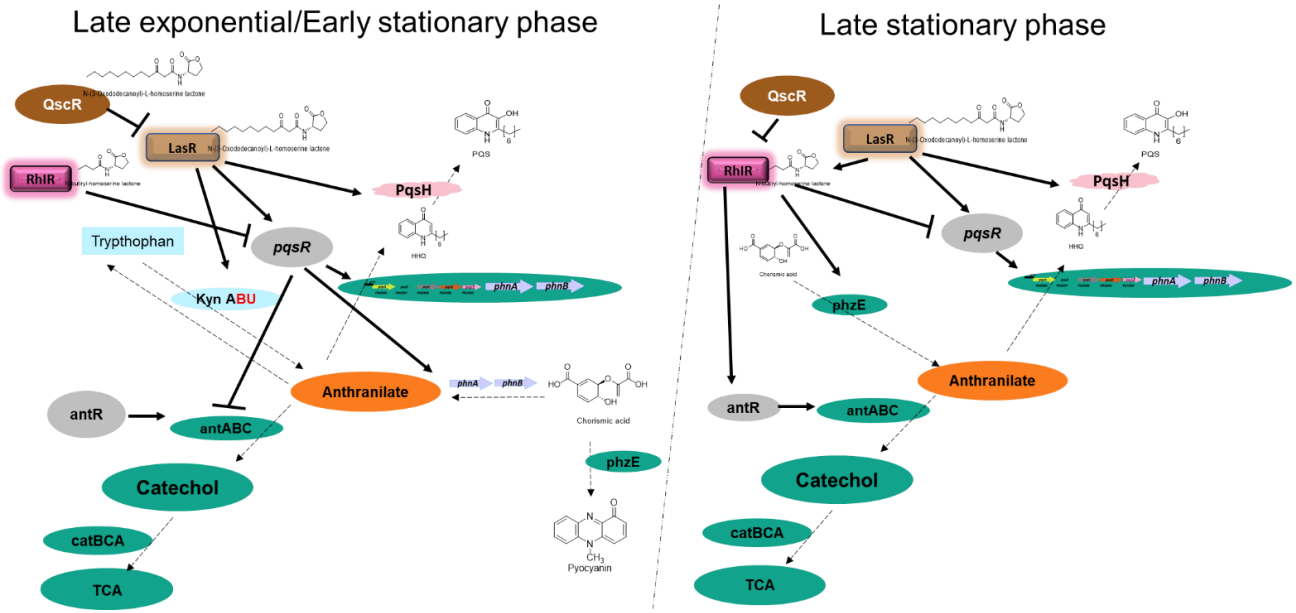


Figure 1.7. Anthranilate as a metabolic branch point in *P. aeruginosa* and its relationship with QS.

During late exponential/early stationary phase the anthranilate production is favoured. (Left panel), QscR controls the expression of *lasR*, which induces *pqsR* and *pqsH* towards the production of PQS. LasR also upregulates the *kynB* and *kynU* enzymes of the kynurenine pathway to provide a source of anthranilate from tryptophan. PqsR represses *antA* to favour the formation of PQS through the increase in anthranilate levels. Anthranilate is also formed from chorismite by the action of *phnAB* and that is under the control of PqsR. During late stationary phase degradation of anthranilate is favoured (Right panel), LasR activates RhlR, which in turn represses *pqsR* and *pqsA*, decreasing the formation of PQS and promoting the accumulation of anthranilate leading to its degradation by AntR, which gene is upregulated by RhlR. The accumulation of anthranilate is believed to be an action mediated by *phzE1*, which is upregulated by *rhlR* during late stationary phase, hence favouring the degradation of anthranilate towards the TCA cycle. Modified from Choi et al., (2011).

1.9 The importance of PqsE in *P. aeruginosa*

PqsE is a metallo β-hydrolase enzyme that shows a β lactamase fold and a Fe(II)/(III) centre in the active site (Yu et al., 2009). In *P. aeruginosa*, the crystal structure of PqsE and its enzymatic role in AQs biosynthesis has been elucidated (Drees & Fetzner, 2015). However, beyond its thioesterase activity, PqsE was named "PQS response protein" as it is required for the production of PQS-controlled virulence factors, such as pyocyanin, elastase, rhamnolipids, and also participates in biofilm formation (Diggle et al., 2003; Farrow et al., 2008; Rampioni et al., 2010). In addition, *pqsE* overexpression abolishes *pqsA* transcription, and thus abrogates AQ biosynthesis (Rampioni et al., 2010). Considering that the activation of *pqsABCDE* transcription results in increased levels of AQs and PqsE, it cannot be discarded that functional effects previously related to be HHQ- and/or PQS-dependent are mediated trough PqsE. On the other hand, since the overexpression of *pqsE* represses the *pqsA* promoter and, as a consequence, abrogates the production of AQs, is not clear whether the altered phenotypes observed under these conditions may be attributed to the lack of AQs or to the induction of *pqsE*.

The fact that PqsE exerts an autoregulatory role upon the *pqs* operon and therefore, its own expression, as well as playing a homeostatic role in limiting AQs accumulation, difficult to understand its physiological role(s). To investigate this role further Rampioni et al., (2010) created a *pqsE* inducible mutant (*pqsE* Ind), where *pqsE* is placed under the control of an IPTG-inducible *ptac* promoter ensuring its expression is independent of the whole *pqsABCDE* operon **(Figure1.8A)**.

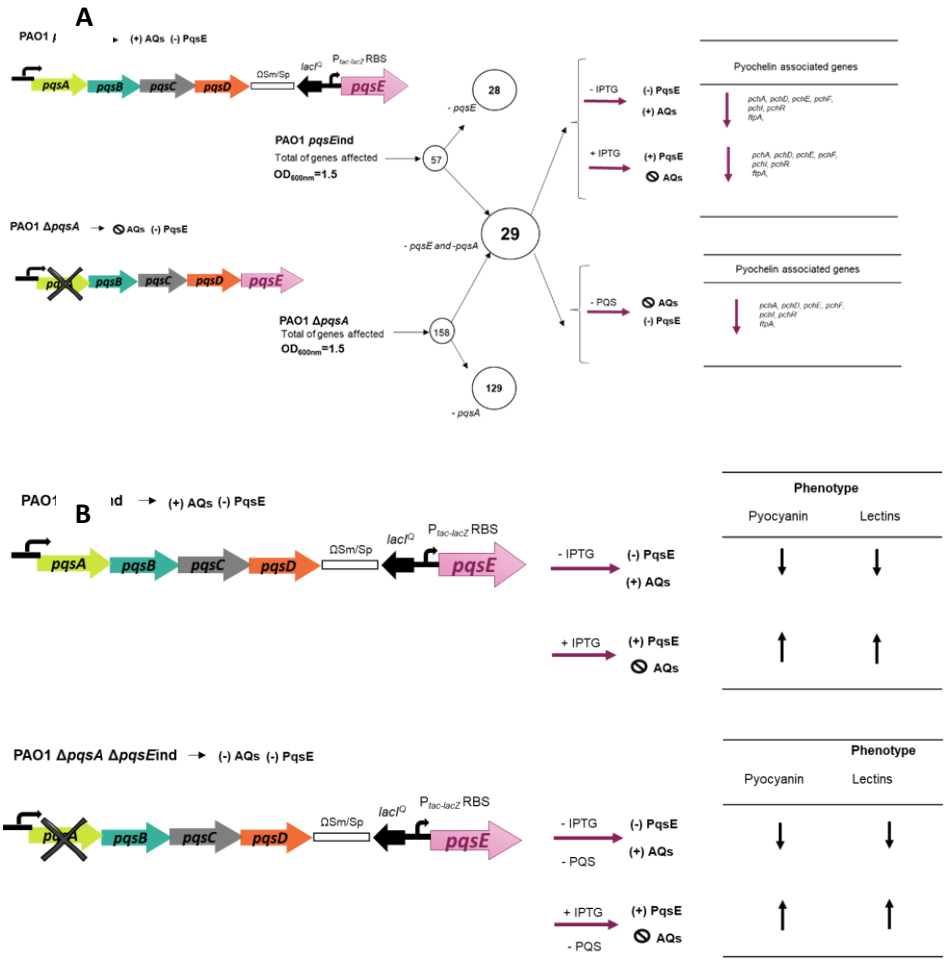


Figure 1.8. Visual representation of the transcriptome and phenotypic analyses performed to elucidate the PQS-independent roles of PqsE.

A) The transcriptome analysis performed at the late exponential phase (OD_{600nm}=1.5) of *P. aeruginosa* growth (Rampioni et al.,2010) comparing PAO1 wild type vs PAO1 *pqsE* Ind construction (top left) and PAO1 wild type vs a *pqsA* isogenic mutant (bottom left) indicates a shared regulatory role for PQS and PqsE towards 29 genes in *P. aeruginosa*. The limitations in attributing PqsE a PQS-independent role in gene regulation is evidenced when the iron entrapment related genes resulted downregulated either in the absence or in the presence of *pqsE*, challenging to associate the regulation of these genes to PqsE, PQS, or both.

B) The independent induction of *pqsE* and the provision of exogenous PQS in a PAO1 *pqsE* Ind (top left) and compared to a PAO1 *pqsA pqsE* Ind (bottom left), further clarified the PQS-independent role played by PqsE towards the production of pyocyanin and lectins in *P. aeruginosa*, which had been previously linked to be under the regulation of PQS. (+) IPTG: 1mM Isopropyl β-d-1-thiogalactopyranoside (-) states for lack. AQs: Alkyl quinolones (+) IPTG: 1mM Isopropyl β- d-1-thiogalactopyranoside PQS: 2-Heptyl-3-hydroxy-4-quinolone

In this work, a transcriptome analysis was performed at late exponential growth (OD_{600nm}=1.5) comparing PAO1 wild type to a *pqsA* mutant (does not produce AQs) and the uninduced to the induced *pqsE* (*pqsE* Ind - IPTG to *pqsE* Ind +IPTG, respectively). The analysis revealed that 29 out of the 57 genes affected by the *pqsE* conditional mutation (-IPTG) in the isogenic PAO1 *pqsE* Ind strain, were are also altered in the *pqsA* mutant (does not express *pqsE*) (**Figure 1.8A**), suggesting that the changes in expression of this subset genes are affected by the lack of either *pqsE,* PQS or both. For instance, when compared to the wild-type, among the downregulated genes in the *pqsA* mutant were found the genes encoding for pyochelin biosynthesis, uptake and regulation of iron (*pchA, pchB, pchC, pchD, pchE, pchF, pchI, pchR, ftpA*) (Rampioni et al.,2010), which in agreement with other studies, have shown to be under the control of PQS (Bredenbruch et al., 2006; Diggle et al., 2007). Interestingly, from the downregulated genes mentioned above, *pchA, pchB, pchD, pchE, pchF, pchI, pchR* and *ftpA* were also downregulated in the *pqsE* conditional mutation (*pqsE* Ind -IPTG) (which still produces PQS), suggesting at first, a probable role of PqsE in iron acquisition. The latter posit however is weakened when the induction of *pqsE* in the conditional mutation (*pqsE* Ind + IPTG) also resulted in the downregulation of a subset of the above mentioned genes , indicating that either the lack of PQS and PqsE in the *pqsA* mutant, or the abrogation of PQS caused by the premature overexpression of *pqsE* is responsible for these changes (Rampioni et al,. 2010) (**Figure 1.8A**). In the same work, the authors revealed that the production of different virulence factors in *P. aeruginosa* involves PqsE-PQS co-dependent and counter-dependent mechanisms. For instance, the biofilm formation in a *pqsA* mutant (which does not produce biofilm) was not restored when *pqsE* was induced alone and it was partially restored under the sole presence of exogenous PQS, indicativng that both elements are required for fully production of this virulence factor (Rampion et al., 2010). On the contrary, the production of lectin and pyocyanin in a *pqsA* mutant, was restored under the sole induction of *pqsE* ,which reveals therefore, a PQS-PqsE counter-dependent mechanism (**Figure 1.8B**), this data, is consistent with previous evidence in the ability of PqsE to act independently of

PQS (Farrow et al., 2008), and that suggest multidisciplinary roles attributed to PqsE.

To clarify further the roles attributed to PQS and/or PqsE in the production of virulence factors in *P. aeruginosa*, Rampioni et al., (2016) created a quadruple mutant, which carries in frame deletions of *pqsA*, *pqsH* and *pqsL* genes (unable to produce AQs), and that incorporates the IPTG inducible *pqsE* gene. The addition of exogenous PQS, HHQ, HQNO or the induction of *pqsE*, contributed to elucidate to some extent, the regulations attributed to each QS signal, as well as the PQS-independent regulated genes in which only PqsE takes part (Rampioni et al., 2016). From the transcriptome analysis performed at late exponential phase, notably, 115 genes were under the control of PqsE, further supporting a positive role on the expression of *lecA*, required for the production of the LecA lectin, *rhlA* and *rhlB*, involved in rhamnolipids biosynthesis, *hcnAB* operon responsible for the production of hydrogen cyanide and also a subset of pyocyanin related genes, among others. Interestingly, only 30 genes were controlled by both PqsE and PQS, from which the vast majority are involved in anaerobic respiration and that were downregulated by either stimulus (+IPTG or exogenous PQS, respectively). The authors also demonstrated that PQS rather than *pqsE* controls the iron-regulated genes, as the sole induction of *pqsE* did not alter this subset of PQS-dependent genes, demonstrating that the impact of PqsE towards the iron acquisition genes observed in Rampioni et al., (2010) was due to the ability of PqsE to affect the PQS levels (Rampioni et al., 2016). Similarly, exogenous PQS in the quadruple mutant did not alter some of the PqsE-controlled virulence factors such as pyocyanin and lectins, hence suggesting that in previous experiments the addition of exogenous PQS or the abrogation of PQS in a *pqsA* mutant led to a dysregulation of *pqsE* expression (Rampioni et al., 2010; Rampioni et al., 2016). Noticeably, an important proportion (29.3%) of the PqsE up-regulated genes encoded hypothetical proteins, suggesting a vast number of unknowns that need further examination.

Besides the independent roles of PqsE in *P. aeruginosa* virulence, evidence strongly suggests that PqsE acts as a regulator that is dependent on RhlR. In a report from Farrow et al., (2008) it was shown that the overexpression of *pqsE* in a *lasR/lasI* mutant, presented delayed but increased production of pyocyanin. Moreover, whilst the overexpression of *pqsE* in a *rhlI* mutant (lacking C4-HSL), partially restored pyocyanin production, this phenotype was complete lost when *rhlR* was absent. These observations evidenced that only RhlR is essential for the PqsE-mediated virulence in the production of pyocyanin in *P. aeruginosa* and it relates to that described in Higgins et al., (2018). The observation that PqsE partially overcomes the absence of the C4-HSL for the production of pyocyanin suggested the existence of an alternative ligand. Mukherjee et al., (2017) found that supernatants of a *rhlI* mutant overexpressing *pqsE* promoted the expression of some RhlR-regulated virulence factors. Based on this, the authors claimed the existence of a ligand produced by PqsE, named "PqsE-alternative ligand" , which can drive the expression of selected classes of RhlR-dependent genes (Mukherjee et al., 2018). The same group, also revealed PqsE sensitizes RhlR to C4-HSL trough protein-protein interaction between PqsE and RhlR (Taylor et al., 2021), evidencing that the E182 residue, which is buried in the core of the PqsE protein is related to the activation of pyocyanin production in vivo. Interestingly, the same work also showed that the catalytic activity of PqsE is dispensable for its role in virulence, which further evidenced its multidisciplinary and independent roles in *P. aeruginosa.* The adaptability of *P. aeruginosa* regulations and the link between PqsE and RhlR was also revealed in García-Reyes et al., (2021). In this work, the authors evidenced that PqsE increases the concentration of RhlR to specifically favor the RhlR regulatory activity towards pyocyanin production in PAO1 and PA7 strain ATTC 9027. Interestingly, in the last strain, which carries a frame-shift mutation in *pqsR*, the authors unveiled that *pqsE* is still expressed and that this phenomenon occurs independently of the PqsR-PQS activation of the *pqsABCDE* operon, which expands the complexity of the regulatory pathways connected to *pqsE*.

1.10 Importance of study and overall aim

Despite the efforts in unveiling the regulatory networks participating in the regulation of the *pqs* system in *P. aeruginosa*, the current understanding of the molecular mechanisms governing this QS system is however limited, to a great extent due to the interdependent, autoregulatory and multi-component nature of the system. With major attention, several questions regarding the pivotal and intriguing player PqsE remain obscure, which is greatly involved in the complex intercellular signaling regulatory scheme in a yet unsolved mechanism. The present study focuses on dissecting further the regulation of the *pqs* system and more specifically to elucidate the mechanisms by which PqsE mediates the negative regulation of the *pqsA* promoter. To achieve the above, different strategies have been undertaken in the different chapters of this work and that are described below:

* Chapter 3 analyses a *pqsA* DNA promoter pull-down carried out at early stage of growth of *P. aeruginosa* and that was performed before this thesis from which, the premature induction of *pqsE* exposed the proteins binding the *pqsA* upstream region. From this study, the main candidates to mediate the action of PqsE towards *pqsA* are described further and one of them is selected to study more in depth. Gene expression and phenotypic experiments, protein purification and EMSA analysis are used to evaluate whether this candidate is required for the PqsE-mediated repression of *pqsA* at the early stage of growth of this pathogen.

* Chapter 4 focuses on the study of a second *pqsA* DNA promoter pull-down carried out at the late stage of growth of *P. aeruginosa* and that was also performed previously to this work. The overexpression of *pqsE* in the setting experiment reveals a few candidates to mediate the action of PqsE. Using gene expression analysis, one candidate is selected to study in more extent. Applying the same rationale and experimental approaches as in Chapter 3, the work performed in this section aims to determine whether

this candidate is essential for the PqsE-mediated repression towards the expression of *pqsA,* at the late stage of growth of *P. aeruginosa*.

* Chapter 5 studies different venues of how the *pqs* operon is regulated, focusing on the study of specific regulatory elements that suggest being pivotal for the accurate understanding of the repression of *pqsA*. In addition, considering the high level of interconnected regulations taking part in the QS regulation, the impact of the main QS regulators towards the expression of *pqsA* is covered and more in detail, the link between RhlR and PqsE is discussed further. Gene expression analysis and phenotypic assays are used in this chapter aiming to unravel key regulatory elements required for the *pqsA* regulation and help to expand the knowledge of this sophisticated regulatory circuit.

Altogether, this study aims to better understand the mechanisms of how PqsE successfully controls virulence in *P. aeruginosa* and specifically, to set new basis in the regulation of *pqsA*, opening new routes of study towards the elucidation of the beyond complex QS system.

2 Chapter Two: Material and Methods

2.1 Bacterial strains and culture techniques

All bacterial strains and plasmids are listed in **Table 2.1.** *P. aeruginosa* and *E.coli* strains were routinely grown in Luria-Bertani broth (LB) and on LB agar. Bacterial cultures were grown at 37 ºC with 200 rpm shaking. Conical flasks were filled to a 10 % of their total volume to provide oxygenation. Anaerobic growth for *ΔnirQ* was carried out in LB medium with 40 mM Arginine. When required, LB was supplemented with 40 µM synthetic HHQ, PQS or mPQS, 10 µM C4-HSL or 3OC12-HSL. Antibiotics were added at the following concentrations: ampicillin (Amp) (100 µg/mL), carbenicillin (Cb) (300 µg/mL), gentamycin (Gm) (20 µg/mL), tetracycline (Tet) (25/125 µg/mL) and chloramphenicol (Cam) (50 µg/mL) in *E. coli* and/or *P. aeruginosa*. Whenever IPTG was used to induce gene expression, the concentration was 1 mM unless otherwise stated.

2.1.1 Pseudomonas isolation agar

Pseudomonas isolation agar (PIA) was used to select Pseudomonas transconjugants. This medium was prepared following the manufacturer's instructions. The media composition was as follow: Agar 13,6 g/L, 1.4 g/L $MgCl_2$, 10 g/L K_2SO_4, 20g/L peptic digest of animal tissue, 0.025 g/L 5-chloro-2-(2,4-dichlorophenoxy) phenol (irgasan) and was made in distilled water supplemented with 20 mL/L glycerol.

2.1.2 Anaerobic growth for *P. aeruginosa.*

Since a *nirQ* isogenic mutant is not able to grow anaerobically neither in the presence of KNO_3 nor KNO_2, aerobic *P. aeruginosa* overnight cultures were used to inoculate 5 mL of LB arginine 40 mM with a starting optical density OD_{600}= 0.01. The resulting cultures were transferred into an anaerobic box with anaerobe gas generation bags (Thermo scientific™

Oxoid™) and incubated at 37^{o}C inside a microaerophilic cabinet with 2% oxygen until mid-exponential phase was reached.

2.1.3 Bacterial storage

Overnight bacterial strains were mixed with glycerol 80% in a ratio 1:1 and stored at -80^{o}C.

Table 2.1. Bacterial strains and plasmids used in this study

Strains	Features	Reference
***Pseudomonas* strains**		
PAO1-L	Wild type PAO1 strain, Lausanne subline	H B. Holloway via D. Haas
PAO1-N	Wild type PAO1 strain, Nottingham subline	Laboratory collection, derived from Holloway's isolate (Holloway., 1955)
PAO1-L *pqsE* Ind	PAO1-L with an IPTG-inducible conditional *pqsE* gene; Sm^R	*
Δ*pqsA-L*	*pqsA* in frame mutant derivate of PAO1-L	Carol Paiva, Laboratory collection
Δ*pqsE-N*	*pqsE* in frame mutant derivate of PAO1-N	Matthew Fletcher, Laboratory collection
Δ*PA2704*	*PA2704* in frame mutant strain derivative of PAO1-L	*
Δ*PA2705*	*PA2705* in frame mutant strain derivative of PAO1-L	*
Δ*PA2705 pqsE* Ind	Double mutant of PAO1-L carrying in frame clear deletion of *PA2705* gene, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δ*rhlR*	*rhlR* in frame mutant strain derivate of PAO1-L	James Lazenby, Laboratory collection
Δ*lasR*	*lasR* in frame mutant strain derivate of PAO1-L	James Lazenby, Laboratory collection.
Δ*rhlI*	*rhlI* in frame mutant strain derivate of PAO1-L	James Lazenby, Laboratory collection.
Δ*lasI*	*lasI* in frame mutant strain derivate of PAO1-L	James Lazenby,

		Laboratory collection.
Δ*lasR* Δ*rhlR*	Double mutant of PAO1-L carrying in frame clear deletions of the *lasR* and *rhlR* genes	James Lazenby, Laboratory collection.
Δ*pqsR*	*pqsR* in frame mutant strain derivative of PAO1-L	*
Δ*pqsR* Δ*rhlR*	Double mutant of PAO1-L carrying in frame clear deletions of the *pqsR* and *rhlR* genes	*
Δ*pqsR* Δ*lasR*	Double mutant of PAO1-L carrying in frame clear deletions of the *pqsR* and *lasR* genes	*
Δ*pqsR* Δ*lasR* Δ*rhlR*	Triple mutant of PAO1-L carrying in frame clear deletions of the *pqsR, lasR and rhlR* genes	*
Δ*rhlR pqsE* Ind	Double mutant of PAO1-L carrying in frame clear deletion of the *rhlR* gene, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δ*pqsR pqsE* Ind	Double mutant of PAO1-L carrying in frame clear deletion of the *pqsR* gene, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δ*lasR pqsE* Ind	Double mutant of PAO1-L carrying in frame clear deletion of the *lasR* gene, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δ*rhlR* Δ*pqsR pqsE* Ind	Triple mutant of PAO1-L carrying in frame clear deletions of the *rhlR* and *pqsR* genes, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δ*lasR* Δ*pqsR pqsE* Ind	Triple mutant of PAO1-L carrying in frame clear deletions of the *lasR* and *pqsR* genes, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δ*lasR ΔrhlR pqsE* Ind	Triple mutant of PAO1-L carrying in frame clear deletions of the *lasR* and *rhlR* genes, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δ*lasR* Δ*rhlR* Δ*pqsR pqsE* Ind	Quadruple mutant of PAO1-L carrying in frame clear deletions of the *lasR, rhlR* and *pqsR* genes, in which *pqsE* is under the control	*

	of an IPTG-inducible promoter; Sm^R	
PAGR31	*clpC* in frame mutant strain derivate of PAO1-N	Giordano Rampioni, Laboratory collection.
PAGR32	*PA0779* in frame mutant strain derivate of PAO1-N	Giordano Rampioni, Laboratory collection.
PAGR33	*pepA* in frame mutant strain derivate of PAO1-N	Giordano Rampioni, Laboratory collection.
PAGR34	*nirQ* in frame mutant strain derivate of PAO1-N	Giordano Rampioni, Laboratory collection.
PAGR35	*PA4843* in frame mutant strain derivate of PAO1-N	Giordano Rampioni, Laboratory collection.
PAGR36	*phaF* in frame mutant strain derivate of PAO1-N	Giordano Rampioni, Laboratory collection.
ΔnirQ	*nirQ* in frame mutant strain derivate of PAO1-L	*
ΔnirQ pqsE Ind	Double mutant of PAO1-L carrying in frame clear deletion of the *nirQ* gene, in which *pqsE* is under the control of an IPTG-inducible promoter; Sm^R	*
Δvfr	*vfr* in frame mutant strain derivative of PAO1-L	Matthew Fletcher, Laboratory collection.
Δ*cyaB*	*cyaB* in frame mutant strain derivative of PAO1-L	*
Δvfr Δ*cyaB*	Double mutant of PAO1-L carrying in frame clear deletions of the *vfr* and *cyaB* genes	*
***E. coli* strains**		
E. coli DH5α	*Escherichia coli* strain used as host of pGEMT-easy vector and pBluescript II KS carrying PCR products	Grant et al., 1990)
E. coli S17-1λpir	*Escherichia coli* Strain for maintenance and mobilization of pMiniCTX-*lux* and vectors	Simon et al., 1983

	pME6032, pEX18, pDM4, pME3087	
E. coli BL21(*DE3)*	F- *hsd*S gal	Chambedin et al., 1990)
Plasmids		
pDM4	R6K-derived suicide vector; sacBR; Gm^R	Milton et al., 1996
pDM4::*pqsE* Ind	pDM4 derivative for the generation of the *pqsE*-inducible strain; Cam^R	Rampioni et al., 2010
pDM4::*pqsE* Ind*	pDM4 derivative for the generation of the *pqsE*-inducible strain; Gm^R	*
pNR1	Upstream region of PA2705 cloned into pBluescript II KS amplified by using primers PA2705-Up-F and PA2705-Up-R	*
pNR2	Downstream region of *PA2705* cloned into pBluescript II KS, amplified by using primers PA2705-Ds-F and PA2705-Ds-R	*
pNR3	Upstream region of *PA2704* cloned into pBluescript II KS, amplified by using primers PA2704-Up-F and PA2704-Up-R	*
pNR4	Downstream region of *PA2704* cloned into pBluescript II KS, amplified by using primers PA2704-Ds-F and PA2704-Ds-R	*
pNR5	Upstream region of *cyaB* cloned into pBluescript II KS, amplified by using primers CyaB-Up-F and CyaB-Up-R	*
pNR6	Downstream region of *cyaB* cloned into pBluescript II KS, amplified by using primers CyaB-Ds-F and CyaB-Ds-R	*
pMiniCTX-*lux**	Gentamicin-resistant variant of pMiniCTX-*lux*	Matthew Fletcher, Laboratory collection
$P_{pqsA\ short}$-*lux*	pMiniCTX::$P_{pqsA\ short}$-*lux*. Plasmid to insert P_{pqsA}-lux transcriptional fusion into the chromosome of *Pseudomonas* ; Gm^R	Matthew Fletcher, Laboratory collection
P_{PA2705}-*lux.*	pMiniCTX::P_{PA2705}-*lux.*Plasmid to insert P_{PA2705}-*lux* transcriptional fusion into the chromosome of *Pseudomonas*; Gm^R Obtained by the insertion of 366 bp upstream PA2705 amplified using primers	*

	Ppa2705-F and *Ppa2705*-R, into EcoRI/BamHI sites in pminiCTX-*lux*, Gm^R	
$P_{pqsA\ long}$-*lux*	pMiniCTX::$P_{pqsA\ long}$ -*lux*. Plasmid to insert P_{pqsA}-*lux* transcriptional fusion into the chromosome of *Pseudomonas* ; Gm^R . Obtained by the insertion of 502 bp upstream *pqsA* amplified using primers *PpqsA*-F and *PpqsA*-R, into EcoRI/PstI sites in pMiniCTX-*lux*, Gm^R	*
$P_{pqsA\ \Delta rhlR\text{-}box}$-*lux*	pMiniCTX::$P_{pqsA\ \Delta rhlR\text{-}box}$-*lux*. Plasmid to insert $P_{pqsA\ \Delta rhlR\text{-}box}$-*lux* transcriptional fusion lacking the *las/rhl* (RhlR) box into the chromosome of *Pseudomonas*; Gm^R . Obtained by the insertion of 486 bp upstream *pqsA* amplified using primers *PpqsA* -F/ *PpqsA* Δ*rhlRbox* -R and *PpqsA* Δ*rhlRbox* -F/ *PpqsA* -R, into EcoRI/PstI sites in pMiniCTX-*lux*, Gm^R	*
P_{pqsE}-*lux*	pMiniCTX::P_{pqsE}-*lux*. Plasmid to insert P_{pqsE}-*lux* transcriptional fusion into the chromosome of *Pseudomonas*; Gm^R . Obtained by the insertion of 500 bp upstream *pqsE* amplified using primers *PpqsE*-F and *PpqsE*-R, into EcoRI/PstI sites in pMiniCTX-*lux*, Gm^R	*
P_{nirQ}-*lux*	pMiniCTX::P_{nirQ}-*lux*. Plasmid to insert P_{nirQ}-*lux* transcriptional fusion into the chromosome of *Pseudomonas*; Gm^R . Obtained by the insertion of 215 bp upstream *nirQ* amplified using primers *PnirQ*-F and *PnirQ*-R, into KpnI/PstI sites in pMiniCTX-*lux*, Gm^R	*
P_{PA2705}-*luxCDABE*	pMiniCTX::P_{PA2705}-lux translational version. In-frame fusion of P_{PA2705} plus the first ATG codon with the *luxC* derivative of pMiniCTX-lux, GmR	*
$P_{pqsA\ long}$-*luxCDABE*	pMiniCTX::$P_{pqsA\ long}$-*lux* translational version. In-frame fusion of P_{pqsA} with the *luxC* derivative of pMiniCTX-lux, Gm^R	*

P_{nirQ} '-' - *luxCDABE*	pMiniCTX::P_{nirQ}-*lux* translational versión. In-frame fusion of 100 bp of P_{nirQ} plus the first ATG codon with the luxC derivative of pMiniCTX-lux, Gm^R	*
pEX18:Δ*pqsR*	Gene replacement vector, oriT+ sacB; Gm^R for in-frame deletion of *pqsR* in *PAO1-L*	Paolo Pantalone, Laboratory collection.
pME3087	Tet^R, suicide vector for allelic replacement; ColE1-replicon, IncP-1, Mob	Voisard et al., 2007
pMESHLasR	pME3087:: *ΔlasR.* Construct for deletion of *lasR* in *P. aeruginosa,* Tet^R	Steve Higgins, Laboratory collection.
pMENR1	pME3087::*ΔPA2705.* Construct for deletion of 388 out of 394 codons of *PA2705* in *P. aeruginosa,* Tet^R. Upstream and downstream region of *PA2705* were digested with XbaI and BamHI from pNR1, and BamHI and KpnI from pNR2, respectively	*
pMENR2	pME3087::*ΔPA2704.* Construct for deletion of 332 out of 340 codons of *PA2704* in *P. aeruginosa,* Tet^R. Upstream and downstream region of *PA2704* were digested with XbaI and BamHI from pNR3, and BamHI and KpnI from pNR4, respectively.	*
pMENR3	pME3087::*ΔcyaB.* Construct for deletion of 439 out of 464 codons of *cyaB* in *P. aeruginosa,* Tet^R. Upstream and downstream region of *cyaB* were digested with XbaI and EcoRI from pNR5, and EcoRI and KpnI from pNR6, respectively.	*
pMENR4	pME3087::*ΔnirQ.* Construct for deletion of 252 out of 261 codons of *nirQ* in *P. aeruginosa,* Tet^R. Truncated *nirQ* was digested with XbaI and KpnI	*
pME6032	pVS1-p15A shuttle expression (IPTG-inducible) vector, Tet^R	Heeb et al., 2002
pMENRC1	pME6032::*PA2705,* Tet^R. For complementation of *PA2705*, using primers PA2705-C-F and PA2705-C-R	*

pMENRC2	pME6032::*PA2704,* Tet^R. For complementation of *PA2704*, using primers PA2704-C-F and PA2704-C-R	*
pMENRC3	pME6032::*nirQ,* Tet^R. For complementation of *nirQ*, using primers NirQ-C-F and NirQ-C-R	*
pMENRC4	pME6032::*vfr,* Tet^R. For complementation of *vfr,* using primers Vfr-C-F and Vfr-C-R	*
pMENRC5	pME6032::*rhlR,* Tet^R. For complementation of *rhlR*, using primers RhlR-C-F and RhlR-C-R	*
pMENRC6	pME6032::*lasR,* Tet^R. For complementation of *lasR*, using primers LasR-C-F and LasR-C-R	*
pMENRC7	pME6032::*pqsR,* Tet^R. For complementation of *pqsR*, using primers PqsR-C-F and PqsR-C-R	*
pqsX-T1	PqsX-T1 region inserted into pME6032, Tet^R. Obtained by the insertion of 258 bp downstream the -399 *pqsA* Transcriptional start site (TSS) using primers RhlR-Term1/2-F and RhlR-Term1-R	*
pqsX-T2	PqsX-T2 region inserted into pME6032, Tet^R. Obtained by the insertion of 337 bp downstream the -399 *pqsA* Transcriptional start site (TSS) using primers RhlR-Term1/2-F and RhlR-Term2-R	*
Pet21a	Amp^R, Bacterial expression vector, T7-lac, C-His tag	Siyu Wu, Laboratory collection
pNR1pet	*Pet21a::nirQ,* Amp^R. *A* Full length nirQ cloned into Pet21a. Obtained by the insertion of 783 bp into Pet21a using primers NirQ-F/R	*
pCold1	Amp^R, Bacterial expression vector, Cold-shock expression vector, *cspA* promoter, adds N-terminal His tag	Qing et al., 2004
pNR1Cold1	*pCold::PA2705,* Amp^R. Full length *PA2705* cloned into pCold. Obtained by the insertion of 1182 bp into pCold using primers PA2705-F/R	*
pNR2Cold1	*pCold::nirQ,* Amp^R.Full length *nirQ* cloned into pCold. Obtained by the insertion of 783bp into pCold suing primers NirQ-F/R	*

pNR3Cold1	*pCold::PA2707,* Amp^R .Full length PA2707 cloned into pCold. Obtained by the insertion of 846bp into pCold suing primers PA2707'F-R-F/R	*

* This study

2.2 DNA Manipulation.

All plasmids generated and/or used in this study are listed in **Table 2.2**. DNA concentration and purity was assessed with the Nanodrop ND-1000 (Nanodrop Technologies). Elution was carried out using molecular grade water.

Table 2.2. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')	Restriction site
In -frame deletions		
PA2705-Up-F	ATA TCTAGA GGAGCGGACGCGGT	XbaI
PA2705-Up-R	ATA GGATCC GAGCAGCATGGCTGCGA	BamHI
PA2705-Ds-F	ATA GGATCC CGCGGTTGAGCCAACTG	BamHI
PA2705-Ds-R	ATA GGTACC GAGAGCAGCGCGTAG	KpnI
PA2704-Up-F	ATA TCTAGA GCGGAGGAGCTGGAC	XbaI
PA2704-Up-R	ATA GGATCC GTCGATCATGGCGTG	BamHI
PA2704-Ds-F	ATA GGATCC ATTTCCGCGCCTGAGC	BamHI
PA2704-Ds-R	ATA GGTACC GCTGAACTCGAACAGTTG	KpnI
Cyab-Up-F	ATA TCTAGA TCGCCGAGTTCTACCC	XbaI
Cyab-Up-R	ATA GAATTC CTTCATGCGCTGGAGAGG	EcoRI
Cyab-Ds-F	ATA GAATTC GTCATCCTCTAAGTTCGTC	EcoRI
Cyab-Ds-R	ATA GGTACC CCTGTATGTCGGGCG	KpnI
NirQ Up-F	ATA TCTAGA TGCTGGGTGATGTCCG	XbaI
NirQ Ds-R	ATA GGTACC AGCACTCCCGACTCCAG	KpnI
Gene complementation		
PPA2705-C-F	TAT GAATTC ATGCTGCTCACCTGTTC	EcoRI
PPA2705-C-R	TAT GAGCTC TCAACCGCGCGAG	SacI
PPA2704-C-F	TAT GAATTC ATGATCGACTCGACCTTC	EcoRI
PPA2704-C-R	TAT GAGCTC TCAGGCGCGGAAATG	SacI
NirQ-C-F	TAT GAATTC ATGCGGGACGCGACA	EcoRI
NirQ-C-R	TAT GAGCTC TCAGGCGACATGGAG	SacI
Vfr-C-F	TAT GAATTC ATG GTAGCTATTACCCACAC	EcoRI
Vfr-C-R	TAT GAGCTC TCAGCGGGTGCCGAA	SacI
RhlR-C-F	TATGAATTC ATGAGGAATGACGGAGGCT	EcoRI
RhlR-C-R	ATA GGTACC TCAGATGAGACCCAGCG	KpnI
LasR-C-F	TAT GAATTC ATGGCCTTGGTTGACGG	EcoRI
LasR-C-R	ATA GGTACC TGGGTCTTATTACTCTCTGA	KpnI
PqsR-C-F	TAT GAATTC ATGCCTATTCATAACCTGA	EcoRI
PqsR-C-R	TAT GAGCTC CTACTCTGGTGCGGC	SacI
Plasmid constructions		
PPA2705-F	GATATC GAATTC GCGAGACGGTGCTGA	EcoRI
PPA2705-R	TATTAA GGATCC AATCCTCGAACGGTAACGCC	BamHI
PPA2705-translational-F	ATAGTTAAACAGCAACTTAAGTTGAAATTACCCCCATTAAGC GAGACGGTGCTGAAGAAC	
PPA2705- translational-R	CCTGGCCGTTAATAATGAATGAAATTTTTTTAGTCATCATAAT CCTCGAACGGTAACGCC	
PPqsA-translational-F	ATAGTTAAACAGCAACTTAAGTTGAAATTACCCCCATTAACTG CAAATGGCAGGCGAG	
PPqsA translational-R	AATAATGAATGAAATTTTTTTAGTCATATTTGCCATCCATGAC ATGACAGAACGTTCCCTCT	
PNirQ-translational-F	CGCAAGATAGTTAAACAGCAACTTAAGTTGAAATTACCCCCA TTAAA GAAAGGATCATAGAAAGCAGG	
PNirQ- translational-R	GAATGAAATTTTTTTAGTCATATTTGCCATCCATatGTCCTACT CCTGCGCT	
PpqsA-F	TATTAA GAATTC CTGCAAATGGCAGGCGAGG	EcoRI
PpqsA-R	TATT CTGCAG TGGACATGACAGAACGTTCCC	PstI
PnirQ-F	TAT GGTACC GGTCTATCTCCTCAGGAGC	KpnI
PnirQ-R	TATT CTGCAG GTCCTACTCCTGCGCTAG	PstI
PpqsA ΔrhlRbox-F	TATTAA GAATTC CTGCAAATGGCAGGCGAGG	EcoRI
PpqsA ΔrhlRbox-R	TATT CTGCAG TGGACATGACAGAACGTTCCC	PstI
PpqsE-F	TATTAA GAATTC AGCGCCGGCGAGAGTCT	EcoRI
PpqsE-R	TATT CTGCAG GGCCGGTTCACCTCCTCAG	PstI
PqsX-T1/T2 -F	ATCATCGGCTCGTATAATGTGTGGCCATCTCATGGGTTCGGA C	
PqsX-T1-R	TACCCGGGAGCTCGAATTAAAAAAAAAACTAGCGGCGCTGG GC	
PqsX-T2-R	TACCCGGGAGCTCGAATTAAAAAAAAAACAGAACGTTCCCTC TTCAGC	
RT-PCR		
pqsA-RTPCR-F	ACGTTCTGTCATGTCCACATTGGC	
pqsA-RTPCR-R	GCCTGGGAGAGAATGTAGGTCCG	
16S-RTPCR-F	CAAAACTACTGAGCTAGAGTACG	Lenz et al., 2008
16-S-RTPCR-R	TAAGATCTCAAGGATCCCAACGGCT	Lenz et al., 2008
ProC-RTPCR-F	CAGGCCGGGCAGTTGCTGTC	Savli et al., 2003
ProC-RTPCR-R	GGTCAGGCGCGAGGCTGTCT	Savli et al., 2003
RpoS-RTPCR-F	CTCCCCGGGCAACTCCAAAG	Savli et al., 2003
RpoS-RTPCR-R	CGATCATCCGCTTCCGACCAG	Savli et al., 2003
RpoD-RTPCR-F	GGGCGAAGAAGGAAATGGTC	Savli et al., 2003
RpoD-RTPCR-R	CAGGTGGCGTAGGTGGAGAA	Savli et al., 2003
Protein expression		

NirQ-F	ATA GGATCC ATGCGGGACGCGACACCC	BamHI
NirQ-R	ATA AAGCTT TCAGGCGACATGGAGATC	HindIII
PA2705-F	ATA GGATCC ATGCTGCTCAACCTGTTC	BamHI
PA2705-R	ATA AAGCTT TCAACCGCGCGAGAG	HindIII
PA2707-F	ATA GGATCC ATGAAGTTCGAAGGCACCCAGTC	BamHI
PA2707-R	ATA AAGCTT TCAGCGGCTGGCGCGG	HindIII
Sequencing analysis		
M13- pUC-F	CCCAGTCACGACGTTGTAAAACG	Flanking primers to sequence insertions into pBluescript II KS
M13- pUC-R	CCTGTGTGAAATTGTTATCCGCT	
pDM4 SEQ-F	ACAGGAACACTTAACGGCTG	Flanking primers to sequence insertions into pDM4
pDM4 SEQ-R	TGTCCCTCCTGTTCAGCTACT	
pTac SEQ-F	GTTGACAATTAATCATCGGCTCG	Primers to verify insertion of the *pqsE* Ind elements into the Chromosome of *P. aeruginosa*
pqsE SEQ-R	AGGCTGGACAGGCCATGC	
pME6032 SEQ-F	CACTTCCCTGTTAAGTATCT	Primers to sequence insertions into pME6032
pME6032 SEQ-R	GGTTCTGGCAAATATTCT	
CTX-lux Seq--F	CGAGGTCGACGGTATCG	Primers to sequence insertions into the MSC of the CTX-*lux*
CTX-lux Seq--R	TAGATTGAATAGCAATCTAATTTTTAC	
KS-CTX-F	CGAGGTCGACGGTATC	Primers to sequence translational insertions into CTX-*lux*
LuxC-CTX-R	CATCACTTTCGGGAAA	
lasR-SEQ-F	CCGTAACAACGTGCCGGATA	Primers to verify *lasR* mutations
lasrR-SEQ--R	AAGCCAGGAAACTTTCTGGAG	
pqsR-SEQ-F	CGGCATGCCAGCGTTAATACTT	Primers to verify *pqsR* mutations (Oton,PhD thesis, 2018)
pqsR-SEQ--R	CATCCCGAGTCGATTCTCACCA	

2.2.1 Isolation of DNA.

DNA isolation was performed accordingly to Wizard® Genomic DNA Purification Kit protocol. Briefly, cells were disrupted using the Nuclei Lysis Solution. After treatment with RNase, a salt precipitation step was performed for the removal of cellular proteins. Finally, the genomic DNA was concentrated and desalted by isopropanol precipitation.

2.2.2 Digestion and Ligation of DNA.

Digestion procedures were performed using NEB or Promega enzymes for 1 hour at 37°C. When possible, enzyme heat inactivation was carried out at 65°C for 15 min, otherwise, the samples were loaded directly into the DNA electrophoretic gel. Ligations were performed overnight at 16°C using NEB T4 ligase enzyme.

2.2.3 DNA Gel Electrophoresis.

Separation of DNA molecules by molecular size was observed using agarose gel electrophoresis. The agarose gel matrixes were prepared

according to Sambrook, J., and Russell 2001. Electrophoretic gels were made between 0.8% to 1.5% depending on the length of DNA sequences. Visualization of DNA bands required UV radiation and the SYBR Safe DNA gel stain (5x). When needed, 6x DNA loading buffer was added to samples prior gel loading. Quick-Load Purple 2-Log DNA Ladder (0.1-10.0 kb) NEB was used to determine the band sizes of DNA.

2.2.4 Polymerase Chain Reaction (PCR).

PCR amplifications were performed according to previously described methods (Saiki et al., 1988) in a final volume of 50 µl unless otherwise stated. The reaction contained 0.25 µl GoTaq polymerase (Promega) or 0.5 µL pfu DNA polymerase, 0.5 µl 10 µM primers, 4 µl 2.5 mM dNTPs , 5 µL buffer and DNA template, with 0.5 µl DMSO in 1× buffer. PCR amplification from colony PCR were prepared using the GoTaq Hot start Polymerase master mix (Promega). The DNA template used was either from a fresh colony or chromosome DNA solution.

2.2.5 Plasmid extractions

For isolation of high or low copy number plasmids DNA from *E. coli* and *P. aeruginosa*, the Qiagen Midi kit (Qiagen Ltd.) and GenElute™ Plasmid Miniprep Kit were used following the supplier instructions.

2.2.6 DNA purification from agarose

DNA fragments purifications from agarose gel were performed using Monarch DNA Gel Extraction Kit (NEB) (T1020S, NEB, USA). Briefly, the fragment of interest was excised using a clean scalpel, placed in a 1.5 mL microcentrifuge tube, resuspended in 4 volumes of Monarch Gel Dissolving Buffer, and melted in a heat block at 50°C. The DNA was then passed through a silica matrix, washed, and eluted in nuclease-free water.

2.2.7 DNA Sequencing.

DNA sample sequencing was performed using Sanger sequencing method by Source Biosciences https://www.sourcebioscience.com/) or at the University of Nottingham DNA sequencing facility. Sequences were then analyzed using Snap Gene or Benchling.

2.3 Conjugative mating experiments

DNA conjugation was performed to transfer plasmids from *E. coli* S17-1λpir to *P. aeruginosa.* Donor cells were grown overnight at 37°C, 200 rpm with the required antibiotics. The recipient strains were grown in a water bath at 42°C to reduce the activity of the restriction-modification system which degrades foreign DNA. Briefly, 1 mL of donor and recipient overnight cultures were pelleted and washed twice in LB broth before mixed at a final resuspension of 100 µL LB broth. From this suspension 25 µL were spotted onto LB plates and incubated at 30°C for 5-6 hours. Selection of *P. aeruginosa* conjugates were performed using Pseudomonas Isolation Agar (PIA) with respective antibiotics.

2.4 Preparation of Electrocompetent Cells.

E. coli DH5α and E. coli S17-1λpir were prepared according to the method described by Sambrook, J., and Russell 2001, with some modifications. All the procedures were carried out at 4°C unless otherwise specified. Cultures, once grown, were placed and transported on ice to ensure the temperature did not rise above 4°C. From an overnight culture, 1 mL was diluted in Fresh LB at a ratio 1:1000 and grown until OD_{600nm} of 0.6-0.8. Flasks were rapidly transferred to the pre-made ice-water bath for 15-30 min and swirled occasionally to ensure rapid cooling. Cells were harvested by centrifugation for 10 min at 5000 rpm and washed with 20 mL of cold glycerol 10% v/v. This step was repeated three times. After the final centrifugation, cells were resuspended in 1 mL glycerol 10% v/v and

aliquoted in pre-cooled tubes at a final volume of 50 µL. Cells were stored at 80°C until needed.

2.5 Transformations.

For transformation procedures, 50 µL of thawed electrocompetent cells were mixed on ice with 2-5 µL of plasmid or ligation previously dialyzed using a nitrocellulose filter and distilled water. The mixture was placed into a 1 mM or 2 mM clean and dry cuvettes previously cooled down. Electroporation was carried out at 1,600 Volt with a time constant (τ) of 5 millisec. Electroporated cells were rapidly re-suspended in 1 mL of fresh LB and incubated for 1-2 hours for recovering. Transformants were selected on LB plates containing respective antibiotics.

2.6 Cloning and Mutagenesis.

2.6.1 Construction of transcriptional and translational bioreporters

The CTX-based system was used to generate six transcriptional *lux* fusions: P_{PA2705}, $P_{pqsA\ long}$, $P_{pqsA\ rhlR\ box}$, P_{pqsE} and P_{nirQ} *lux* and three additional translational *lux* fusions P_{PA2705} , P_{nirQ} and $P_{pqsA\ long}$-*lux*.

Using the primer pair P_{PA2705} F/R, a 366 bp fragment upstream of PA2705 containing its promoter region was amplified by PCR from PAO1-L chromosomal DNA, digested with EcoRI and BamHI, and cloned between the corresponding restriction sites of pMiniCTX-*lux*, resulting in pMiniCTX::P_{PA2705}-*lux* (P_{PA2705}-*lux*). The P_{PA2705}-*lux* fusion was then integrated as a single copy into the chromosome of PAO1-L. The same approach was applied to obtain the pMiniCTX::P_{pqsA}-*lux* (P_{pqsA}-*lux*), pMiniCTX::P_{pqsE}-*lux* (P_{pqsE}-*lux*) and pMiniCTX::P_{nirQ}-*lux* (P_{nirQ}-*lux*) constructs using respective enzymes **(Table 2.1)**. The pMiniCTX:: $P_{pqsA\ rhlR\ box}$-*lux* ($P_{pqsA\ rhlR\ box}$-*lux*) was constructed as described in **section 2.6.5.**

To construct P_{PA2705}, P_{nirQ} and $P_{pqsA\ long}$ -*lux* translational fusions, the promoter DNA region was generated from PAO1-L chromosomal DNA by PCR with primers P*PA2705*-translational-F/R, P*nirQ*-translational F/R and

P*pqsA*-translational-F/R, respectively, cloned into XcmI-cut pMiniCTX-*lux* (Gm^R) plasmid by *in vitro* homologous recombination using a Gibson assembly kit (New England BioLabs) as per manufacturer's instructions. The mini-CTX elements were inserted in the chromosome of PAO1-L and in the corresponding mutant conditions by mating using *E. coli* S17-1 λpir as a donor.

2.6.2 Construction of in-frame deletion mutants

The suicide vector pME3087 (Tet^R) was used to generate in-frame deletion mutants of *PA2705*, *PA2704* and *cyaB* in *P. aeruginosa*. To achieve this, 295 bp upstream and 409 bp downstream of *PA2705*, 782 bp upstream and 457 bp downstream of PA2704 and 575 bp upstream and 512 bp downstream of *cyaB* were PCR amplified from chromosomal PAO1-L DNA using primers PA2705-Up-F/R and PA2705-Ds-F, PA2704-Up-F/R and PA2704-Ds-F/R and CyaB-Up-F/R and CyaB-Ds-F/R, respectively. The PCR fragments containing the upstream and downstream region of the above genes were cloned into pBluescript II KS, resulting in the plasmids pNR1/ pNR2 (For *PA2705*), pNR3/ pNR4(For *PA2704*) and pNR5/ pNR6(For *cyaB*) **(Table 2.1).** The upstream and downstream region of PA2705, corresponding to pNR1 and pNR2 were double digested with XbaI/BamH, BamHI/XpnI, respectively. Similarly, pNR3 and pNR4 were double digested using the same enzymes as above, whereas pNR5 and pNR6, were double digested with XbaI/EcoRI, and EcoRI/KpnI, respectively. The digested fragments were ligated and incorporated into a previously double-digested XbaI/KpnI suicide vector pME3087, resulting in the creation of pMENR1, pMENR2 and pMENR3**(Table 2.1).** For the construction of *nirQ* mutant, an in-frame deletion mutant was available in PAO1-N. The truncated gene was amplified using primer pairs NirQ Up/Ds, digested with enzymes XbaI and KpnI and cloned into the suicide vector pME3087, resulting in pMENR4.

The respective in-frame deletion mutants were created in PAO1-Lausanne genetic background under a two steps allelic exchange (Hmelo

et al., 2015). In the first step, the resulting vectors pMENR1, pMENR2, pMENR3 and pMENR4 were mobilized by conjugation into *P. aeruginosa* recipient strain using *E. coli* S17.1 λpir as a donor. Successful homologous recombination gave resistance to tetracycline and transformants were verified by PCR. In the second step of allelic exchange, the plasmid backbone was excised from the chromosome through double-crossover and applying tetracycline enrichment **(Section 2.6.3)**. The double-crossover mutants were isolated by counter-selection. Mutants were identified by replica plating to identify loss of tetracycline resistance, followed by PCR screening and sequencing.

2.6.3 Enrichments of tetracycline-sensitive cells

The creation of mutants using the suicide plasmid pME3087 (Tet^R) was used for tetracycline enrichments. Briefly, strains grown in LB for 2 hours were inoculated with a bacteriostatic concentration of tetracycline (10 µg/mL) and incubated at 37°C with shaking for 1 hour. Carbenicillin was then added at a bactericidal concentration of 2 mg/mL and incubated at 37°C with shaking for further 6 hours. Following 3 cycles of enrichment, serial dilutions of the cultures were plated and grown on LB agar, and the colonies obtained were then replica-plated on LB agar with or without tetracycline (125 µg/mL) to screen for loss of tetracycline resistance. Tetracycline sensitive colonies were then screened by PCR for the presence of the mutated gene.

2.6.4 Sucrose Counter Selection of *pqsE* Ind conditional and *pqsR* mutant

To generate the *pqsE* Ind derivatives in PAO1-L background, the *pqsE* inducible elements from the chloramphenicol resistant (Cam^R) suicide plasmid pDM4, were excised using the restriction enzymes XbaI and XhoI, gel purified and transferred into a previously XbaI- XhoI digested gentamicin resistant (Gm^R) pDM4. This step conferred compatibility with

PAO1-L genetic background as this subline presents intrinsic resistance to chloramphenicol, given the presence of the Chloramphenicol transferase gene (CAT). The insertion of the *pqsE* Ind elements into the pDM4 (Gm^R) resulted in the formation of pDM4::*pqsE* Ind*. The constructed suicide plasmid was then mobilized by conjugation into *P. aeruginosa* recipient strain using *E. coli* S17.1 λpir as a donor. Successful homologous recombination gave resistance to gentamicin and transconjugants were selected by using gentamicin 20 µg/mL in a LB plate. Following screening for the presence of pDM4::*pqsE* Ind*, sucrose counter selection was carried out using a method adapted from Westfall et al., (2004) using 12% w/v sucrose in LB media to obtain the required conditional mutation in which allelic exchange had occurred by recombination. The recombinants were identify using replica plating to select for the loss of gentamicin resistance, followed by PCR screening using primers p-Tac-SEQ-F and pqsE-SEQ-R **(Table 2.2)**. To induce *pqsE* expression, 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture unless otherwise stated.

Insertion of the mutation of *pqsR* in the PAO1-L genetic background was carried out using the already constructed suicide plasmid pEX18::*ΔpqsR* and applying sucrose counterselection as described above.

2.6.5 Site directed mutagenesis using PCR overlap extension.

Site directed mutagenesis of the *rhlR*-box centred at -311 bp upstream of the *pqsA* transcriptional initiation site (Xiao et al., 2006) was achieved by overlap extension PCR, a technique used for substitution, addition, and deletion of specific base sequences in DNA (Urban et al., 1997). In separate PCR reactions, the two fragments of the target sequence were amplified using primers pairs PpqsA-F/ PpqsA ΔrhlRbox-R and PpqsA ΔrhlRbox-F/ PpqsA-R **(Table 2.2)**, from which each reaction contains a primer with the desired mutation. The two intermediate products with terminal complementarity were combined in a ratio 1:1 and re-amplified using the primers pair PpqsA-F/R. The re-amplified sequence containing

the inserted mutation was digested with EcoRI and PstI, ligated into a previously EcoRI/ PstI cut pMiniCTX-*lux* (Gm^R), resulting in the creation of pMiniCTX::$P_{pqsA\ rhlR\ box}$-*lux* ($P_{pqsA\ rhlR\ box}$-*lux*). The removal of the entire *rhlR*-box was confirmed by DNA sequencing.

2.6.6 Gene complementation and cloning in pME6032

Chromosomal DNA fragments from *P. aeruginosa* PAO1-L were amplified by PCR and cloned into the shuttle expression vector pME6032 (Tet^R), which incorporates the *ptac* promoter. Using specific forward and reverse primers (**table 2.2**), the open reading frames (ORFs) of *PA2705, PA2704, nirQ, vfr* and *pqsR* were amplified by PCR and inserted between the EcoRI and SacI restriction sites of pME6032, and the resulting plasmids were termed pMENRC1, pMENRC2, pMENRC3, pMENRC4, and pMENRC7, respectively. Similarly, the ORF of *lasR and rhlR* were amplified by PCR and inserted between the EcoRI and KpnI restriction sites of pME6032, resulting in the formation of pMENRC5 and pMENRC6.

For the construction of the plasmids *pqsX-T1* and *pqsX-T2* chromosomal DNA fragments from *P. aeruginosa* PAO1-L were amplified using primers pairs PqsX-T1/T2 -F and PqsX-T1-R and PqsX-T1/T2 -F and PqsX-T2-R, respectively. The resulting fragments were cloned into a EcoRI-cut pME6032 (Tet^R) by *in vitro* homologous recombination using a Gibson assembly kit (New England BioLabs). Plasmids obtained were mobilized from *E. coli* S17-1 λpir.

2.7 Phenotypic assays

2.7.1 Alkyl quinolone (AQs) extractions

P. aeruginosa PAO1-L and respective mutants were grown in 5 mL cultures for overnight growth. These cultures were then used to inoculate fresh 10 mL subcultures to an OD_{600nm} of 0.01. After 7, 12 or 16 hours of growth, the cell-free supernatant was extracted three times with equal

volumes of acidified ethyl acetate (0.01% glacial acetic acid [v/v]). Samples were stored at -20°C until LC-MS analysis was performed.

2.7.2 Pyocyanin quantification

Pyocyanin quantification was carried out using sterile supernatants from 7, 12 and/or 16 hours flask overnight cultures. Pyocyanin was extracted from cell-free filtrate using chloroform according to Samanta Saha, 2008 with some modifications. Pyocyanin was measured using the absorbance of pyocyanin in the acidic form at 520 nm according to the following equation (Essar et al., 1990). To obtain quantitative data in µg/mL, the absorbance measurement was then multiplied by the extinction coefficient of the pigment, ε=17.072 m^2/mol

Concentration of pyocyanin (µg/mL) = OD_{520} x 17.072

2.8 Gene expression, bioluminescence and growth measurement

The single copy fusion of the different promoters previously bound to the *luxCDABE* genes were introduced into *P. aeruginosa* PAO1 strains by conjugation. Overnights cultures of the different *P. aeruginosa* conditions were washed and diluted in LB media to an OD_{600nm} ~0.01. Next, 200 µL of the diluted culture were grown in a 96 well black microplate flat clear bottom at 37°C for 16 hours with a 5 sec pre-read orbital shaking. Luminescence and turbidity were measured every 30 min in an automated luminometer-spectrometer (Infinite 200 PRO microplate reader, TECAN). Bioluminescence was normalized as a function of population density and the promoter activity per cell is given as relative light units divided by $OD_{600nm.}$ When needed, strains with inducible genes were grown with or without 1 mM IPTG unless otherwise stated.

2.9 Protein purification

2.9.1 Cloning, expression and solubility analysis

For protein expression of PA2705, NirQ and PA2707 carrying a his-tag at the N-terminus, full-length *PA2705*, *nirQ* and *PA2707* were PCR-amplified from PAO1-L genomic DNA using primers PA2705-F/R, NirQ-F/R and PA2707-F/R, respectively. Digestion of each fragment was performed with enzymes BamHI and HindIII and cloned into a pre-digested pCold1 expression vector, resulting in the formation of pNR1Cold1, pNR2Cold1 and pNR3Cold1 (**Table 2.1**). For protein expression of NirQ carrying a his-tag at the C-terminus, full-length *nirQ* was inserted in a pre-digested pet21a vector, using the same enzymes as above, resulting in pNR1pet. PA2705 and NirQ proteins were expressed in *E. coli* BL21 (DE3) and using Terrific Broth. NirQ (pNR1pet) was expressed as described in Hayashi & Igarashi, (2002) with some modifications. Briefly, cells were grown overnight at 30°C and 200 rpm. Reseeded cultures were grown until OD_{600nm} ~0,6, and expressed at 37°C for 3 hours with IPTG to a final concentration of 0.1 mM. For the expression of PA2705 (pNR1Cold1), NirQ(pNR2Cold1) and PA2707(pNR3Cold1*)*, cells were grown overnight at 30°C and 200 rpm. When the reseeded cultures reached an OD_{600nm} ~0,6 was reached, the cultures were immediately transferred to a water-ice bucket for 30 min and induced at 16°C for 16 hours with IPTG to a final concentration of 0.1 mM. Successful expression of the desired proteins was confirmed by SDS-PAGE. Samples were loaded at an adjusted OD_{600nm} 0.6. For protein solubilization, a range of mild solubilizing agents were incorporated in the insoluble fractions as described in by Gaberc-porekar et al., (2005). Briefly, Cells contained expressed protein were disrupted by sonication with a 15% amplitude pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in different solubilizing agents (**Table 2.3**) overnight at 4°C in a rocker platform. Soluble fractions were obtained from resulting supernatant after centrifugation at 12.000 rpm for 10 min.

Table 2.3. Treatments for protein solubilization

50mM Tris Urea 8M , 5% Glycerol pH=8
40mM Tris HCL 5% propanol, 2M Urea pH=
40mM Tris HCL 0,5% Triton X 100 pH=8
40mM Tris HCL 5% DMSO pH=8
40mM Tris HCL 2M Urea pH=8
40mM Tris HCL 0,2% n lauryl sarcosine
40mM Tris Urea 2M, pH=12

2.9.2 Purification of PA2705 and NirQ

PA2705 and NirQ were purified using ÄKTA pure protein purification equipment from GE Healthcare. Briefly, pellets from 500 mL cultures expressing the desired proteins were resuspended in 40 mL of Tris cL buffer pH8 (25 mM Tris HCl, 150 mM NaCl, 5% Glycerol and EDTA-free protease inhibitor cOmplete ™ cocktail tablet), disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in 40 mM Tris HCL 0,2% n lauryl sarcosine (or sarkosyl) overnight at 4°C in a rocker platform. The lysates were centrifuged at 15000 rcf for 15 min at 4°C and the cleared lysate containing soluble protein was loaded into a Nickel his trap column (HisTrap™ HP 5 mL purification column, GE healthcare). The steps for protein loading were as follow: a wash with ethanol 20% to eliminate bubbles from the pumping system at high flow rate of 4 mL/min; a second wash with 10 mL of dH_2O at a flow rate of 4 mL/min followed by a third wash with 25 mL and a flow rate of 2 mL/min. During this step, the nickel column was connected to the pumping system avoiding the formation of bubbles. Binding buffer A (25 mM Tris HCl, 150 mM NaCl, 5% Glycerol, imidazole 5 mM and EDTA-free protease inhibitor cOmplete ™ cocktail tablet with the addition of 0.2 % n-lauryl sarcosine was used to equilibrate the column at a flow rate of 2 mL/min. Next, the supernatant containing the soluble protein was loaded onto the equilibrated column with a 1

ml/min flow rate and the flow through was collected and kept on ice as it could carry the protein of interest if it failed to bind the Nickel resin. After the binding steps, 25 mL of buffer A was passed through at a flow rate of 1 mL/min to eliminate any non-specific binding. The Nickel column with loaded protein was then closed properly without formation of bubbles. Good care was taken to ensure the column matrix did not take any air during the whole process.

2.9.3 ÄKTA pure

The ÄKTA pure inlets A & B as well as the ÄKTA system were initially washed with 35 mL of milliQ water using a flow rate of 2.5 mL/min. Next, the inlets A and B were introduced into their respective buffers and equilibrated with 35 mL of buffer A (25 mM Tris HCl pH=8, 150 mM NaCl, 5% Glycerol, 5 mM Imidazole, 0.2 % n-lauryl sarcosine and EDTA-free protease inhibitor cOmplete™ cocktail tablet (Roche)) and buffer B (25 mM Tris HCl pH=8, 200 mM NaCl, 5% Glycerol, 500 mM Imidazole, 0.2 % n-lauryl sarcosine and EDTA-free protease inhibitor cOmplete™ cocktail tablet (Roche)). The ÄKTA system was then equilibrated with 35 mL of buffer A. Whilst buffer A was running, the Nickel column was carefully connected to the V9-C5 inlet. The fractionation step was performed at 100%B for 40 mL length with a pressure alarm of 0.5 mPa and a flow rate of 0.5 mL/min. Fractions of 2 mL were collected until 100% B was reached. After gradient completion, 25 µL of each collected sample was analysed in an SDS-PAGE gel. A final wash was performed to both inlets and the ÄKTA system with 35 mL of milliQ water followed by 20% ethanol. Inlets A and B were kept in ethanol 20%. Purified proteins where subjected to membrane dialysis ensuring the elimination of imidazole as well as reduction of salt content.

2.9.4 Size exclusion

The ÄKTA pure inlets A & B as well as the ÄKTA system were initially washed with 35 mL of milliQ water using a flow rate of 2.5 mL/min. Column Superdex 200 10/300 GL was connected to the V9-C5 system and washed with milliQ water for 35mL with an alarm pressure of 1.5 mPa. Inlet A was placed in size exclusion buffer (25 mM Tris-HCL pH=8, 150 mM NaCL, 0.2 % n-lauryl sarcosine and EDTA-free protease inhibitor cOmplete ™ cocktail tablet (Roche)) and inlet B remained in water. The column was equilibrated with 36 mL of size exclusion buffer with a system flow of 0.7 mL/min. The sample injection valve V9-Inj was washed twice with milliQ water and buffer prior injection of the ÄKTA sample containing purified protein. Fractions of 2 mL were collected and run in an SDS-PAGE for analysis. Protein dialysis was performed at 4^{o}C on a plate platform against size exclusion buffer containing 25 mM Tris-HCL pH=8, 150 mM NaCL and EDTA-free protease inhibitor cOmplete ™ cocktail tablet (Roche), followed by spin concentration and storage at -80^{o}C.

2.9.5 Western blotting for the presence of PA2705 and NirQ

Western blot analysis was carried out using the Penta·His HRP Conjugate Kit from Qiagen, which consists of the QIAexpress mouse IgG1 Anti·His Antibodies chemically coupled to the reporter enzyme horseradish peroxidase (HRP). HRP catalyses the oxidation of the substrate luminol peroxide producing chemiluminescence, hence allowing the sensitive detection of recombinant proteins carrying His tags, without the need for secondary antibodies. Pure PA2705 and NirQ as well as PA2707 from cell extracts were run in an electrophoretic SDS-PAGE gel and transferred to a nitrocellulose membrane at 4°C for 1.5 hours at 150 volts. The membrane was incubated in Ponceau S staining solution for 2 min, doubled washed for 10 min each time with 1X TBS buffer and incubated in blocking buffer for 1 hour at room temperature. After blocking procedures, the membrane was washed twice for 10 min in 1X TBST

followed by one wash with 1X TBS and incubated overnight with gentle agitation at 4°C in the presence of the primary antibody solution. Subsequently, the membrane was washed twice for 10 min each time in 1X TBST buffer at room temperature, followed by one was in 1X TBS. For the immunodetection of the desired proteins anti-His HRP Conjugates were used for chemiluminescence detection. High sensitivity visualization of the PA2705, NirQ and PA2707 western blots were achieved by using the Amersham™ ECL Select™ reagents from GE Healthcareand, using X-ray film.

2.9.6 BCA assay

Protein quantification was carried out using Pierce™ BCA Protein Assay Kit (Thermo scientific). Albumin (BSA) standards were diluted using the same diluent as the samples and according to protocol for microplate procedures. Working reagent (WR) was added to 25 µL of triplicate samples in a ratio 1:8, mixed by shaking and incubated at 37 °C for 30 min. The absorbance at 562 nm was measured using an Infinite 200 PRO microplate reader (TECAN). The average 562 nm absorbance measurement of the Blank standard replicates was subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates. Protein concentration (µg/ml) of each unknown sample was determined using the BSA standard curve.

2.9.7 Electrophoretic mobility shift assay (EMSA)

Protein-DNA interaction were tested using the Electrophoretic Mobility-Shift Assay (EMSA) Kit, with SYBR™ Green & SYPRO™ Ruby EMSA stains (Invitrogen). The 502bp upstream region of the *pqsA* gene was PCR amplified, gel-purified and quantified. A total of 500fmol of the DNA promoter was added per reaction. Increasing protein concentration of 65, 195, 390, 780 and 1040 ng were added to the binding reaction buffer for 40 min. Native PAGE gel at 7.5% and 6% were equilibrated with glycerol

and 6X EMSA loading dye solution for 40 min prior loading the samples. Samples were run at 4°C for 3-4 hours and 70 volts. Gels were then incubated in 1X SYBR® Green EMSA gel stain for 20 min protected from light, washed twice for 10 sec in 50 mL of dH_2O, visualized in a Bio-Rad Universal Hood II Gel Documentation System. For protein visualization, the gels were then incubated overnight in SYPRO® Ruby EMSA protein gel stain with TCA protected from light. The resulting gels were washed in 150 mL of dH_2O, distained in a 10% methanol and 7% acetic acid solution for 60 min, and visualized in a Bio-Rad Universal Hood II Gel Documentation System.

2.10 RNA extraction and expression profiling experiments

RNA from *P. aeruginosa* wild type and the *nirQ* isogenic mutant grown under anaerobic conditions was extracted at an OD_{600} = 0.6 (mid-exponential phase). Cells were immediately treated with RNAprotect® Bacteria Reagent (Qiagen) and pelleted down at 5000 rpm for 5 min. The resulting pellets were stored at -80°C until needed. Total RNA extraction was performed in a MSC class II cabinet and using the RNeasy Midi Kit (Qiagen) as per the manufacturer instructions. Briefly, 100 µL of TE buffer containing lysozyme and Proteinase K was added to the pelleted cultures and mixed by pipetting, followed by 10 sec of vortexing and incubation at room temperature for 10 min with agitation. Samples were lysed with 350 µL of buffer RLT, centrifuged for 2 min at maximum speed and 250 µL of ethanol 100% was added to the sample supernatants. 700 µl of lysate, were transferred to a RNeasy Mini spin column placed in a 2 mL collection tube and centrifuged for 15 sec at ≥8000 x rcf. Samples were treated with DNase digestion using the RNase-Free DNase Set (Qiagen). Next, 500 µl of Buffer RPE were added to the RNeasy Mini spin column and centrifuged once for 15 sec and 2 min at ≥8000 x rcf. RNA was eluted with 30 µl of RNase-free water.

2.10.1 Purity and Integrity of RNA:

Purity of RNA was validated by the ratio of the readings at 260 nm and 280 nm. Pure RNA had an A260/A280 ratio of 1.9–2.1. The integrity of RNA was validated using gel electrophoresis. Intact total RNA was run in denaturing agarose gel at 60 volts for 50 min at 4^{o}C in TBE buffer. Water for buffer solutions was treated with DEPC 0.1% overnight at 37^{o}C and autoclaved prior use. Electrophoresis components were washed with SDS 0.5% and pure ethanol prior electrophoresis.

2.10.2 Reverse transcription

The cDNA from purified RNA was obtained using the Quantitec Reverse transcription kit (Qiagen). Genomic DNA was first removed by mixing 1 µg of RNA sample with 2 µL of gDNA wipe out buffer. The reaction was incubated at 42^{o}C for 2 min and placed immediately on ice. For retro-transcription procedures, the samples were incubated with Quantiscript Reverse Transcriptase enzyme, RT buffer and RT primer at 42^{o}C for 30 min, followed by 3 min at 95^{o}C to inactivate Quantiscript Reverse Transcriptase. Samples were used directly for Real time PCR procedures.

2.10.3 Real time PCR

Real time PCR was performed using the QuantiTect SYBR Green PCR Kit. Master mix reactions were prepared for every endogenous gene with 1x PCR Master Mix Master, and appropriate concentration of each primer (**Table 2.2**), 0.5 units of UNG and 100-0.001 ng of cDNA. The reaction was made up to a final volume of 25 µl with water. A negative amplification control was used for each primer set. A standard curve with 10-fold dilution of cDNA allowed the selection of appropriate endogenous genes. Cycling was performed on a 7500 Real Time PCR System (Applied Biosystem), with an initial hold at 50°C for 2 min and 95°C for 15 min,

followed by 40 cycles at 95°C for 15 sec, 57°C for 30 sec and 72°C for 30 sec.

2.11 Statistical Tests

All statistical analyses were performed with the use of GraphPad Prism version 9.1.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Standard deviation of the mean of the three biological replicates is reported. Paired t-test was used to compare the averages/means and standard deviations of two related groups. Unpaired t-test was used to compare the averages/means and standard deviations of two independent groups. A P-value of ≤0.05 was considered significant. For qRT-PCR analysis, the relative expression was calculated using the comparative cycle threshold method ΔΔCT. PCR efficiency was calculated in based on the slope of each standard curve.

3 Chapter Three: PA2705 as a potential candidate to mediate the action of PqsE during early growth

3.1. Introduction

To date, PqsE has gained major attention not only due to its biosynthetic role in the formation of PQS but, most importantly, the roles it plays in the regulation of virulence in *P. aeruginosa*. Among its regulatory roles, PqsE has been shown to modulate the production of the QS signal PQS by downregulating the expression of *pqsA* (Rampioni et al., 2010). Nevertheless, since PqsE does not interact with DNA (Yu et al., 2009), and due to the high level of interconnected regulatory pathways impacting on the activity of the *pqsA* promoter (P_{pqsA}), the effect of PqsE towards *pqsA* expression is likely to be indirect and as the result of a chain of events possibly involving additional mediators.

3.2. PA2705 as a main candidate to mediate the action of PqsE

Promoter pull down experiments were previously carried out in this laboratory using the *P. aeruginosa* PAO1-N *pqsE* Ind conditional mutant under non-inducible (*pqsE* - IPTG) and inducible (*pqsE* + IPTG) conditions. Cytoplasmic cell extracts harvested from exponential cultures of this strain at OD_{600nm} 0.5 were incubated with the P_{pqsA} region illustrated in **Figure 3.1** for the pull down experiments. This OD was selected by the author (Robyn Bates) as it corresponded to the peak in the transcriptional activity of the P_{pqsA} used under the conditions tested (**Figure 3.2**). This region included the first and second TSS at -71 bp and -339 relative to the *pqsA* translational start codon, which has been shown to be under the positive control of PqsR (McGrath et al., 2004) and negative control of RhlR, respectively (Xiao et al., 2006). The two *lux* box-like sequences were also included. Xiao et al., (2006) had demonstrated that the distal *lux*-box placed at -311 bp from the *pqsA* transcription initiation site is the binding site for RhlR and causes the final repression of this promoter, whereas the second *lux* box-like is not required for the *pqsA* regulation (**Figure 3.1**).

Figure 3.1. The P_{pqsA}.

The region directly upstream of the *pqsA* open reading frame (ORF) contains several regulatory elements. Two transcriptional start sites (highlighted in light blue) have been identified at -71 and -339 positions relative to the translational start site (underlined) of *pqsA*. An LTTR-specific motif (yellow) is present 121 bp upstream of the *pqsA* start codon, allowing PqsR interaction with the P_{pqsA}. Two lux boxes have also been identified, (orange) although only the distal *lux box* 1 is believed to be involved in the regulation of *pqsA* by RhlR. Modified from Bates , PhD Thesis , (2013).

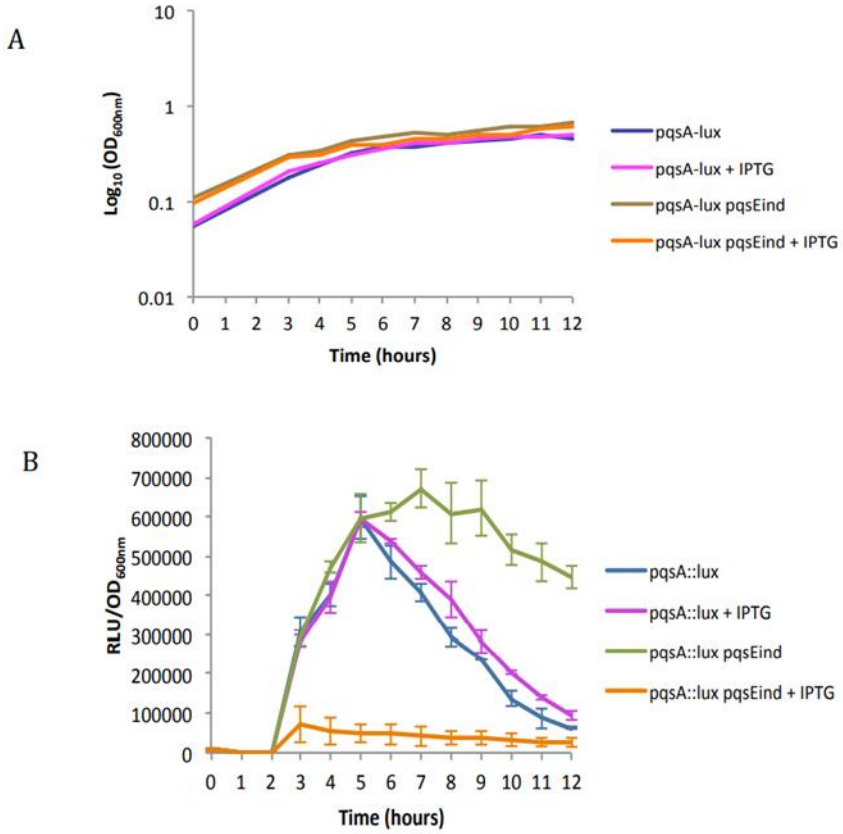


Figure 3.2. The P_{pqsA} activity.

P_{pqsA} activity in PAO1-N and PAO1-N *pqsE* Ind grown in culture flasks at 37 °C, in the presence (+IPTG) or absence (-IPTG) of 1 mM IPTG. Both OD_{600nm} and the relative light units (RLU) were measured. A) Log10 growth curves showed that all strains grew similarly. B) The P_{pqsA} activity curves indicated that the peak occurred after ~5 hours of growth for the PAO1-N cultures and approximately at 7.5 hours for PAO1-N *pqsE* Ind. Error bars show 2x standard error calculated across three experimental replicates. Bates , PhD Thesis,(2013).

The promoter pull-down is illustrated in **Figure 3.3.** From the large amount of protein bands observed, only the numbered bands were analysed by LCMS-MS. This selection was based on the difference in band intensity when *pqsE* was not expressed (*pqsE* Ind - IPTG) compared to when it was overexpressed (*pqsE* Ind + IPTG) (Bates , PhD Thesis, 2013). The full list of proteins found binding the P_{pqsA} were adapted from the original work and are listed in **Table 3.1**.

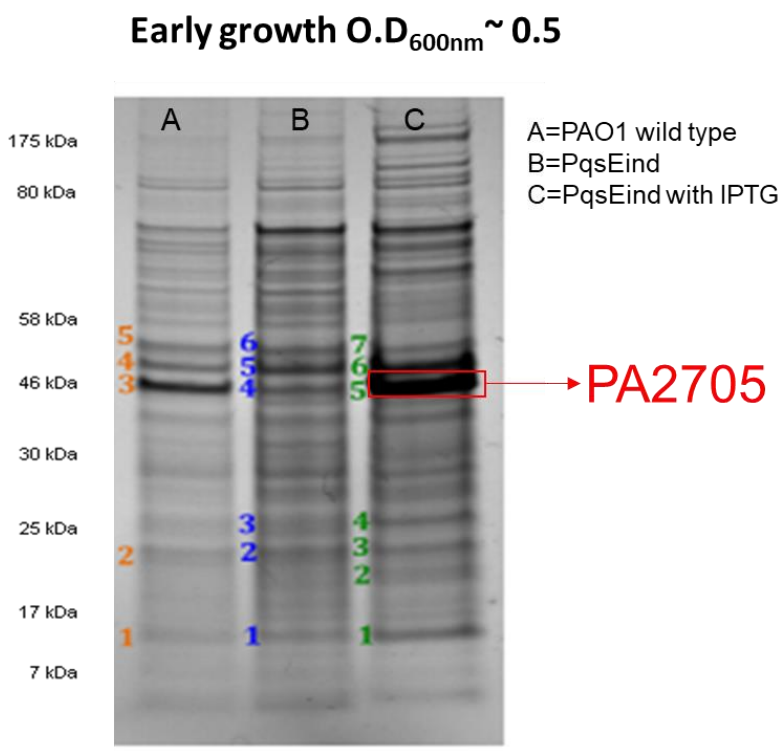


Figure 3.3. The effect of PqsE on the protein profile at the P_{pqsA}.

Promoter pulldowns were performed using the P_{pqsA} region in combination with cell extracts harvested from PAO1-N wildtype, PAO1-N *pqsE* Ind with an unexpressed (-IPTG) or expressed *pqsE* (+IPTG). Cell extracts were harvested at OD_{600nm} 0.5. Control experiments included the P_{pqsA} or cell extracts alone. Protein bands analysed by LCMS-MS are colour enumerated. Orange: PAO1-N bands; Blue: PAO1-N *pqsE* Ind – IPTG; Green: PAO1-N *pqsE* Ind + IPTG. The colour coded numbers also reflect the LCMS-MS protein profile shown in **table 3.1**. Bates , PhD Thesis, (2013).

Table 3.1. LCMS/MS Identification of the proteins bound to the P_{pqsA} with different levels of pqsE.

Proteins are classified in three colours as in **Figure 3.3**. Orange: PAO1-N wild type; Blue: PAO1-N *pqsE* Ind -IPTG; Green: PAO1-N *pqsE* Ind + IPTG. Strike line indicates proteins that are no longer present in a specific condition. Proteins included in this section correspond only to those with confirmed regulatory functions or uncharacterized proteins. Function description is according to PseudoCap function. Individual proteins have assigned a relative abundance according to their unweighted LCMS/MS spectrum counts. Values in brackets represent highest protein abundance value within the band. The LCMS/MS tables are continued on the subsequent pages.

PAO1-N					
Band number	**Band size (kDa)**	**Protein**	**Relative abundance**		**Function**
1	15	No protein of interest			
2	23	Vfr	10	(11)	Transcriptional regulator
		AguR	9		Transcriptional regulator
		RoxR	8		Transcriptional regulator
		PA3801	6		Uncharacterized protein
3	46	PA2705	7	(21)	Uncharacterized protein
4	50	ClpX	19	(33)	ATP-dependent protease subunit
		AlgP	15		Transcriptional regulator
		AlgB	6		Transcriptional regulator
		PilR	6		Transcriptional regulator
		SadB	5		Transcriptional regulator
5	57	FleQ	33	(28)	Transcriptional regulator
		PA3455	11		Predicted polyphosphate kinase

PAO1-N - IPTG

Band number	Band size (kDa)	Protein	Relative abundance		Function
1	15	No protein of interest			
2	23	**Vfr**	9	(11)	Transcriptional regulator
		RoxR	8		Transcriptional regulator
		ErdR	7		Transcriptional regulator
		PA1894	7		Uncharacterized protein
		Dnr	5		Transcriptional regulator
3	27	**RhlR**	21	(21)	Transcriptional regulator
		BfmR	6		Transcriptional regulator
		PA5208	6		Predicted Transcriptional regulator
		ParR	6		Transcriptional regulator
		AmgR	6		Transcriptional regulator
		PhoB	6		Transcriptional regulator
		Anr	5		Transcriptional regulator

5	50	**ClpX**	23	(33)	ATP-dependent protease subunit
		AlgB	18		Transcriptional regulator
		AlgP	17		Transcriptional regulator
		DctD	13		Transcriptional regulator
		PilR	8		Transcriptional regulator
		SadB	6		Transcriptional regulator
6	57	**FleQ**	15	(28)	Transcriptional regulator
		PA3455	13		Predicted polyphosphate kinase
		FleR	9		Transcriptional regulator
		CbrB	6		Transcriptional regulator

PAO1-N + IPTG

Band number	Band size (kDa)	Protein	Relative abundance		Function
1	15	No protein of interest			
2	20	**PA0171**	9	(16)	Uncharacterized protein
		PA4352	8		Predicted stress protein
		DadR	6		Transcriptional regulator
3	23	**ClpP2**	14	(14)	ATP-dependent protease subunit
		ErdR	8		Transcriptional regulator
		PA2731	7		Uncharacterized protein
		Vfr	7		Transcriptional regulator
		PA2066	6		Uncharacterized protein
		RoxR	6		Uncharacterized protein
4	27	**RhlR**	21	(21)	Transcriptional regulator
		Anr	5		Transcriptional regulator
		BfmR	5		Transcriptional regulator
5	46	**PA2705**	5	(55)	Uncharacterized protein

6	50	**ClpX**	33	(33)	ATP-dependent protease subunit
		AlgB	15		Transcriptional regulator
		AlgP	8		Transcriptional regulator
		DctD	8		Transcriptional regulator
		PA5209	7		Uncharacterized protein
		PilR	6		Transcriptional regulator
		SadB	5		Transcriptional regulator
7	57	**FleQ**	20	(29)	Transcriptional regulator
		CbrB	10		Transcriptional regulator
		PA3455	6		Predicted polyphosphate kinase
		PA4132	6		Predicted Transcriptional regulator

A stringy complex protein profile at the early stage of growth in both the presence and absence of PqsE was obtained. Within 18 bands, a total of 169 proteins were identified. Among them, Vfr, RoxR, ClpX, SadB, AlgB, AlgP, PilR and FleQ were found bound to the P_{pqsA} in all backgrounds tested, along with the uncharacterised protein PA3455, suggesting that any potential effect upon the expression of *pqsA* is not affected by PqsE. On the other hand, It was interesting to observe that in the PqsE negative background, the vast majority of the proteins binding *pqsA*, which included ErdR, BfmR, ParR, AmgR, PhoB, DctD, FleR and CbrB, were response regulators (RRs) from two-component systems (TCSs) (**Table 3.1**) (Francis et al., 2017). This observation suggests that PqsE modulates the expression of several transcriptional regulators that in its absence may cause their derepression and possibly the subsequent induction of the activity of P_{pqsA} (**Figure 3.2**), hence, propounding that PqsE has a pivotal role in orchestrating the sensing of its environment to decide upon an appropriate response and modify its behavior accordingly to better suited prevailing conditions. Moreover, it is plausible that PqsE may have different binding partners placing PqsE not as a final but an intermediate effector in the middle of independent branches of regulatory cascades that impact on the regulation of *pqsA*. RhlR was also binding the P_{pqsA} when *pqsE* was absent, this was interesting as this regulator was not present in the wild type condition and it is known to be a repressor of *pqsA* (Xiao et al., 2006; Brouwer et al., 2014). Anr and Dnr were also binding the P_{pqsA} in the absence of *pqsE*, which further support the connection between the oxygen environment, denitrification regulations and the *pqs* system (Toyofuku et al., 2008, 2012). The overexpression of *pqsE* facilitated the binding of several additional proteins to the promoter. These were largely uncharacterized proteins PA0171, PA4352, PA2066, PA2705, PA5209, PA4132 and DadR, a transcriptional regulator of the AsnC/Lrp family that regulates the expression of *dadAX* for amino acid catabolism (Boulette et al., 2009; He et al., 2011) and the biofilm promoter homologue ClpP2(Mawla et al., 2021). Major attention caused the hypothetical protein PA2705 (**Table 3.1**), as it was the only one binding the P_{pqsA} in the wild type

condition with an intact *pqsE,* as well as only when *pqsE* was overexpressed. This suggested that PA2705 is dependent on the presence of PqsE and hence, it could become an attractive target as a potential mediator of the action of this effector.

3.3. PA2705 background

The Pseudomonas Genome database http://www. pseudomonas.com (Winsor et al., 2016), placed PA2705 as a hypothetical protein of 45.6 kDa encoded by a 1182 bp open reading frame. It is predicted to be localized in the cytoplasm and in its structure contains a von Willebrand factor type A (vWFA) domain CoxE-like.

The vWFA domains are commonly found in eukaryotic signalling proteins (Hohenester & Engel, 2002; Whittaker & Hynes, 2002). Phylogenetic distribution of eukaryotic signal domains evidenced that the vWFA domain family was present in genomes from each of the three divisions of cellular life: Eukaryotic, Prokaryotic and Archea and that vWFA domain was already present in the last common ancestor (Ponting et al., 1999). The majority of prokaryotic vWFA domains show conservation of aspartic acid and serine residues known from structures of eukaryotic vWFA domains to interact with divalent cations, which correspond to a non-contiguous sequence motif called metal ion-dependent adhesion site (MIDAS). The functions of the majority of prokaryotic vWFA homologues remain unknown, however, it is suggested that the vWFA domain participates in protein binding as well as other diverse functions, as their architectures and cellular localisations are widespread within the cell (Neuwald et al., 1999; Pelzmann et al., 2009; Raynaud et al., 2021).

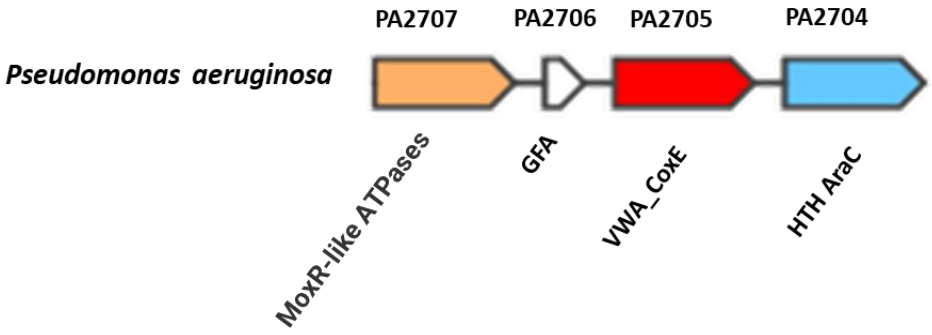


Figure 3.4. Illustration of the *PA2705* gene neighbourhood in *P. aeruginosa*.

The *PA2705-PA2707* operon is composed of three hypothetical proteins. PA2705 and PA2707 have predicted cytoplasmic localisation, whereas PA2706 is unknown. PA2705 belongs to the Von Willebran Factor CoxE like (vWFA CoxE) family domain. PA2706 represents a Glutathione-dependent Formaldehyde Activating enzyme (GFA) whereas PA2707 belongs to the AAA+ superfamily of ATPases. PA2704 is a probable AraC transcriptional regulator with DNA binding HTH domain and it is found immediately after the *PA2705-PA2707* operon.

In *P. aeruginosa*, PA2705 is part of a three genes operon (**Figure 3.4**), including PA2706 and PA2707, both hypothetical proteins of the Mss4-like superfamily and Ploop ATPases, respectively. This operon is followed by PA2704, a probable AraC transcription regulator, which by close vicinity it may be related to the regulation of this operon (Junier & Rivoire, 2016). NCBI Diamond blastx showed a total of 552 orthologues of PA2705 within the *Pseudomonas* group. Noticeably, the presence of PA2705 together with PA2706 and PA2707 was conserved among the *P. aeruginosa* strains, as well as the AraC transcriptional regulator PA2704, nevertheless, in other *Pseudomonas* orthologues (i.e., *syringae, putida, fluorescencens*), whilst PA2705 was found next to PA2707, the other two ORFs were missing suggesting that PA2706 and PA2704 are strain specific, or that were acquired later during the evolution of *Pseudomonas* or were product of ancestral rearrangements. This is supported by the fact that a group of vWFA in eukaryotes and prokaryotes are commonly related to ATPases associated with various cellular activities (AAA). Two proteins in the human genome contain a combination of AAA and vWFA domains (Reviewed in Whittaker & Hynes (2002). In prokaryotes, PA2707 belongs to one of the seven subfamilies of the MoxR AAA+ ATPases, that also includes the MoxR

Proper (MRP), TM0930, RavA, CGN (CbbQ/GvpN/NorQ), APE2220 and YehL subfamilies. AAA modules contain a variety of conserved sequence motifs that are responsible for ATP sensing and hydrolysis. Walker A and B, Sensor I and II correspond to the major motifs among these proteins (Neuwald et al., 1999) and they usually function as oligomers forming hexamer rings (Hanson & Whiteheart, 2005). In a work performed by Snider & Houry, 2006, it was found that only the subfamilies corresponding to MRP, GCN and PA2707 were present in the *Pseudomonas* genomes. PA2707 was found in *P. aeruginosa* PAO1, *P. fluorescence* PfO-1 and *P. putida* KT2440. Besides Proteobacteria, the PA2707 subfamily was also found in other phyla such as Acinetobacteria, Chlorobi, Cyanobacteria and Spirochaetes, whereas no member were detected in Archeas (Snider & Houry, 2006). In the same work, they showed that 70% of the PA2707 gene homologues were near genes encoding vWFA proteins, with 60% of them found adjacent to PA2707. Moreover, no PA2707 was found in proximity to more than a single vWF encoding gene. Except for 3 members, all the vWFA were identified as CoxE type and more specifically COG3552 CoxE vWFA (NCBI Clusters of Orthologous Groups), which are like the vWFA members of the APE2220 subfamily of ATPases. Members of this COG are of unknown function, but their genes are often found as part of a carbon monoxide dehydrogenase (Cox) gene cluster. However, in contrast to these members, the vWFA CoxE type related to PA2707 did not suggest to be part of the *cox* gene clusters (Pelzmann et al., 2009), albeit 23% of them were close to genes encoding a cytochrome *c*.

The Pseudomonas Genome database http://www. pseudomonas.com (Winsor et al., 2016), annotates PA2706 as a hypothetical protein with potential carbon sulfurylase activity. It presents 3 orthologues in *P. aeruginosa* PAO1 that are also found in strain PA14. All of them encode for hypothetical proteins belonging to the Mss4-like superfamily and more specifically to the Glutathione-dependent formaldehyde-activating enzyme family (GFA). GFA from *Paracaocuss denitrificans* has been purified and shown to participate in the degradation of formaldehyde and glutathione to produce S-hydroxymethylglutathione, hence contributing to the degradation of

formaldehyde, known to be highly toxic due to nonspecific reactivity with proteins and nucleic acid (Goenrich et al., 2002).

Some of the PA2706 orthologues in *P. aeruginosa* are predicted enzymes with ribulose-phosphate 3-epimerase (Rpe) activity. Rpe is an enzyme of the non-oxidative branch of the pentose phosphate, it binds ferrous iron for its activity and its substitution for manganese prevents it from inactivation caused by H_2O_2 (Sobota & Imlay, 2011). No evidence suggests an interaction between GFA and vWFA. The protein-protein interaction (PPI) STRING database (https://string-db.org) predicted with a high level of confidence interaction score (>0.7) PA2707 as the only interacting partner, which may suggest that participates together with AAA ATPases.

3.4. *PA2704/05/06/07* promoter analysis

The promoter analysis of the PA2705/06/07 operon and PA2704 gene was performed, using Prodoric database (http://prodoric.tu-bs.de/) and *P. aeruginosa* PAO1 as reference strain, in order to gain a better understanding of their regulation and how they may integrate with the QS regulatory network. Binding sites for the AlgU, NarL, LasR and RhlR transcriptional regulators were found in the promoter region of the PA2705-PA2707 operon.

AlgU is an extracytoplasmic sigma factor responsible for the production of mucoidy. *P. aeruginosa.* AlgU regulates the transcription of the operon responsible of alginate biosynthesis (Potvin et al., 2008) and was also found to bind the *pqsA* and *lecA* promoters (Bates, PhD Thesis*., 2*013)

NarL is a key component in the anaerobic growth of *P. aeruginosa*. It is part of the nitrate-responding two-component regulatory system NarX-NarL. Interestingly, this system regulates NirQ, a putative ATP-binding protein from the MoxR ATPase family (Schreiber et al., 2007), which suggests that that the ATPase PA2707 could also be regulated by anaerobic regulators.

LasR and RhlR are key transcriptional regulators in QS system. Both regulatory proteins regulate the production of many virulence factors (**See Chapter 1**). Indeed, RhlR and PqsE have been shown to act in a concerted fashion (Farrow et al., 2008; Mukherjee et al., 2017, 2018; Groleau & Pereira., 2020; Taylor et

al., 2021) and it is possible that via these interactions, PA2705 may also be interconnected with the QS system in *P. aeruginosa*

3.5. Aims of the chapter

As seen in previous data, the overexpression of *pqsE* led to several proteins bind the P_{pqsA}. In high relative abundance, the hypothetical protein PA2705 was found posing the hypothesis that this protein may be a mediator of the action of PqsE. In this chapter, the interlinks between PqsE, PA2705 and the P_{pqsA} are studied and hence the main aims are to:

- Elucidate if PA2705 is essential for the PqsE-mediated repression towards *pqsA* by:
  - Establishing whether *pqsA* is regulated by PA2705.
  - Determining whether PqsE regulates PA2705.
  - Validating whether PA2705 binds the P_{pqsA}.

Since little is known about PA2705, the study of its gene neighborhood may contribute towards the understanding of the relationship between PA2705 and the regulation of *pqsA*. Hence in this chapter we study whether the predicted transcriptional regulator PA2704 controls the expression of PA2705.

3.6. Results

For the present work, the PAO1-L subline from Lausanne (D. Haas's collection) has been selected as this strain appears to be the closest to the original isolate from Bruce Holloway's laboratory 60 years ago (Holloway, 1955), which has since been distributed and replicated many times in different laboratories around the world. The work in this chapter is all based on PAO1-L unless otherwise stated.

3.6.1 Validating the regulation of PA2705 towards P_{pqsA} and its regulatory link with PqsE.

As previously observed, PA2705 was shown to bind to the P_{pqsA} in pull down experiments when PqsE was overexpressed, propounding that PA2705 may be a mediator in the action of PqsE. To validate this hypothesis, however, it was necessary to dissect this regulation in more detail and analyze in first place whether PA2705 had an impact on the activity of $P_{pqsA.}$

3.6.1.1 PA2705 modulates the expression of *pqsA*.

Whether PA2705 is a mediator of the action of PqsE, its mutation was expected to alter the expression levels of *pqsA*. To validate this, in-frame mutagenesis of the *PA2705* was carried out by deleting the central region of this gene as described in **Chapter 2, sections 2.6.2 and 2.6.3**, and verified as shown in **Supplementary data, Figure S7.1**. Moreover, since little is known about this candidate gene, a similar approach was undertaken for *PA2704*, encoding the predicted transcriptional regulator.

For the analysis of the gene expression activity in these mutants, the *lux*-based bioluminescence pMiniCTX-*lux* (Gm^R) reporters were used. For a first screening, the chromosomal-based transcriptional reporter *P. aeruginosa* PAO1-L pMiniCTX::P_{pqsA}-*lux* available from the laboratory collection was used. This reporter holds 309 bp upstream from the transcriptional initiation site of *pqsA* (**Figure 3.5**), hence, lacking 115 bp of the P_{pqsA} included in the promoter pull-down analysis (**Figure 3.1**). This reporter was named as $P_{pqsA\text{-}short}$-*lux*. For a

more accurate analysis of the PqsE regulation towards P_{pqsA}, a new P_{pqsA}-*lux* was created in parallel as described in **Chapter 2, section 2.6.1** and validated as shown in **Supplementary data, Figure S7.4**. The new P_{pqsA}-*lux* included the same P_{pqsA} region employed in the promoter pull-down analysis (**Figure 3.1**), including 424 bp upstream from transcriptional initiation site of *pqsA* (**Figure 3.5**) and it was named $P_{pqsA\ long}$-*lux*.

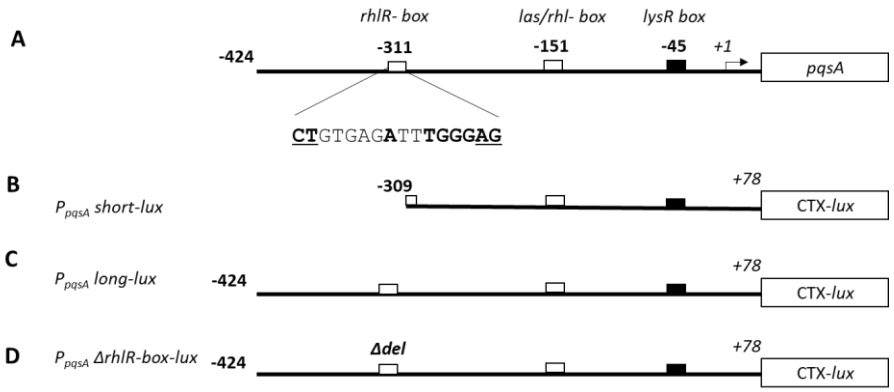


Figure 3.5. Schematic representation of the P_{pqsA} in different pMiniCTX-*lux* reporters.

A) Localization in the *pqsA* intergenic region of the putative *las/rhl* (-311), (-151) and *lysR* boxes (-45), and the putative *rhl/las* box sequence. The -311 *las/rhl box* is under the negative regulation of RhlR (Xiao et al., 2006), hence named *rhlR-box.* The *rhl*-responsive motif is shown in bold (M. Schuster et al., 2004). The highly conserved consensus sequence in CT-[N]12-AG is shown in bold and underlined. The *pqsA* transcriptional (+1) start occurs 71 bp upstream of the translational start codon (McGrath et al., 2004). B) Representation of the P_{pqsA} region included in the $P_{pqsA\text{-}short}$ -*lux* reporter, included 309 bp upstream of the *pqsA* transcriptional start C) Representation of the P_{pqsA} region included in the $P_{pqsA\text{-}long}$ -*lux,* included 424 bp upstream of the *pqsA* transcriptional start D) The entire -311 *rhlR-box* was deleted *(Δdel)* from $P_{pqsA\text{-}long}$ -*lux,* to create the $P_{pqsA\ \Delta rhlR\ box}$-*lux.*

Next, $P_{pqsA\text{-}short}$-*lux* and $P_{pqsA\ long}$-*lux* were inserted into the chromosome of the *PA2705* and *PA2704* deletion mutants by conjugation as well as in the PAO1-L wild-type and the transcriptional activity of P_{pqsA} monitored over time.

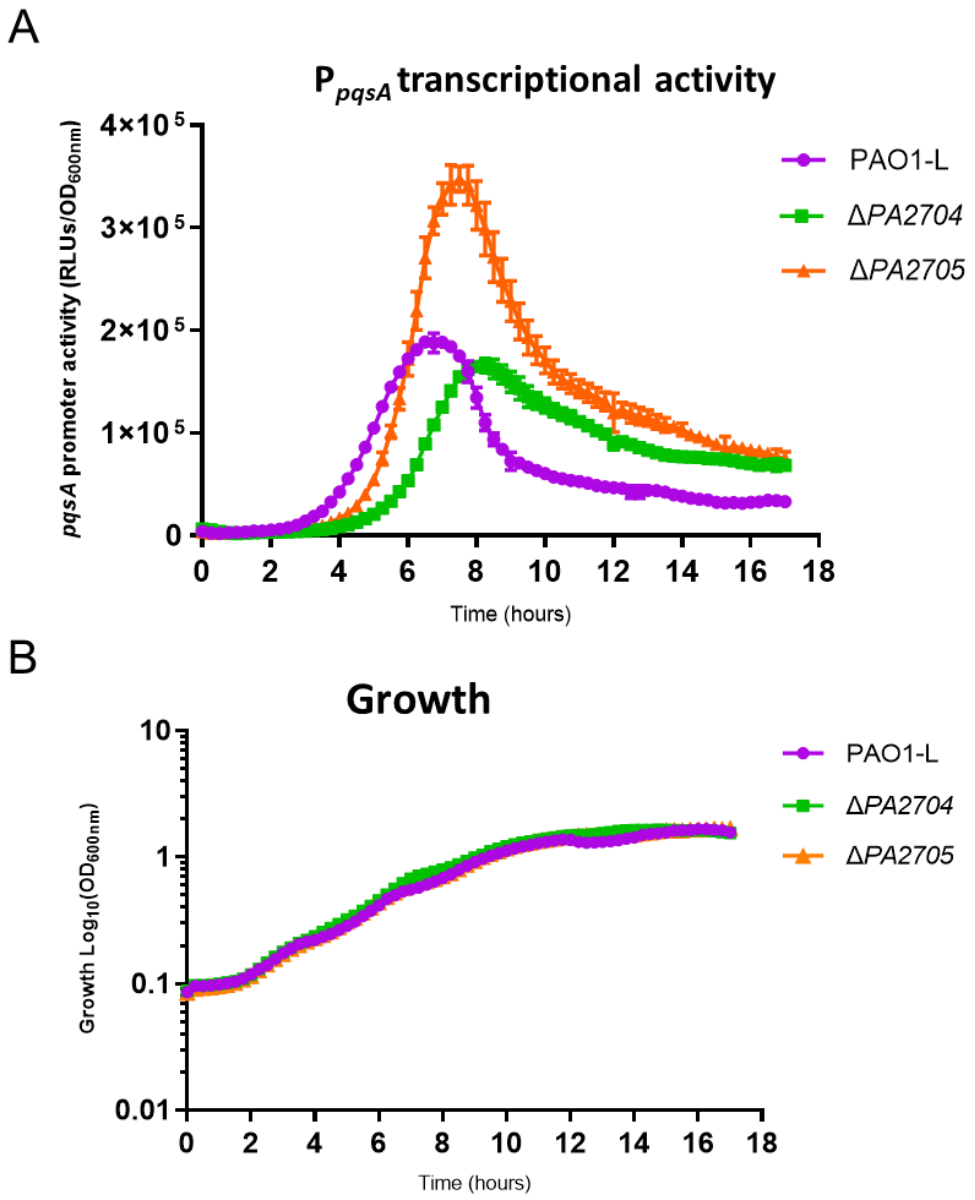


Figure 3.6. The transcriptional activity of P_{pqsA} in *P. aeruginosa PA2704* and *PA2705* mutants using a laboratory collection $P_{pqsA\ short}$-*lux*.

A) Normalised bioluminescence production for PAO1-L, Δ*PA2704* and Δ*PA2705* carrying the $P_{pqsA\ short}$-*lux* (Gm^R) in LB broth. Both OD_{600nm} and the relative light units (RLUs) were measured. Strains were grown at 37°C for 18 hours. B) The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars show 2x standard error calculated across three biological replicates.

Analysis using the $P_{pqsA\ short}$-*lux* showed that, when compared to the wild type condition, the transcriptional activity of P_{pqsA} increased in the absence of *PA2705* (**Figure 3.6**), indicative of a regulatory link where PA2705 has a negative

impact on this promoter. Interestingly, the mutation of *PA2704* also altered the P_{pqsA} activity, causing a delay on its expression that peaked 2 hours later than the wild type, followed by higher levels of activity of P_{pqsA} that remained during the growth of *P. aeruginosa*. Curiously, the mutation of *PA2704* reached similar levels to that observed in the absence of *PA2705*. Although this was only observed at the end of the stationary phase, a probable regulatory link between the two cannot be discarded.

Interestingly, analysis using the $P_{pqsA\ long}$-*lux* (**Figure 3.7**), revealed that when compared to the wild-type condition, the mutation of *PA2705* resulted in a lower transcriptional activity of P_{pqsA}, which started early in the growth of *P. aeruginosa* and continued until the end of stationary phase, hence, further supporting a regulatory link between PA2705 and the P_{pqsA} but, in contrast to the short reporter, as a positive modulator. Interestingly, during late stationary phase, the activity of P_{pqsA} reached temporary wild type levels, suggesting the possibility that this mutation could be compensated later in growth probable via another regulator.

On the other hand, mutation of *PA2704* also altered the transcriptional activity of P_{pqsA} in the long construct which, interestingly, followed the same pattern as the *PA2705* mutant (**Figure 3.7**) Intriguingly, no wild-type levels of activity of P_{pqsA} were reached in the absence of *PA2704* at any time during the growth of *P. aeruginosa*, suggesting that this downregulation may not be compensated via another regulator.

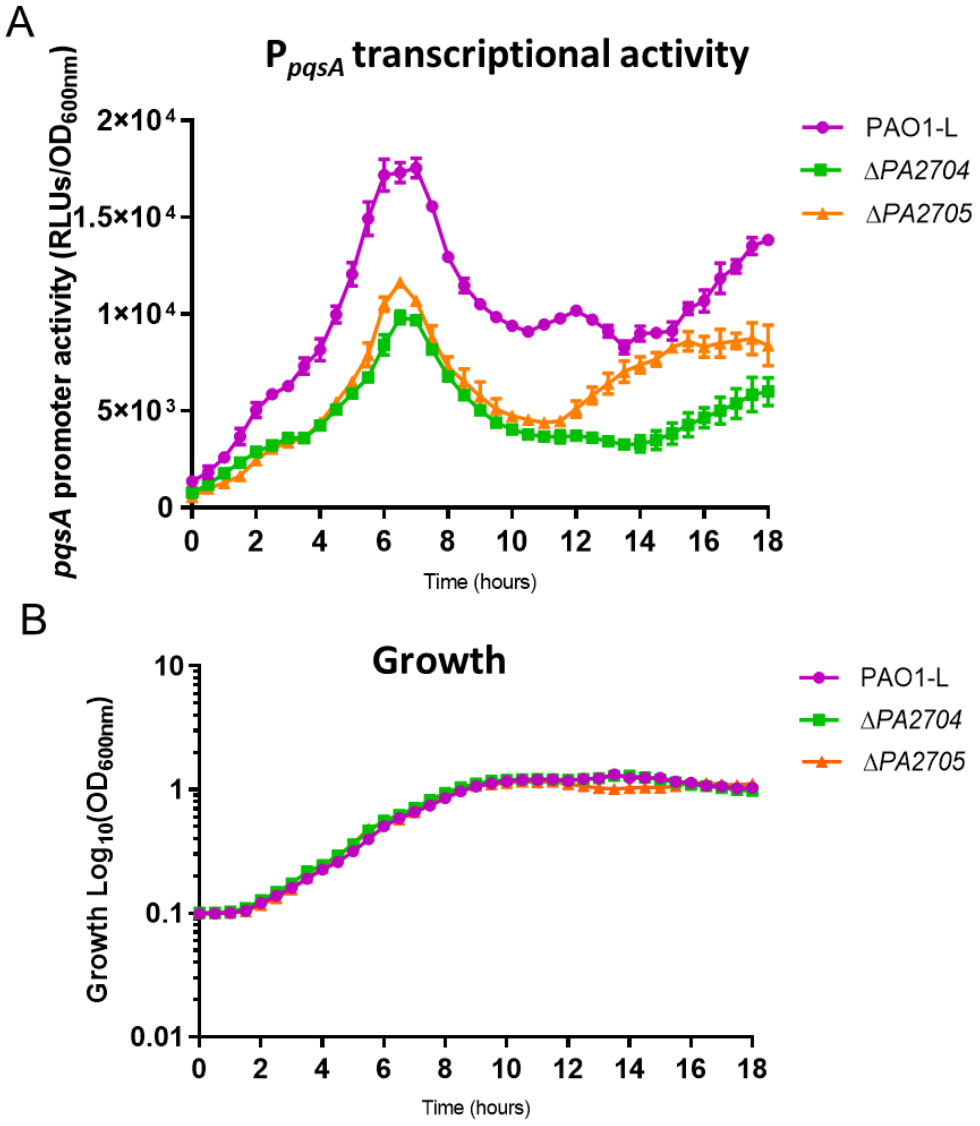


Figure 3.7. The transcriptional activity of P_{pqsA} in *P. aeruginosa PA2704* and *PA2705* mutants using new bioreporter $P_{pqsA\ long}$-*lux*.

A) Normalised bioluminescence production for PAO1-L, Δ*PA2704* and Δ*PA2705* $P_{pqsA\ long}$-*lux* (Gm^R) in LB broth. Both OD_{600nm} and the relative light units (RLUs) were measured. Strains were grown at 37 °C for 18 hours. B) The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars show 2x standard error calculated across three biological replicates.

For the accurate analysis of the potential regulation of PA2705 towards $P_{pqsA,}$ it was necessary to understand the impact of the addition of those 115 bp present in the transcriptional $P_{pqsA\ long}$-*lux* that led to the significant difference between the transcriptional activity of P_{pqsA} in the *PA2704* and *PA2705* mutants using the two different reporters (**Figure 3.6 and 3.7**). Sequence analysis of the two P_{pqsA} sequences evidenced that the *rhlR-box* 5' CTGTGAGATTTGGGAG 3' centred at -311 bp upstream of the *pqsA* transcriptional initiation site (**Figure 3.5)**, was interrupted in the $P_{pqsA\ short}$-*lux* reporter, more specifically, 9 bp of this genetic element were absent in this reporter. Based on this observation, the question arose to whether the interrupted *rhlR-box was* responsible for these changes in P_{pqsA} activity. To address this question, deletion of the entire *rhlR-box* was carried out within the same region included in $P_{pqsA\ long}$-*lux* as described in **Chapter 2, section 2.6.5**. The resulting construct was named $P_{pqsA\ \Delta rhlR\text{-}box}$-*lux* (**Figure 3.5**) and the construction of this vector can be found in **Supplementary data, Figure S7.5**.

Afterwards, the transcriptional activity of $P_{pqsA\ long}$-*lux* and $P_{pqsA\ \Delta rhlR\text{-}box}$-*lux* was monitored using the same mutations and conditions as in Rampioni, et al., (2010)., that included: *P. aeruginosa* PAO1-N, PAO1-N *pqsE* Ind, *rhlR* and *rhlR pqsE* Ind mutants but in PAO1-L. As the work presented in this thesis has been focused on the PAO1-L genetic background, the construction of the *pqsE* Ind in the PAO1-L derivative strains was carried out first as described in **Chapter2, section 2.6.4**, and verified as shown in **Supplementary data, Figure S7.2**. The analysis of the transcriptional activity of P_{pqsA} in PAO1-L, PAO1-L *pqsE* Ind, *rhlR* and *rhlR pqsE* Ind mutants using the $P_{pqsA\ \Delta rhlR\text{-}box}$-*lux* and the $P_{pqsA\ long}$ -*lux* are presented in **Figure 3.8** and **Figure 3.9**, respectively.

The transcriptional activity of P_{pqsA} with a deleted *rhlR-box* in wild type parent strain reached its peak at 7,5 hours of growth **(Figure 3.8).** Uninduced *pqsE* within the *pqsE* Ind condition (*pqsE* Ind) slightly increased the transcriptional activity of P_{pqsA}, placing PqsE as an apparent repressor of *pqsA* under these conditions. Following IPTG-induced expression of *pqsE (pqsE* Ind + IPTG*),* the transcriptional activity of P_{pqsA} was completely abrogated, validating the latter

assumption. In addition, mutation of *rhlR* in the wild type condition slightly increased the transcriptional activity of P_{pqsA}, suggesting that RhlR is a repressor of this promoter at the transcriptional level. Furthermore, uninduced *pqsE* in the *rhlR* mutant condition not only increased, but also advanced the transcriptional activity of P_{pqsA} by nearly 2 hours, and the IPTG-induced expression of *pqsE (pqsE* Ind + IPTG), drastically diminished these levels, propounding that the *pqsE*-mediated repression is independent on RhlR. These results are comparable to those observed by Rampioni et al., (2010) and suggests that the final outcome towards the transcriptional activity of P_{pqsA} was due to the interruption of the *rhlR-box present in the* $P_{pqsA\ short}$-lux used for that study .

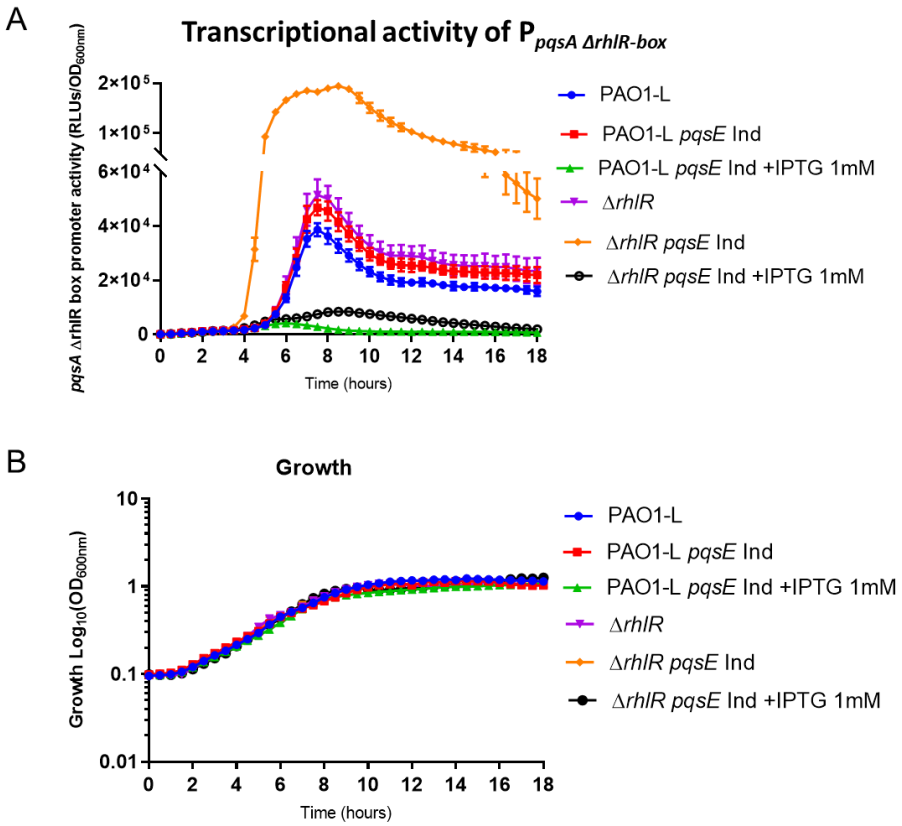


Figure 3.8. The transcriptional activity of P_{pqsA} with a mutated *rhlR-box* in *P. aeruginosa rhlR* mutant and inducing *pqsE*.

A) Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*rhlR* and Δ*rhlR pqsE* Ind strains harboring the $P_{pqsA\ \Delta rhlR\text{-}box}$-lux ($Gm^R$) bioreporter in LB broth. B) The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars show 2x standard error calculated across three biological replicates.

On the other hand, the transcriptional activity of P_{pqsA} with an intact *rhlR-box* in the wild-type parent strain PAO1-L reached its peak at 7 hours of growth and presented lower levels of P_{pqsA} compared to those observed with a mutated *rhlR-box* (**Figure 3.9**). Insertion of the conditional mutation of *pqsE (pqsE* Ind*)* in the wild-type genetic background significantly reduced the transcriptional activity of P_{pqsA}, placing PqsE as an inductor of this promoter. Strikingly, although the increment in IPTG concentration used in the *pqsE* Ind strain slightly advanced the peak and augmented the activity of P_{pqsA}, this induction was not significant and the wild type levels of activity of P_{pqsA} were not restored, indeed, they remained similar to the levels observed in the uninduced *pqsE* Ind condition (**Figure 3.9**). The sole mutation of *rhlR* slightly increased the transcriptional activity of P_{pqsA} when compared to the wild type, whereas the mutation of *rhlR* in the uninduced *pqsE* Ind condition presented similar activity levels of P_{pqsA} until late stationary phase. Surprisingly, the IPTG-induced expression of *pqsE* (*pqsE* Ind + IPTG) in the last condition considerably increased the transcriptional activity of P_{pqsA}, suggesting that PqsE behaves as a positive regulator of *pqsA* when *rhlR* is absent.

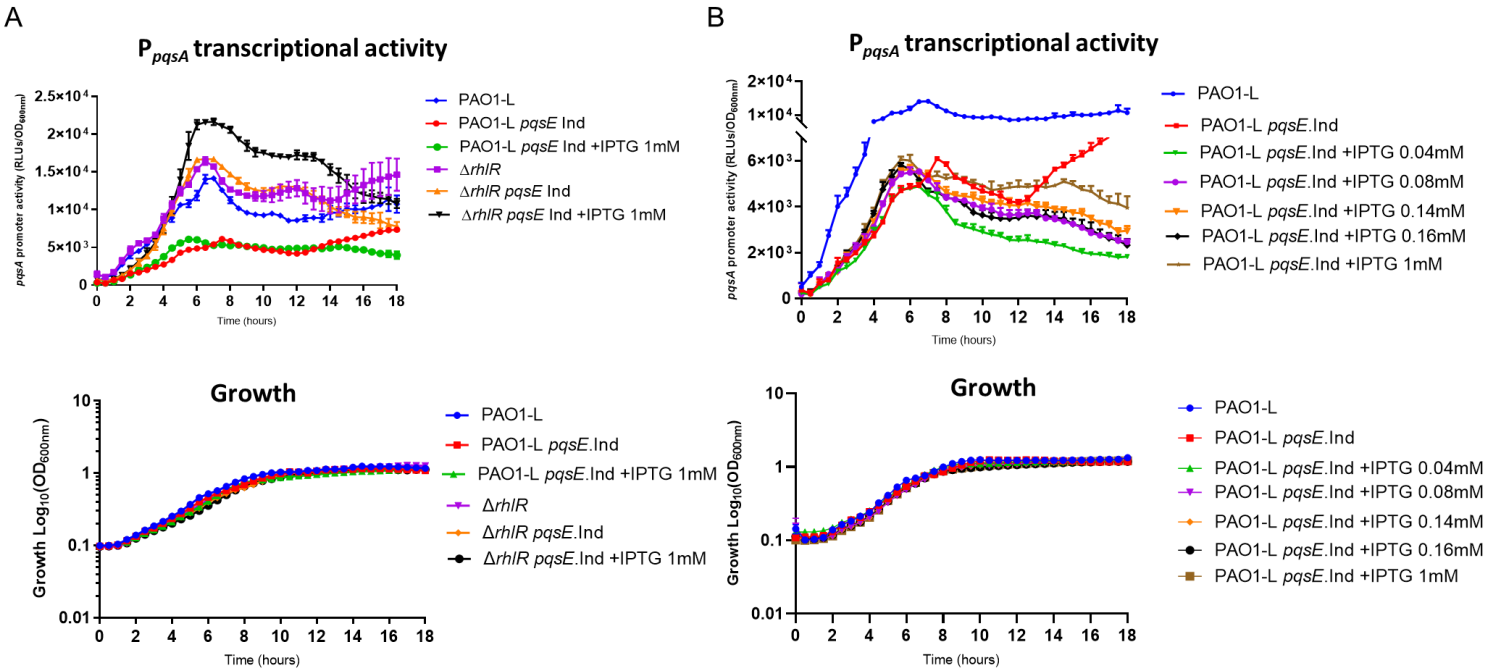


Figure 3.9. The transcriptional activity of P_{pqsA} with an intact *rhlR-box* in *P. aeruginosa rhlR* mutant and inducing *pqsE*.

A) Top: Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*rhlR* and Δ*rhlR pqsE* Ind strains harboring the $P_{pqsA\ \Delta rhlR\text{-}box}$-*lux* ($Gm^R$) bioreporter in LB broth. Bottom: The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars show 2x standard error calculated across three biological replicates. B) Top: Induction of *pqsE* under different IPTG concentrations. Normalized bioluminescence production for strains PAO1-L *pqsE* Ind carrying the reporter $P_{pqsA\text{-}long}$-lux (Gm^R) exposed to increasing concentration of IPTG. Strains were grown at 37 °C for 18 hours in a 96-well TECAN plate. Bottom: The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars show 2x standard error calculated across three biological replicates.

Taking these analyses together, it can be concluded that the presence of the *rhlR-box* within the P_{pqsA} is an essential element to fully understand the mechanisms underlying this regulation and, therefore, it is suggested that the use of the $P_{pqsA\ long}$-*lux* should be used for an accurate analysis towards the P_{pqsA} regulation. Based on this observation, it can also be concluded that PA2705 and PA2704 are positive regulators of P_{pqsA} ***(*Figure 3.7*).***

3.6.1.2 PqsE represses *pqsA* at a post-transcriptional level

Considering that: (i) the regulation of PqsE towards the transcription of *pqsA* expression, using an intact *rhlR-box* vs truncated or absent, did show a clear picture of this regulation, and (ii) that Rampioni et al., (2010) demonstrated that the mutation of *pqsE* in PAO1-N increased the production of PQS whereas the induction of this effector almost abrogated the production of this AQs, it was paramount to unravel whether the regulation observed by PqsE could be mediated at the post-transcriptional level.

To address this, PQS levels were first measured in the PAO1-L genetic background strains with a view to elucidate whether PqsE behaves in the same manner as in the PAO1-N strain. To do this, the quantitation of PQS was carried out at 7 hours of growth in PAO1-L and PAO1-L *pqsE* Ind strains as this time showed the maximal transcriptional activity of P_{pqsA} (**Figure 3.9B**). Results are shown in **figure 3.10**. When compared to the wildtype parental strain, mutation of *pqsE* in the *pqsE* Ind condition resulted in increased levels of PQS (**Figure 3.10),** suggesting that the ultimate effect of PqsE is the repression of the production of PQS, moreover, following IPTG-induced expression of *pqsE* completely abrogated the production of this AQs. This is in line with the results obtained by Rampioni et al., (2010).

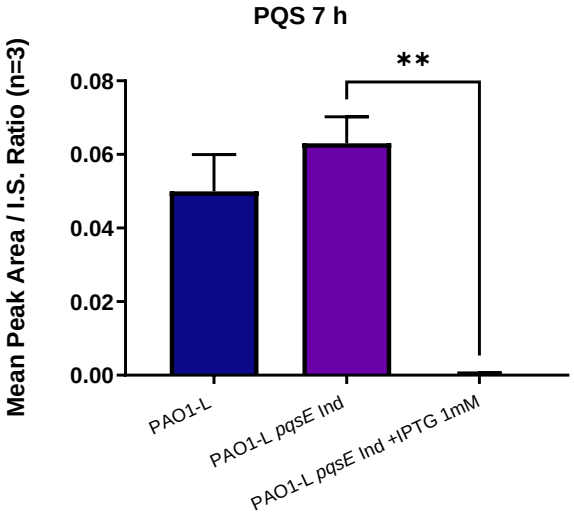


Figure 3.10. PQS quantification in *P. aeruginosa* at 7 hours of growth.

Bacteria were grown in flasks for 7 hours at 37 °C in LB media. Quantification of PQS was performed from sterile supernatants extracted with ethyl acetate. Error bars represent standard deviation of three biological triplicates. T-tests were used to assess for statistical significance.

A P_{pqsA} translational reporter was then constructed in the pMiniCTX-*lux* (Gm^R) vector as described in **Chapter 2, section 2.6.1** and validated as shown in **Supplementary data, Figure S7.4**. This reporter was named as $P_{pqsA\ long}$ '-' -*luxCDABE* and it was designed so that the P_{pqsA} is fused in frame to the *luxCDABE* operon, hence reflecting when the target gene *pqsA* was transcribed and translated (Thomas., 2001). The translation of *pqsA* was monitored in PAO1-L and PAO1-L *pqsE* Ind. Results are shown in **Figure 3.11**. Briefly, the translation of *pqsA* in the PAO1-L wild type condition peaked at 7 hours of growth. In addition, in agreement to that observed with the quantification of PQS (**Figure 3.10**), uninduced *pqsE* in the *pqsE* Ind condition caused a significant increase in the translation of *pqsA*, that was then dramatically reduced when *pqsE* was overexpressed (*pqsE* Ind + IPTG) (**Figure 3.10**), hence, validating that PqsE acts a repressor of *pqsA* and that this regulation takes place at the post-transcriptional level.

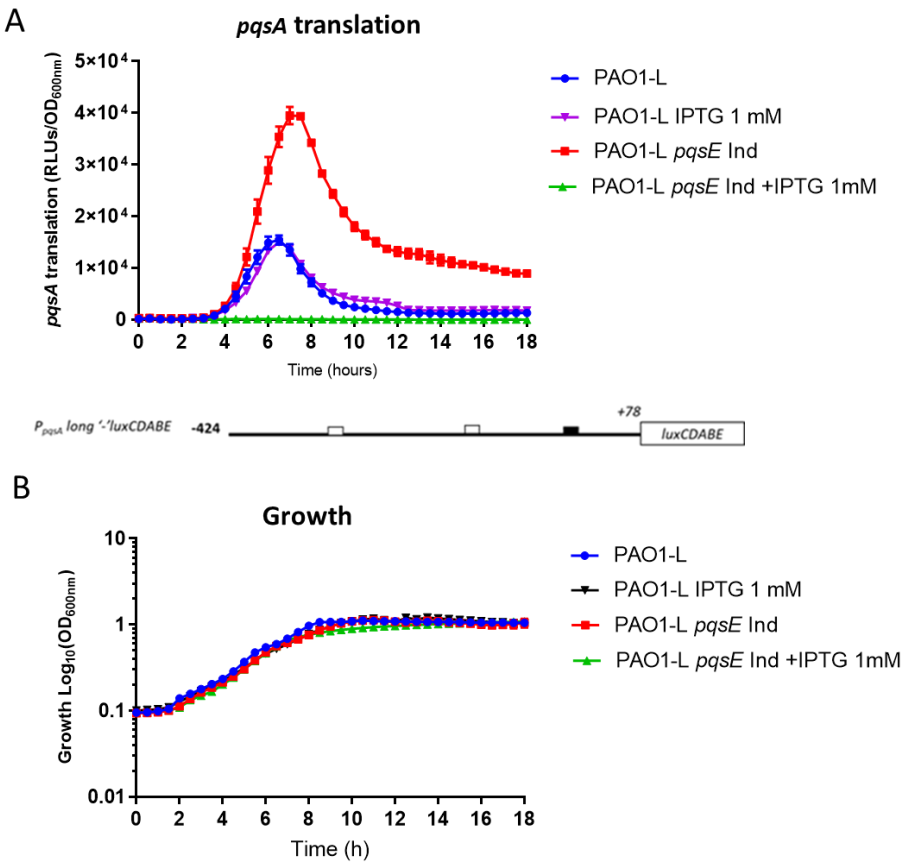


Figure 3.11. The translational levels of *pqsA* in *P. aeruginosa pqsE* Ind.

A) Normalised bioluminescence production for strains PAO1-L PAO1-L and PAO1-L *pqsE* Ind carrying the reporter $P_{pqsA\ long}$*'-'-luxCDABE* (Gm^R) (illustrated). IPTG alone did not cause a significant impact on the translation of *pqsA* in PAO1-L. Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. B) The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars show 2x standard error calculated across three biological replicates

3.6.1.3 PqsE induces *PA2705* at the post-transcriptional level.

As seen in the original P_{pqsA} pull down, PA2705 was found binding this promoter when PqsE was overexpressed (**Table 3.1**), hence, it would be expected that PqsE regulates PA2705 in a positive manner.

Since this work has demonstrated that the regulation mediated by PqsE occurs in a post-transcriptional event the impact of PqsE towards *PA2705* was studied at both transcriptional and post-transcriptional levels. To do this, a PA2705 translational reporter was constructed using the pMiniCTX-*lux* (Gm^R) vector as described in **Chapter 2, section 2.6.1**, and validated as shown in **Supplementary data, Figure S7.4**. This translational reporter was named as P_{PA2705}'-'-*luxCDABE* and included 366 bp upstream of PA2705-PA2707 operon plus the ATG of *PA2705*.

The use of a translational fusion shows a degree of both transcriptional and translational regulation, on that account, the comparison of results with a transcriptional fusion would identify how much regulation is taken place at each level. To do this, a transcriptional reporter of *PA2705* was also constructed in pMiniCTX-lux (Gm^R) as described in **Chapter 2, section 2.6.1**, and validated as shown in **Supplementary data, Figure S7.4.** This reporter included 366 bp of the upstream region of PA2705 gene and was named P_{PA2705}-*lux*. Both reporters were separately introduced in the chromosome of PAO1-L and PAO1-L *pqsE* Ind strains by conjugation and the transcription and translation of *PA2705* was monitored over time. Results are shown in **Figure 3.12**.

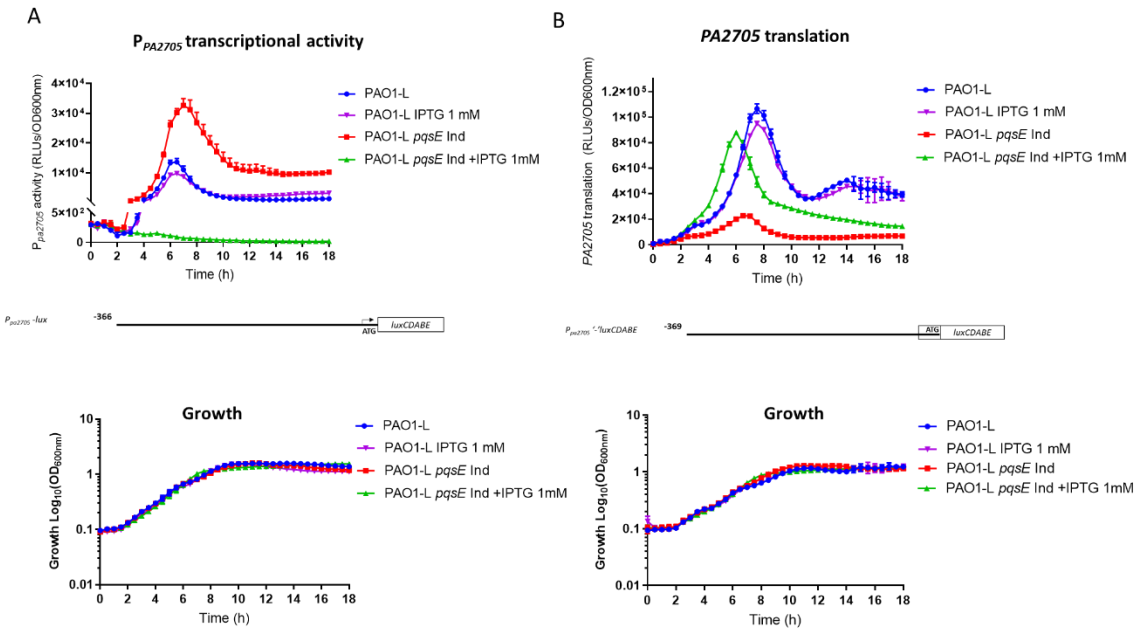


Figure 3.12. The transcription and translation of *PA2705* under induction of *pqsE*.

Top: Normalised bioluminescence production for PAO1-L and PAO1-L *pqsE* Ind carrying the A) P_{PA2705}-*lux* (Gm^R) and B) $P_{PA2705'-''}$ *luxCDABE* bioreporters (illustrated) in LB broth. IPTG alone did not cause a significant impact on the transcription or translation of *PA2705* in PAO1-L. Bottom: All strains in A) and B) were grown at 37 °C for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. The Log10 growth curve shows that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

As seen in **Figure 3.12**, the regulation at the transcriptional level was opposite to that observed at the translational one. On one hand, the transcriptional activity of P_{PA2705} in the wildtype hit its highest levels at 6,5 hours of growth (**Figure 3.12A**). Uninduced *pqsE* in the *pqsE* Ind condition showed a small advancement but a significant increase in the transcriptional activity of P_{PA2705} that was almost abrogated when *pqsE* was induced (*pqsE* Ind + IPTG). On the other hand, the translation of *PA2705* in the wild type reached higher levels to those observed at the transcriptional level peaking later at 8 hours of growth (**Figure 3.12B**). Lack of IPTG-induced *pqsE* expression in the *pqsE* Ind condition dramatically decreased the translation *PA2705* with a peak slightly advanced in comparison to the wildtype. These data indicated that PqsE exerts a positive impact on PA2705 translation, supporting the observations inferred from the P_{pqsA} pull down. These results further evidence the role of PqsE in gene regulation at the post-transcriptional level which requires further investigation.

3.6.1.4 PA2705 is dispensable for the PqsE-mediated repression of *pqsA.*

This work has evidenced that PA2705 plays a role in the regulation of P_{pqsA} via PqsE, however, it remained unclear as to whether PqsE requires PA2705 for the translational control of *pqsA*. To elucidate this, the translation of *pqsA* was monitored in PAO1- L, PAO1-L *pqsE* Ind, *PA2705* and *PA2705 pqsE* Ind mutant strains. In first instance the construction of the *PA2705 pqsE* Ind strain was carried out in PAO1-L as described in **Chapter 2, section 2.6.4** and verified as shown in **Supplementary data, Figure S7.2.**

As seen in **Figure 3.13**, the uninduced (*pqsE* Ind) and IPTG-induced (*pqsE* Ind + IPTG) expression of *pqsE* elevated and diminished the translation of *pqsA*, respectively, ratifying its repressor role towards *pqsA*. Mutation of *PA2705* within the uninduced *pqsE* Ind condition slightly decreased the translation of *pqsA* when compared to the parent strain, probably due to the lack of its positive role towards *pqsA*. Subsequent IPTG-induced expression of *pqsE*, however, significantly repressed the translation of *pqsA*. These results show

that although PA2705 is involved in the regulation of *pqsA*, is not essential for the PqsE-mediated repression of *pqsA* expression.

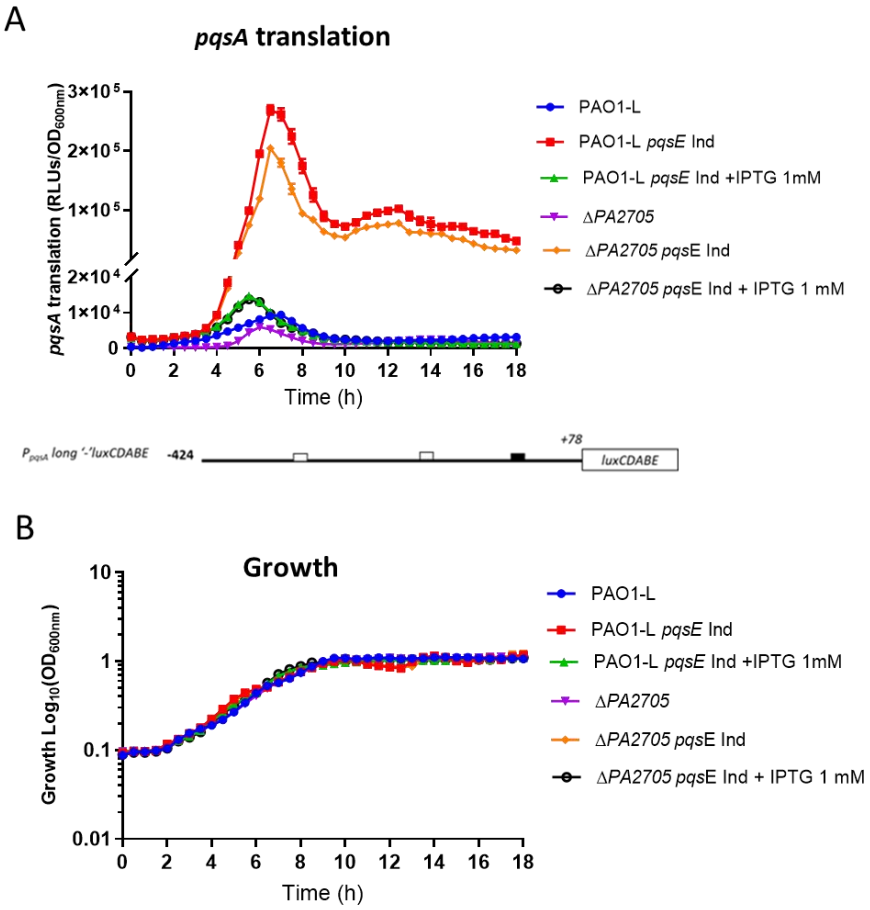


Figure 3.13. The translation of *pqsA* in *P. aeruginosa PA2705* mutant and inducing *pqsE*.

A) Normalised bioluminescence production for strains PAO1-L, PAO1-L *pqsE* Ind, Δ*PA2705* and Δ*PA2705 pqsE* Ind carrying the construct $P_{pqsA\ long}$'-'- *luxCDABE* (Gm^R) (illustrated). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. B) The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

(*) Note that IPTG alone did not cause a significant impact on the translation of *pqsA* in PAO-L or *PA2705* mutant **(Supplementary data, Figure S7.6.1**)

3.6.2. New insights in the regulation of *PA2705*

This work has shown that PA2705 participates in the regulation of *pqsA* and that is under the control of PqsE. In addition, albeit PA2705 is dispensable for the PqsE-mediated repression of *pqsA*, its close interactions with the *pqs*

QS system prompted the design of further studies to gain a better understanding of the nature of these relationships.

3.6.2.1. The mutation of *PA2705* reduces the production of alkyl quinolones in *P. aeruginosa*.

To confirm further whether PA2705 positively regulated *pqsA* and to characterize the impact of its mutation in *P. aeruginosa* pathogenesis, phenotypic assays were carried out in this strain. As seen in **Chapter 1**, PQS is an alkyl quinolone which biosynthesis requires the *pqsABCDE* operon in addition to *pqsH*. Based on this and, considering that the mutation of *PA2705* resulted in a declined *pqsA* transcription and translation, this should also result in reduced levels of PQS production. To validate this, PQS was quantified after 16 hours of growth in PAO1-L and *PA2705* mutant. Results show that, compared to the wild type, mutation of *PA2705* decreased the production of PQS although this difference was not statistically significant. Addition of the empty plasmid pME6032, used to later express *PA2705* for complementation, to both strains in the presence of IPTG slightly increased the production of PQS in the wild type (not significant), whereas in *PA2705* mutant significantly reduced the production of this signal molecule. Complementation with pMENRC1 (pME6032 overexpressing *PA2705*) restored the wild type levels of PQS in *PA2705* mutant (**Figure 3.14**). Overall, this result suggests that PA2705 has a positive impact in the production of PQS.

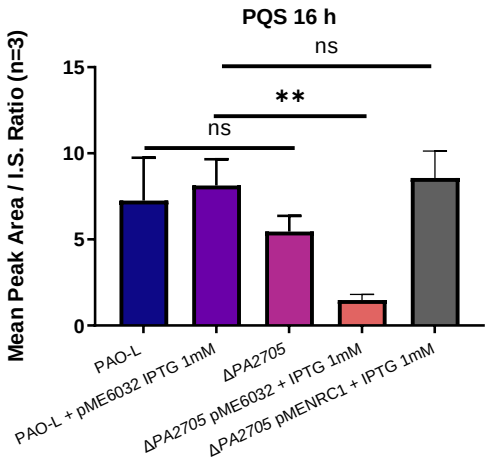


Figure 3.14. PQS quantification in *P. aeruginosa PA2705* mutant.

Bacteria were grown in flasks for 16 hours at 37 °C in LB media. Quantitation of PQS was performed from sterile supernatants extracted with ethyl acetate. Error bars represent standard deviation of three biological triplicates. T-tests were used to determine the statistical significance.

3.6.2.2. The mutation of *PA2705* impacts the production of pyocyanin in *P. aeruginosa.*

Pyocyanin is a crucial virulence factor produced by *P. aeruginosa* which regulation is highly complex and diverse due to its link with multiple QS systems (Gallagher et al., 2002; Diggle et al., 2003). Research has shown that PqsE regulates pyocyanin production in a AQs-independent whilst in a RhlR-C4-HSL-dependent manner, and mutants of *pqsE* abrogates its production, highlighting the tight relationship between PqsE and the production of this virulence factor (Farrow et al., 2008; Hazan et al., 2010; Rampioni, et al., 2010; Rampioni et al., 2016). Albeit pyocyanin is widely regulated, since PqsE has shown to regulate *PA2705*, it is probable that the mutation of *PA2705* impacts the production of pyocyanin.

As seen in **Figure 3.15**, pyocyanin production in the absence of *PA2705* was strongly reduced when compared to the wild type condition, whereas its complementation with pMENRC1 (pME6032 overexpressing *PA2705*) partially restored the pyocyanin levels. This data suggests that PA2705 modulates pyocyanin production in a positive manner and that its absence

may potentially cause a major disbalance in the production of this virulence factor due to its link with PqsE.

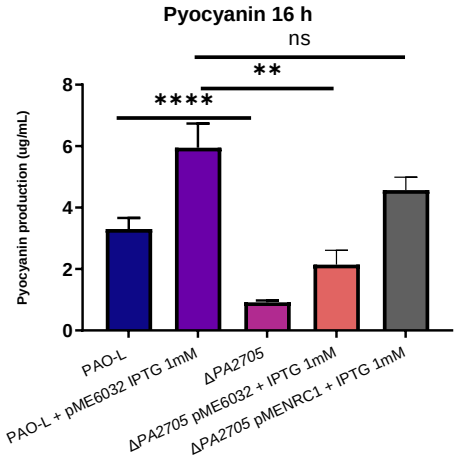


Figure 3.15. Pyocyanin production in *P. aeruginosa PA2705* mutant.

Bacteria were grown in flasks for 16 hours at 37 °C in LB media. Pyocyanin quantification was performed from sterile supernatants extracted with chloroform and measured reading at OD_{520nm}. Error bars represent standard deviation of three biological triplicates. T-tests were used to assess for statistical significance.

3.6.2.3. *PA2705* is regulated by PA2704

Since gene proximity can result in a possible functional link (Junier and Rivoire. 2016) and considering that no data has been reported about any regulators controlling *PA2705* expression, it became logical to investigate whether PA2704, encoding a putative transcriptional regulator, could be involved in the regulation of PA2705. To investigate this, the pMiniCTX-based P_{PA2705}-*lux* fusion was introduced in the chromosome of *PA2704* mutant and the transcriptional activity of P_{PA2705} was measured in this strain.

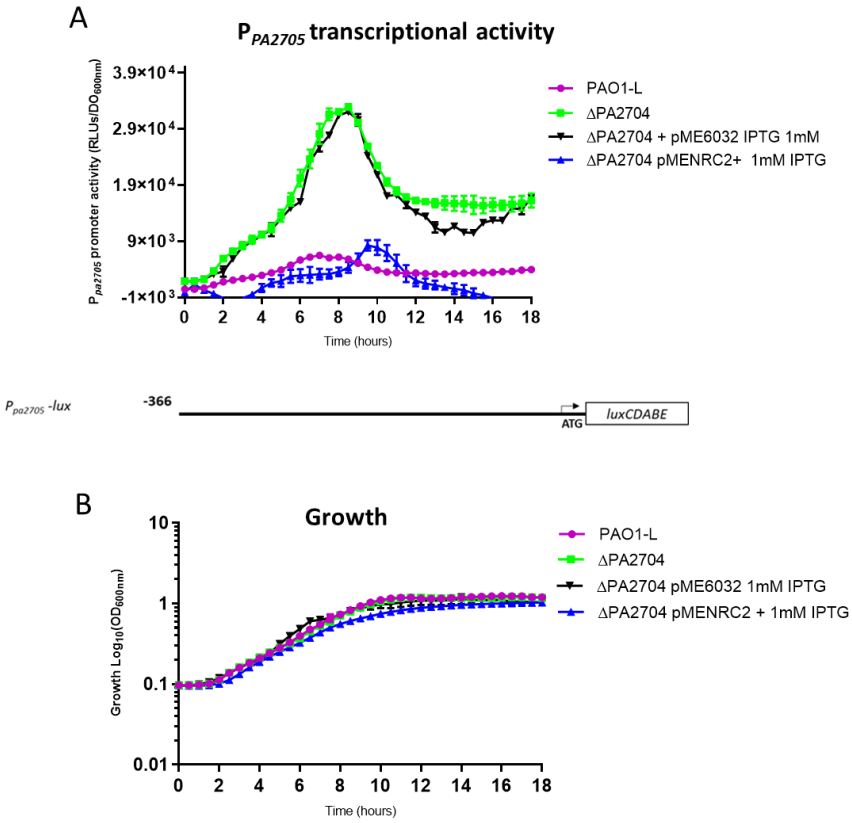


Figure 3.16. Effect of the mutation of *PA2704* upon the transcriptional activity of P_{PA2705}.

A) Normalised bioluminescence production for PAO1-L and Δ*PA2704* carrying the P_{PA2705}-*lux* (Gm^R) (illustrated) in LB broth. Strains were grown at 37 °C for 18 hours. B) The growth curves are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

Results show that when compared to the wild type, mutation of *PA2704* caused a large increase in the transcriptional activity of P_{PA2705} which is

reduced to maximal levels close to those from the parental strain upon *PA2704* complementation (pMENRC2 + IPTG) (**Figure 3.16**), indicating that PA2704 regulates *PA2705* in a negative manner, hence, validating the regulatory link between the two.

3.6.2.4. *PA2705* is regulated by the *las/rhl* system

The three QS systems have been shown to be interconnected in multiple ways. In addition, research has shown that these interconnections are more complex and dynamic than originally anticipated (McGrath et al., 2004; Dekimpe & Déziel, 2009; Hazan et al., 2010**;** Allegretta et al., 2017)**.** With this in mind and based on the PA2705 promoter analysis (**section 3.4**), it was thought that PA2705 expression may also be affected by the *las/rhl* systems. To investigate this, the pMiniCTX-based P_{PA2705}-*lux* reporter was introduced into the *lasI* and *rhlI* mutants and the transcriptional activity of P_{PA2705} was monitored over time.

Results indicated that when compared to the wildtype condition, the transcriptional activity of P_{PA2705} significantly increased in the absence of *lasI* or *rhlI* which was partially complemented upon addition of the QS molecules responsible for the synthesis of 3OC12-HSL and 4-HSL, respectively (**Figure 3.17**). These results evidence that both the *las/rhl* system have a negative impact on the expression of *PA2705.*

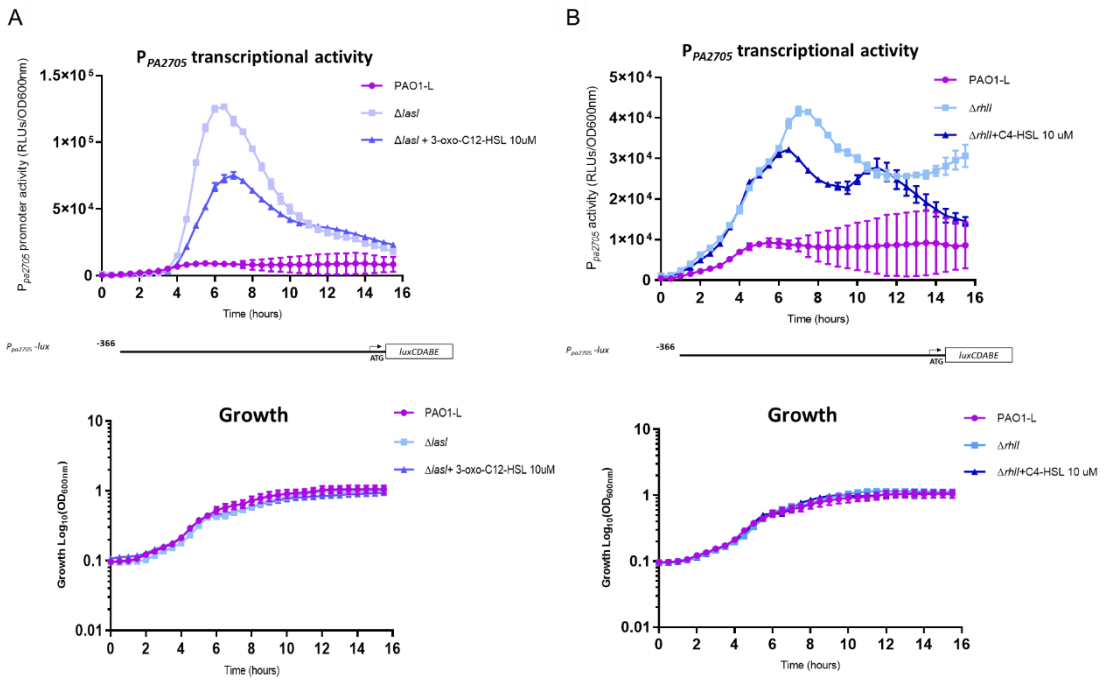


Figure 3.17. QS canonical autoinducers partially restore the *PA2705* expression in *P. aeruginosa lasI* and *rhlI* mutants.

Top: Normalised bioluminescence production for A) Δ*lasI* and B) Δ*rhlI* P_{PA2705}-*lux* (Gm^R) (illustrated) in LB broth. Addition of 3OC12-HSL10 uM and C4-HSL 10 uM showed to decrease the transcriptional activity of P_{PA2705} when compared to the mutant conditions. Bottom: The growth curves in A) and B) are represented as Log10 of the OD_{600nm} showing that all strains grew similarly. Error bars show 2x standard error calculated across three biological replicates.

3.7. Elucidating whether PA2705 interacts with P_{pqsA} in isolation

During this work, PA2705 has been shown to be involved in the P_{pqsA} regulation. However, albeit it was not essential for the PqsE-mediated repression, the fact that *Bate, PhD Thesis*, (2013) identified this protein in the P_{pqsA} pull down suggested that PA2705 binds to this promoter. Intriguingly, no apparent DNA binding domain was found in within the PA2705 sequence, hence it was paramount to investigate whether this hypothetical protein binds, on its own, the P_{pqsA}

To achieve this, PA2705 was expressed and purified. The following sections present this process and an attempt to study the interaction of this protein with P_{pqsA}.

3.7.1. PA2705 protein expression and purification

The purification of recombinant target proteins can be achieved using a poly histidine tag usually consisting of 6 histidine residues that allows binding of target proteins to resins/agarose beads containing immobilized divalent metal ions such as cobalt, nickel, copper, or zinc (Porath et al., 1975; Nilsson et al., 1997) .

Using this approach, the purification of PA2705 (45,6 kDa) was performed using metal affinity chromatography (IMAC) (Jerker Porath, Jan carlsson. 1975). To achieve this, whole *PA2705* gene was cloned into an empty pCold vector as described in **Chapter 2, section 2.9.1**. This expression vector carries a poly histidine residue that allowed to his-tag PA2705 at its N-terminus. Albeit the optimal placement of the tag is protein specific, N-terminal his-tags often improve the yield of recombinant proteins by providing a reliable context for efficient translation initiation (Aslantas & Surmeli., 2019). The resulting vector named pNR1Cold1, was subsequently transformed into *E. coli* BL21 (DE3) strain, that is routinely used in protein purification due to its deficiency in both *lon* and *ompT* proteases and that is compatible with the T7 *lac*O promoter system (Grodbergl & Dunn, 1988; Chambedin et al., 1990). Competent cells carrying pNR1Cold1 were expressed at 16^{o}C as described in **Chapter 2, section**

2.9.1. Whole pellet cells expressing the his-tagged PA2705 are shown in **Figure 3.18**. Polyacrylamide gel revealed a band with high density nearly at ~50 kDa, which was absent in the cell expressing the empty vector, suggesting that the expressed protein corresponded to PA2705.

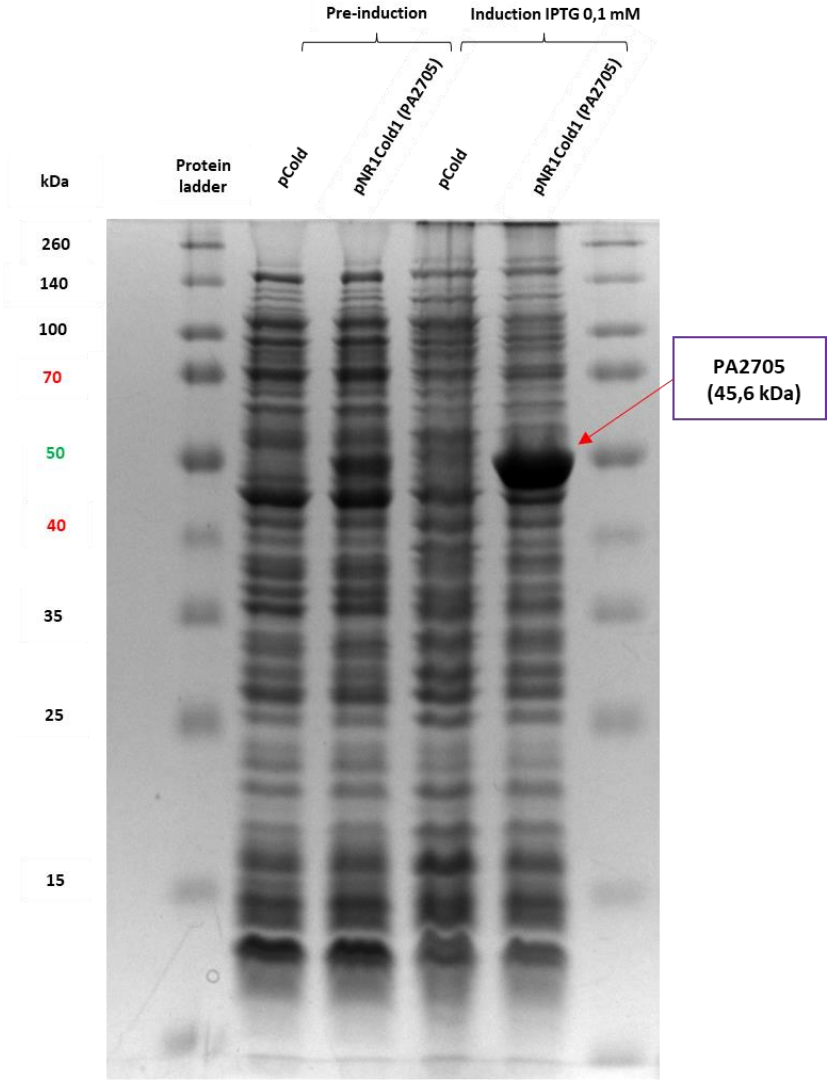


Figure 3.18. SDS-PAGE of his-tagged PA2705 expression.

E. coli BL21 (DE3) carrying the vector pNR1Cold1 was induced at 16°C with 0,1 mM IPTG for 16 hours. The pellets were adjusted to an OD_{600nm}=0.6, treated with 1XSDS, boiled at 100°C for 10 min and then separated on a 12% polyacrylamide gel at 100 volts for 90 min. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).

Disruption of the cell pellets by sonication resulted in PA2705 located in the insoluble fraction (**Figure 3.19A**). For protein solubilization, a range of mild solubilizing agents were incorporated in the insoluble fractions as described in **Chapter 2, section 2.9.1.** The soluble and insoluble fractions were analyzed by gel electrophoresis. For this analysis, urea 8 M, a chaotropic compound that disrupts hydrogen bonds and hydrophobic interactions both between and within proteins (Salvi, Rios, & Vendruscolo., 2005) was used as a denaturing agent, serving as a positive control for the effective solubilization of PA2705. As seen in **Figure 3.19B**, among the milder conditions tested, n-lauryl sarcosine (sarkosyl) at 0.2% (**Figure 3.19B, Lane 6**) was the most effective in partially solubilizing PA2705, hence, this detergent was used for further purification steps.

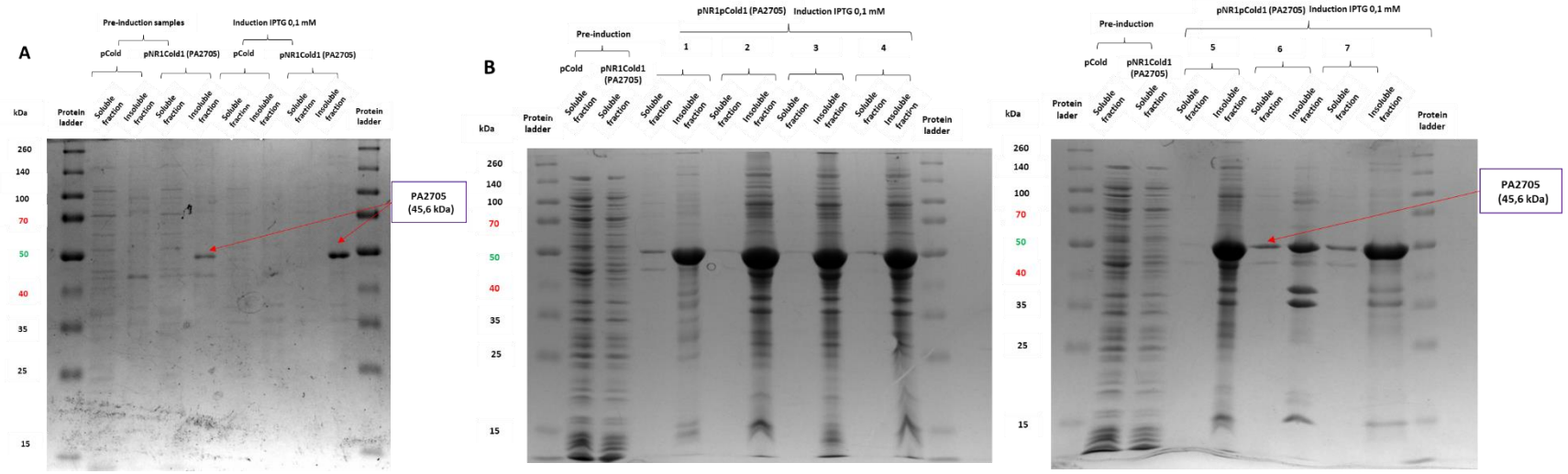


Figure 3.19. SDS-PAGE of PA2705 solubility analysis using different nondenaturing and mild solubilizing agents.

A) *E. coli* BL21 (DE3) carrying the vector pNR1Cold1 was induced at 16°C with 0,1 mM IPTG for 16 hours. The resulting pellets were adjusted to an OD_{600nm}= 0.6 and resuspended in Tris HCL buffer pH 8. Cells were disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each and the soluble and insoluble fractions were collected after centrifugation at 12.000 rpm for 10 min.

B) *E. coli* BL21 (DE3) carrying the vector pNR1Cold1 was expressed as above. Cells were disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in different solubilizing agents overnight at 4°C in a rocker platform. Soluble and insoluble fractions were obtained after centrifugation at 12.000 rpm for 10 min, treated with 2XSDS loading buffer, boiled 100°C for 10 min and then separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa). **Lane 1**: 50 mM Tris Urea 8 M, 5% Glycerol pH 8. **Lane 2:** 40 mM Tris HCL 5% propanol, 2 M Urea pH 8. **Lane 3**: 40 mM Tris HCL 0,5% Triton X 100 pH 8. **Lane 4**: 40 mM Tris HCL 5% DMSO pH 8. **Lane 5**: 40 mM Tris HCL 2 M Urea pH 8. **Lane 6**: 40 mM Tris HCL 0,2% n lauryl sarcosine. **Lane 7**: 40 mM Tris Urea 2 M, pH 12.

Next, to determine whether PA2705 could be eluted using imidazole, the common competitive agent for elution of histidine-tagged proteins, the soluble fraction of PA2705 was first mixed with HisPur™ Ni-NTA nickel resin overnight at 4^{o}C in a rocking platform, thus, allowing the binding of the protein to the metal resin. Subsequently, an imidazole titration using increasing concentrations of this agent allowed the elution of PA2705 (**Figure 3.20**), with maximum efficiency at concentrations that ranged between 80 and 100 mM of imidazole.

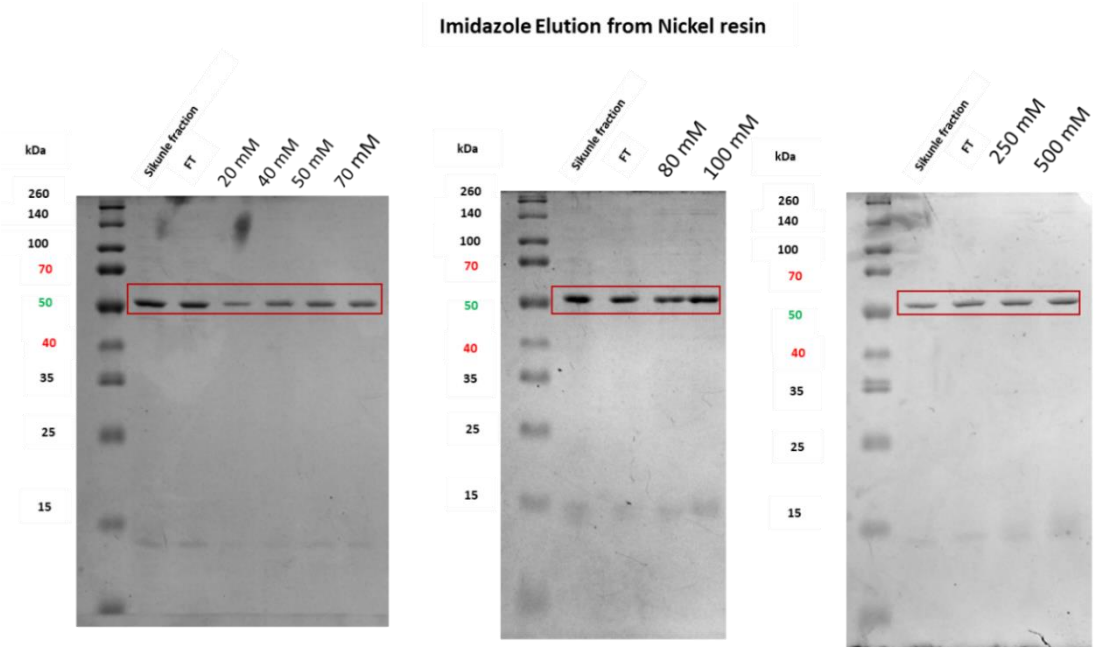


Figure 3.20. SDS-PAGE of the PA2705 imidazole elution.

E. coli BL21 (DE3) carrying the vector pNR1Cold1 was induced at 16°C with 0,1 mM IPTG for 16 hours. The resulting pellets were adjusted to an OD_{600nm}= 0.6 and resuspended in Tris HCL buffer pH 8. Cells were disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in 40 mM Tris HCL 0,2% n-lauryl sarcosine overnight at 4°C in a rocking platform. Soluble fractions were collected and incubated with HisPur™ Ni-NTA overnight at 4°C. PA2705 was eluted using increasing concentrations of imidazole (20-500 mM). Samples were treated with 2XSDS loading buffer and then separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Soluble fraction corresponds to the sample containing soluble protein prior imidazole elution. FT denotes flow through and correspond to the unbound fractions of protein after incubation with Ni-NTA. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).

Following the pre-purification steps, PA2705 was expressed as described in **Chapter 2, section 2.9.2** and subsequently purified in an ÄKTA pure system. To achieve the latter, supernatants containing the soluble protein were pre-loaded into a Nickel immobilized nitrilotriacetic acid (NTA). Note that due to some endogenous proteins present in IMAC media can show weak binding to the column (Bolanos-garcia & Davies, 2006), a low concentration of imidazole of 5 mM was added in the washing buffer A and passed through the column with bound PA2705 to lessen nonspecific binding to nickel. Pure PA2705 was eluted by using an imidazole gradient as described in **Chapter 2, section 2.9.3**. IMAC chromatogram showed a sharp peak between the fractionation samples numbers 6 and 10, that corresponded to the elution of PA2705 as evidenced followed polyacrylamide gel electrophoresis (**Figure 3.21**).

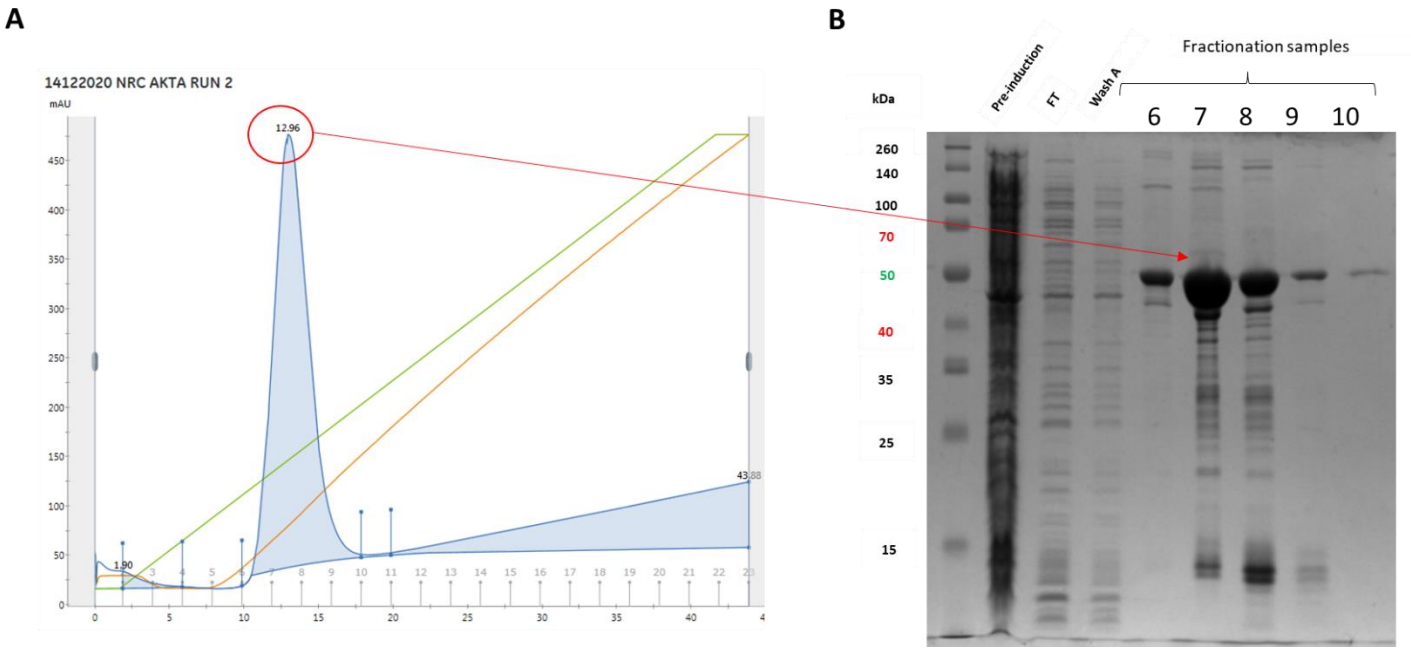


Figure 3.21. PA2705 ÄKTA purification.

E. coli BL21 (DE3) carrying the vector pNR1Cold1 was induced at 16°C with 0,1 mM IPTG for 16 hours. The resulting pellet was resuspended in Tris HCL buffer pH 8, disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in 40 mM Tris HCL 0,2% n-lauryl sarcosine overnight at 4°C in a rocker platform. The soluble fraction was collected and loaded in a Nickel Column - HisTrap™. PA2705 was eluted in fractions of 2 mL in an ÄKTA pure purification system. A) Chromatogram shows a sharp peak between fractionation samples 6-10. B) Visualization of fractionation samples 6-10 after ÄKTA purification in a 12% polyacrylamide gel. Pre-induction: Soluble fraction prior expression of PA2705. FT: Flow through with unbound fractions of protein after Ni-NTA column. Wash A: Ni-NTA column wash with Buffer A prior ÄKTA purification. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).

3.7.2. His-tag detection of PA2705

After the protein purification procedures, the fractionation samples containing a purified protein with molecular weight in the region of 50kDa were assumed to have PA2705 in them (**Figure 3.22**). To confirm this, the immunodetection of this protein was carried out by western blotting as described in **Chapter 2, section 2.9.5.**

Wet-transfer of the fractionation sample containing the pure protein to a nitrocellulose membrane was performed followed by the detection of his-tagged protein on X-Ray film. As a positive control, the pNR1Cold1 expressing PA2705 from a cell extract was included. As seen in **Figure 3.22**, PA2705 was observed in the cell extract as well as in the fractionation sample containing the pure protein. As expected, this protein was not observed in the cell extracts expressing pCold alone, hence validating the purification of PA2705.

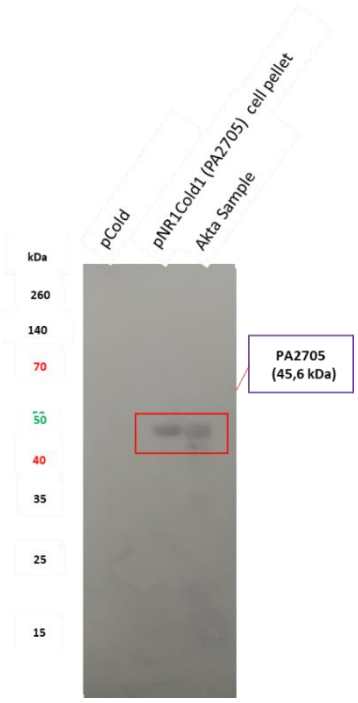


Figure 3.22. Western blot of PA2705.

His-tag detection using Penta-His HRP conjugate detected the presence of his-tag in PA2705 after ÄKTA purification. Negative Control: whole lysate carrying the pCold empty plasmid induced with IPTG 0.1 mM. Positive control: whole pellet cell lysate carrying the pNR1Cold1 expressing PA2705 induced with 0.1 mM IPTG.

3.7.3. Size exclusion chromatography/Gel filtration

Followed purification and subsequent confirmation of the presence of PA2705 by Western-blot, size exclusion chromatography (SEC) was performed as described in **Chapter 2, section 2.9.4**, hence, to enable further purification by molecular weight separation. A Superdex 200 10/300 GL column was used allowing a separation range for molecules with molecular weights between 10 000 and 600 000 Da. As seen in **Figure 3.23A**, a sharp peak in fraction 12 was observed corresponding to PA2705 as shown by polyacrylamide gel electrophoresis (**Figure 3.23B**). Based on a typical chromatogram from a function test of Superdex 200 10/300 GL (**Figure 3.23C**), it is suggested that PA2705 is found in one molecular state, more specifically, in an octameric or nonameric form, as its molecular weight is at 440.000 Da. Samples containing purified PA2705 were subjected to dialysis as described in **Chapter 2, section 2.9.4**

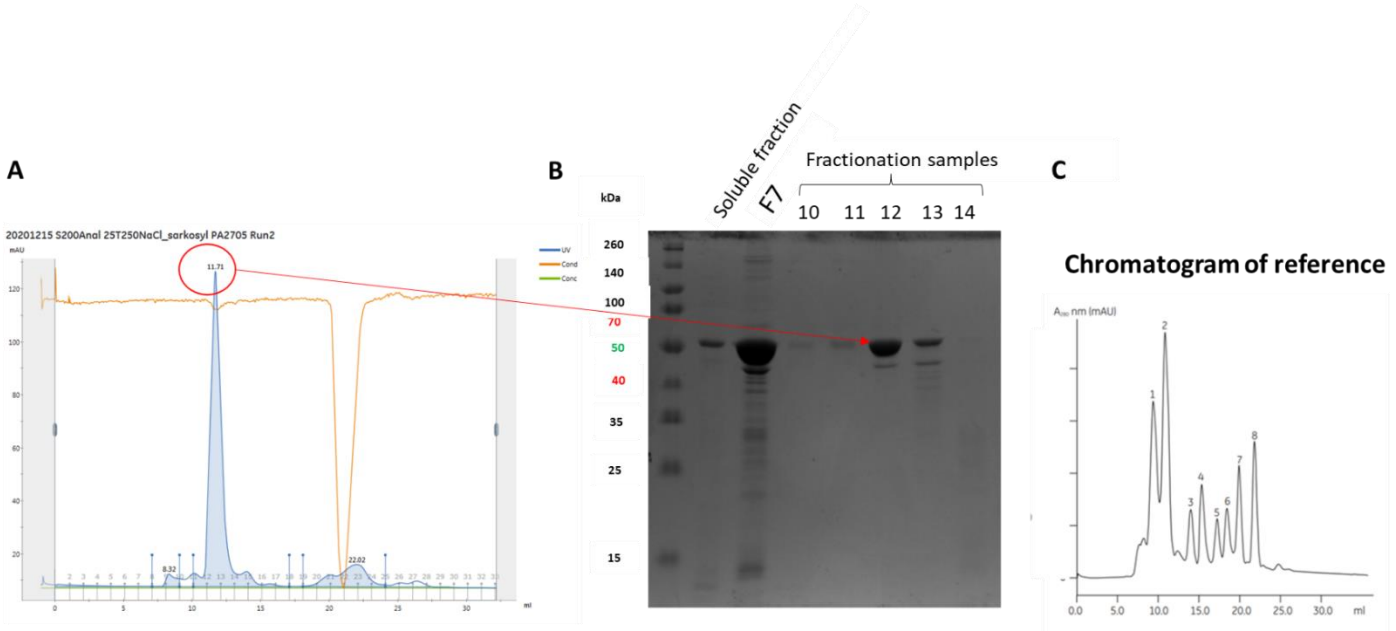


Figure 3.23. Size exclusion chromatography of PA2705.

Purified PA2705 was analysed by size exclusion chromatography in 25 mM Tris-HCl, NaCl 150 mM, 0,2% n-lauryl sarcosine pH 8.0. Chromatography was carried out on a Superdex 200 10/300 GL at a flow rate of 0.7 mL/min. The volume of protein injected was 50 µL with a fractionation volume of 1 mL. A) Chromatogram shows a sharp peak between fractionation examples 10-14 B) Visualization of fractionation samples 10-14 after size exclusion in a 12% polyacrylamide gel. Soluble fraction: Supernatant containing soluble PA2705 prior Ni-NTA column. F7: Purified PA2705 from fractionation sample 7 after ÄKTA purification. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa). C) Typical chromatogram from a function test of Superdex 200 10/300 GL. 1) Thyroglobulin (M, 669,000) 5 mg/ml. 2) Ferritin (M, 440,000) 0.4 mg/ml. 3) BSA (M, 67,000) 8 mg/ml. 4) ß-lactoglobulin (M, 35,000) 2.5 mg/ml 5) Ribonuclease A (M, 13,000) 5 mg/ml 6) Cytochrome C (M, 13,600) 1.5 mg/ml 7) Aprotinin (M, 6,512) 2 mg/ml 8) Vitamin B12 (M, 1,355) 0.1 mg/ml. Peak 5 and 6 are separated from each other only for difference in shape.

Considering that PA2705 alone may not bind the $P_{pqsA,,}$ the expression of its potential interacting partner PA2707 (reviewed in **Section 3.3)** was carried out as described in **Chapter 2. Section 2.9.1**. Besides, if PA2705 participates in multicomponent complexes (Whittaker & Hynes., 2002), it is not expected that PA2707 is the only participant missing in this regulation. Based on this, the expression of PA2707 was carried out in *E. coli*, which also contains WFA interacting proteins with ATPases, hence the use of culture extracts containing PA2707 may provide with required elements that perhaps allow protein-DNA interaction. *E. coli* BL21 carrying the pNR3Cold1 vector expressing PA2707 from cell pellets is shown in **Figure 3.24**. Western blot analysis detected the presence of PA2707 at 34 kDa (**Figure 3.25**). This band was absent when pCold was expressed alone. Solubility analysis showed that PA2707 remained in the insoluble fraction after sonication, whereas the use of mild-solubilizing agent n-lauryl sarcosine at 0.2% partially solubilized PA2707 (**Figure 3.26**)

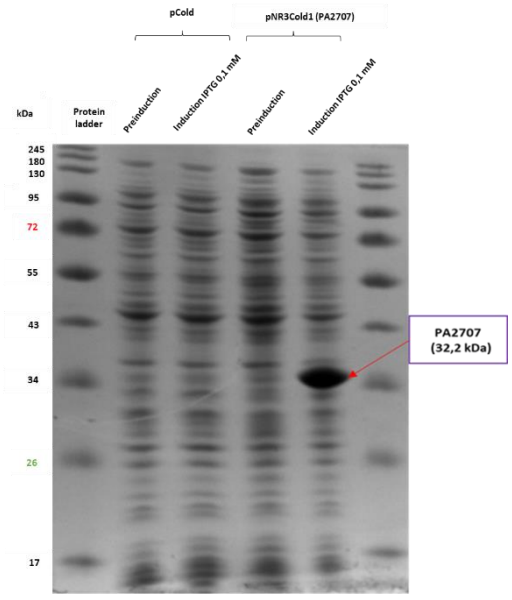


Figure 3.24. SDS-PAGE of overexpressed PA2707 in *E. coli* BL21.

E. coli BL21 harboring pNR3Cold1 was grown overnight at 30°C, 200 rpm. Once the reseeded cultured of an OD_{600nm} ~0.01 reached middle exponential phase (OD_{600nm} ~0.6), it was incubated on ice for 30 min and induced for 16 hours with IPTG 0.1 mM at 16°C, 200 rpm. The pellets were adjusted to an OD_{600nm}= 0.6, treated with 1XSDS, boiled at 100°C for 10 min and then separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Negative Control: whole lysate carrying the pCold empty plasmid pre-induced and induced with IPTG 0.1 mM. Samples were run in reference to the Colour Prestained Protein Standard, Broad Range (10-245 kDa).

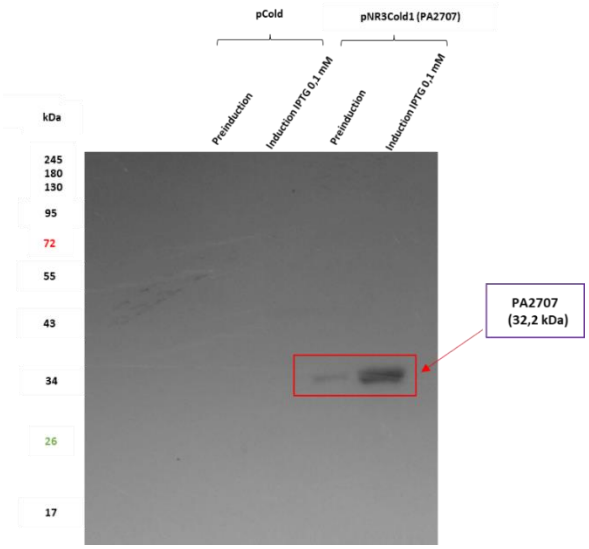


Figure 3.25. Western blot of overexpressed PA2707.

His-tag detection using Penta-His HRP conjugate detected the presence of his-tag in *E.coli* BL21(DE3) cell lysates carrying the plasmid pNR3Cold1 expressing PA2707. Negative Control: whole lysate carrying the pCold empty plasmid pre-induced and induced with IPTG 0.1 mM.

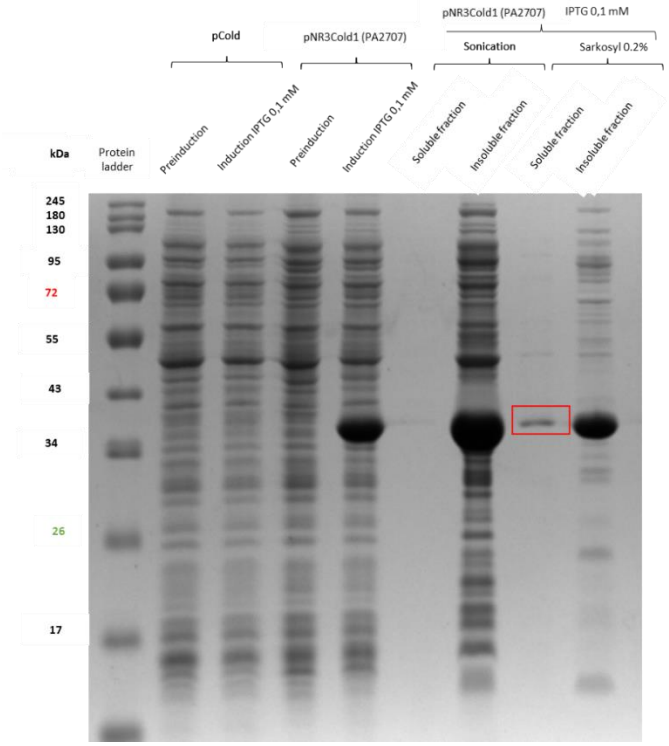
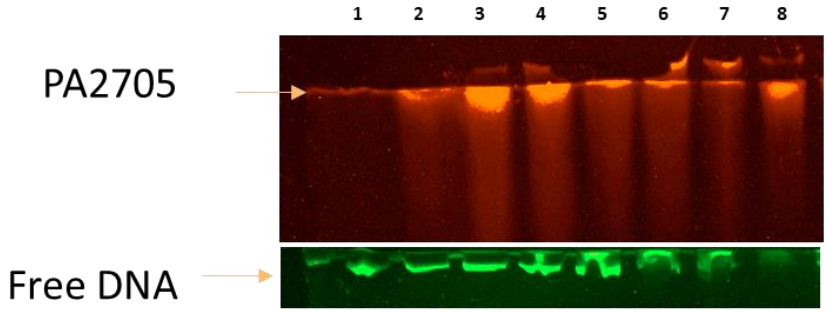


Figure 3.26. SDS-PAGE of solubility analysis of PA2707 in *E. coli* BL21.

E. coli BL21 harboring pNR3Cold1 was induced for 16 hours with IPTG 0.1 mM at 16^{o}C, 200 rpm. 1 mL samples at OD_{600nm}~ 0.6 were processed for solubility analysis. Soluble and insoluble fractions are shown from sonication, and sonication followed by solubilization with 0,2% n-lauryl sarcosine (sarkosyl). Samples were run in reference to the Colour Prestained Protein Standard, Broad Range (10-245 kDa).

3.7.4. Electrophoretic mobility shift assays (EMSA)

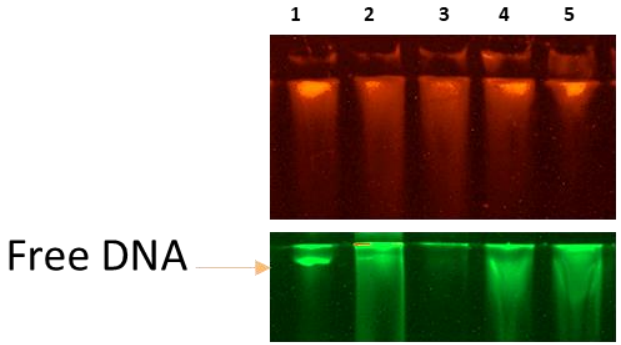
vWFA domains interact with divalent cations through a metal ion dependent adhesion site termed as the MIDAS motif, that are usually found interacting with magnesium (Ponting et al., 1999; Whittaker & Hynes. 2002). In prokaryotes, vWFA/ MIDAS domain has also been reported, suggesting to participate in protein-protein interaction and formation of protein complexes (Pelzmann et al., 2009). Albeit there are no reports of these proteins interacting with DNA, the finding of PA2705 binding the P_{pqsA} when PqsE was overexpressed was intriguing (Bates , PhD Thesis, 2013). To this end, a DNA shift assay was carried out as described in **Chapter 2, section 2.9.7**. PA2705 was quantified using BCA assay as described in **Chapter 2, section 2.9.6.** PA2705 stock concentration was at 250 µg/mL. The P_{pqsA} region corresponding to that used in Bates , PhD Thesis *(*2013) promoter pull-down was used at 500 fmol. Increasing concentration of PA2705 alone was added to the binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM Tris, pH 7.4) containing a constant concentration of the DNA fragment and including the addition of MgCl 5 mM. The loading of the sample was performed quickly to avoid loss of chemical equilibrium. As seen in **Figure 3.27**, PA2705 was present in all the condition tested and remained at the top of gel. Intriguingly, this protein is displaced upwards as its concentration is increased, suggesting that PA2705 interacted with the $P_{pqsA..}$ No other attempt was possible due to time limitations; therefore, this attempt did not allow to reach any conclusion. The addition of soluble supernatants containing PA2707, and 3 mM of ATP did not show any shift band in this experimental set up (**Figure 3.28**), Like above, this assay could not be optimized, hence, whether PA2705 binds P_{pqsA} remains to be elucidated.



Lane 1= DNA 500 fmol
Lane 2 = DNA 500 fmol + MgcL 5 mM
Lane 3 = DNA 500 fmol + MgcL 5 mM + PA2705 65ng
Lane 4 = DNA 500 fmol + MgcL 5 mM + PA2705 195 ng
Lane 5 = DNA 500 fmol + MgcL 5 mM + PA2705 390 ng
Lane 6 = DNA 500 fmol + MgcL 5 mM + PA2705 780 ng
Lane 7 = DNA 500 fmol + MgcL 5 mM + PA2705 1040 ng
Lane 8 = PA2705 1040 ng

Figure 3.27. Titration of the P_{pqsA} DNA with PA2705 protein.

Increasing amounts of PA2705 were mixed with 500 fmol of the P_{pqsA} DNA, incubated for 40 min in binding buffer with MgCl 5 mM, and then separated on a 7.5% nondenaturing polyacrylamide gel at 70 Volts in 0.5X TBE for 3 hours at 4^{o}C. The gel was stained with SYBR® Green EMSA stain (green) followed by SYPRO® Ruby EMSA stain (red), components of the Electrophoretic Mobility-Shift Assay Kit (Thermofisher). After each staining, the image was documented using a Biorad Gel Doc XR Imaging System and the digital images pseudocoloured and overlaid.



Lane 1= DNA 500 fmol + soluble fraction of induced pCOLD empty vector
Lane 2 = DNA 500 fmol + soluble fraction PA2707
Lane 3 = DNA 500 fmol + MgcL 5 mM + ATP 3 mM + soluble fraction PA2707
Lane 6 = DNA 500 fmol + MgcL 5 mM + ATP 3 mM + soluble fraction PA2707 + PA2705 780 ng
Lane 7 = DNA 500 fmol + MgcL 5 mM + ATP 3 mM + soluble fraction PA2707+ PA2705 1040 ng

Figure 3.28. Titration of the P_{pqsA} DNA with PA2705 protein and soluble extract of PA2707.

The soluble extract containing expressed PA2707 was incubated with increasing concentration of pure PA2705 and mixed with 500 fmol of the P_{pqsA} DNA for 40 min in the presence of MgCl 5 mM and ATP 3 mM. Samples were separated on a 7.5% nondenaturing polyacrylamide gel at 70 Volts in 0.5X TBE for 3 hours at 4^{o}C. The gel was stained with SYBR® Green EMSA stain (green) followed by SYPRO® Ruby EMSA stain (red), components of the Electrophoretic Mobility-Shift Assay Kit (Thermofisher). After each staining, the image was documented using a Biorad Gel Doc XR Imaging System and the digital images pseudocolored and overlaid.

3.8. Discussion

In this chapter, a DNA promoter pull-down was used as reference in the search for a protein candidate which could mediate the action of PqsE. PA2705 was chosen as it was the only protein absent when *pqsE* was mutated and bound the P_{pqsA} when *pqsE* was overexpressed (**Table 3.1**). An in-frame deletion mutant of PA2705 in PAO1-L confirmed its role in the modulation of *pqsA* expression, however, the use of two different pMiniCTX::P_{pqsA}-lux reporters challenged to discern whether PA2705 had a positive or a negative effect on this promoter(**Figure 3.7** and **Figure 3.7**). Sequence analysis (**Figure 3.5**) along with gene expression assays **(Figure 3.8** and **Figure 3.9)** demonstrated that the interruption of the -311 r*hlR-box* in one of the reporters was responsible of these changes and revealed its pivotal role in the accurate understanding of the regulation of *pqsA*.

On one hand, it contributed to elucidate that PqsE is an activator of *pqsA* expression in the absence of *rhlR* and/or other elements controlled by this regulator **(Figure 3.8).** This result agreed to that observed by Hazan et al, 2010., in which the constitutive expression of *pqsE* in a *rhlR* mutant increased the transcriptional activity of a P_{pqsA} GFP-based transcriptional reporter, indicating that the PqsE negative control of the activity of the P_{pqsA} is dependent on RhlR. Strikingly, the fact that *pqsE* still downregulated the P_{pqsA} in the absence of the *rhlR-box* in PAO1 wild type suggested that RhlR is not essential in this regulation (**Figure 3.8**). This is because the RhlR binding site was absent. One explanation for this event could be that the PqsE-RhlR regulation towards *pqsA* can take place in an alternative *lux-box* when the *rhlR-box* is absent, which arises the question to whether the first *rhl-box* placed at -151 from the *pqsA* TSS (**Figure 3.1**) may act as an alternative regulatory point at the transcriptional level. On the other hand, the analysis of the *rhlR-box* contributed to unveil that the PqsE-mediated repression of *pqsA* occurs in a post-transcriptional event, which was evident when the translation of *pqsA* in the absence

(uninduced *pqsE* Ind) and presence of *pqsE* (*pqsE* Ind + IPTG) was similar to that observed at the phenotypical level (**Figure 3.11** and **Figure 3.10).** Furthermore, it could be concluded that PA2705 as well as PA2704 are activators of *pqsA* (**Figure 3.7**). Noticeably, both mutations had a similar impact towards this promoter with some small variations (**Figure 3.7**). This suggested that these genes were related, which can be expected due to their contiguous localization in the genome (Galperin & Koonin, 2000; Junier & Rivoire, 2016). The temporary *pqsA* wild type levels observed in P*A2705* mutant could be a result of a regulation mediated by PA2704. This is because the latter encodes for an AraC transcriptional regulator that appears to regulate the expression of the PA2705-PA2707 operon (**Figure 3.16**) and may perhaps control the expression of other genes involved in this regulation.

The fact that PA2705 modulated the expression of *pqsA* led to analyze whether it was under the control of PqsE. Since the PqsE regulation showed to occur at the post-transcriptional level (**Figure 3.11**), this regulation was analyzed using a translational reporter. In this scenario, PqsE was shown to positively regulate *PA2705* (**Figure 3.12B)** explaining why PA2705 was found in the pull-down experiments of the P_{pqsA} only when *pqsE* was overexpressed (**Table 3.1**). It was puzzling to observe however, that at the time point at which the pull down was carried out (OD_{600nm} 0.5), PA2705 which is an activator of *pqsA* (**Figure 3.7**) was binding the P_{pqsA} when *pqsE* was overexpressed *(pqsE* Ind + IPTG) (**Table 3.1**). This interrogates (i) whether PqsE may participate in both, activation, and repression of *pqsA* and (ii) whether at this $OD_{600nm,}$ corresponding to the maximum expression levels of *pqsA* (**Figure 3.2),** may yet not show the protein profile that participate in the repression of this promoter. Yet, it cannot be discarded that PqsE by activating *PA2705*, may perhaps control the expression of other genes that could ultimately repress *pqsA.*

Unfortunately, *PA2705* was dispensable for the PqsE mediated repression of *pqsA* (**Figure 3.13**) which indicates that PA2705 is not the candidate

that transduces the action of PqsE to this promoter, or that *P. aeruginosa* can adapt and replace PA2705 in this role. Remarkably, no other proteins were binding the P_{pqsA} only when *pqsE* was overexpressed *(pqsE* Ind + IPTG) (**Table 3.1**). This recalls the above interrogate described in number (ii), or perhaps propounds that the PqsE-mediated repression of *pqsA* could be exerted not by activating but rather repressing an activator of *pqsA*. In this scenario, the candidate protein is likely to bind the P_{pqsA} only when *pqsE* is mutated (uninduced *pqsE* Ind). Under this condition, the proteins binding the P_{pqsA} were the hypothetical protein PA1894, ParR, AmgR, PhoB and Dnr. PA1894 is found adjacent to the operon PA1895-1897. Ding et al., (2018) trough chromatin immunoprecipitation analysis found QscR to regulate global QS through only regulation of this single operon. Though PA1894 has a separate transcription start site from the PA1895-1897 operon , microarray analysis showed PA1894 together with PA1895-PA1897 differentially expressed in strains deficient in 3OC12-HSL and C4-HSL production (Martin Schuster et al., 2003a; Wagner et al., 2003). Besides, as described in **section 3.2**, the presence of regulators belonging to two-component systems ParR, AmgR and PhoB found binding the P_{pqsA} only when *pqsE* was absent (uninduced *pqsE* Ind) indicated that PqsE orchestrates the regulation of several transcriptional regulators and suggests that the repression of *pqsA* is more complex and perhaps involves the presence of multiple participants that impact the expression of *pqsA* in a concerted manner.

It was clear however, that PA2705 plays a role in the *pqs* regulation, as the levels of PQS were reduced in the absence of this gene (**Figure 3.14**). Nevertheless, these results were somehow masked by the fact that pME6032 alone had a great impact on this phenotype, something that deserves further investigation. In addition, PA2705 showed to promote virulence in *P. aeruginosa* through its positive impact on pyocyanin production (**Figure 3.15**). This further supports the close relationship between PA2705 and PqsE, but it also suggests that it may interact with other areas of the QS network that contribute to the control of pyocyanin

production (Gallagher et al., 2002; Farrow et al., 2008; Mavrodi et al., 2001, 2010; Rampioni et al., 2010; Recinos et al., 2012; Higgins et al., 2018). Indeed, the expression of *PA2705* was controlled by the *las/rhl* system (**Figure 3.17**), supporting the latter observation, however, this gene was not among the statistically differentiated expressed genes in the QS regulome performed at Schuster et al., (2003). Moreover, whether *PA2705* was differentially expressed in the QS regulome performed at Wagner et al., (2003) could not be possible due to an unfunctional link that failed to show the full list of QS regulated genes. The fact that in the pull down PA2705 was binding the P_{pqsA} when *pqsE* was overexpressed by IPTG, suggested that PA2705 can interact with DNA and particularly with the P_{pqsA}. To test whether PA2705 interacts with this promoter required the purification of the PA2705 protein. Strong promoter system used to express recombinant proteins increase the metabolic burden which increases the probability of protein aggregation into inclusion bodies (Chrunyk et al., 1993; Rodríguez-Carmona et al., 2010). The use of lower temperatures has shown to decrease their formation (Gaberc-porekar et al., 2005), however the expression of PA2705 at a low temperature of 16^{o}C did not prevent the location of this protein in the insoluble fraction (**Figure 3.19A**). Based on this, the solubilization of PA2705 was achieved using the anionic mild biosurfactant n-lauryl sarcosine. This agent was chosen because it was the most effective in solubilising PA2705 in this experimental set-up (**Figure 3.19B**). In addition, it is a non-denaturing surfactant and has been shown to retain the secondary structure of native proteins, it does not interfere with spectroscopic concentration measurement, and it can be removed by simple dialysis (Gaberc-porekar et al., 2005; Chisnall et al., 2014). N-lauryl sarcosine was added to the samples at a final concentration of 0.2% to keep it below its critic micellar concentration (CMC) at all the temperatures used for this purpose (Gad et al., 1997; Gaberc-porekar et al., 2005). PA2705 was successfully purified after ÄKTA purification and size exclusion (**Figure 3.21** and **Figure 3.23**). The latter

separates the protein sample content by molecular weight and it is effective in removing traces of nucleic acid contamination, likely to be present when using n-lauryl sarcosine solubilization (Chisnall et al., 2014). Purified PA2705 was dialyzed against size exclusion buffer to remove n-lauryl sarcosine from the sample.

In this work, attempts to elucidate whether PA2705 or PA2705 together with PA2707 interacted with the P_{pqsA} could not be achieved (**Figure 3.27 and Figure 3.28**) due to the extensive optimizations that are usually required (Hellman & Fried, 2007) and the limited time to execute them caused by the COVID-19 restrictions. Albeit no apparent DNA binding domain was present in PA2705, the increasing addition of this protein to the P_{pqsA} DNA seemed to cause a protein-DNA shift in the EMSA analysis (**Figure 3.27)**, however, this was not clearly observed due to the poor migration of the protein towards the anode. The fact that the protein as well as the target DNA did not migrate further even with extended electrophoresis time could be due to the pH used. Tris buffers change their pH at different temperatures, becoming higher at lower temperatures, therefore the buffer used at 4^{o}C could increase the negative charge of PA2705 and hence run closely to the DNA molecule (Carey, 1988). In addition, it was not clear whether the DNA bands became distorted due a band-shift or to other experimental conditions. Similarly, smeared bands of DNA in the presence of cell extracts expressing PA2707 (**Figure 3.28**) may reflect high levels of conductivity of the sample, which can be enhanced in the presence of divalent ions like magnesium and ATP (Hellman & Fried., 2007; Stickle, Liu, & Fried., 1994). Important to consider is that albeit the use of n-lauryl sarcosine was effective to solubilize PA2705 (**Figure 3.19B**), and considering that purified proteins using this detergent at the same concentration like in this work had exhibited relatively high biological activity (Gaberc-porekar et al., 2005), n-lauryl sarcosine can bind proteins tightly and residual content may remain in the sample after dialysis, which could ultimately impact on the native state and hence the bioactivity of this protein.

(Burgess, 1996; Chisnall et al., 2014). Therefore, it becomes paramount to test whether PA2705 is properly folded and whether it is active enough to ultimately validate the interaction with the P_{pqsA}.

Protein-protein interaction analysis using STRING database (https://string-db.org) suggested PA2707 the most likely candidate to interact with PA2705. Nevertheless, since PA2707 was not present in the P_{pqsA} pull down indicates that this protein may not bind P_{pqsA} or DNA, therefore it was hypothesized other elements are required for the final repression of this promoter. Based on this, *E. coli* soluble cell extract containing expressed his-tagged PA2707 was used, hence, expecting that those missing molecules are present in this bacterium. This rationale was based on the fact that like PA2707, the RavA protein which belongs to the subfamily of the MoxR AAA family act together with ViaA, a protein containing vWFA domain in *E. coli* (Snider et al., 2006). Several studies have indicated that the RavA-ViaA participates in different roles in aerobic and anaerobic environments. For example, the RavA-ViaA has been shown to interact strongly with the inducible lysine decarboxylase enzyme LdcI (Snider et al., 2006; El Bakkouri et al., 2010), a major acid stress response protein in *E. coli* (Kanjee et al., 2011). The *ravA-viaA* genes are regulated by the anaerobic transcriptional regulator Fnr under oxygen-limited conditions (Wong et al., 2017). In the same work, it was elucidated that ViaA interacts with the flavin-containing subunit FrdA, which is part of the of the anaerobic respiratory complex fumarate reductase Frd (Cecchini et al., 2002). Albeit in *P. aeruginosa* the RavA-ViaA is not present in the accessory genome, the structural similarities between RavA-ViaA and PA2705-PA2707 suggest participation in related roles in *P. aeruginosa* and perhaps their binding partners could also be found in *E. coli* cell extracts. Clearly, however, this approach has the drawback of missing some parts of the regulatory network that are not present, or are different in *E. coli*, therefore, finding the specific interacting partners of PA2705, in particular when *pqsA* is being

repressed, will still be necessary to ultimately corroborate whether PA2705 interacts with the P_{pqsA}

3.9. Conclusion and future directions

Overall, this chapter showed that PA2705 is involved in the regulation of *pqsA* but is not a PqsE effector protein in this regulation as originally hypothesised. It also shows that PA2705 is integrated within the QS regulatory cascades at different levels although the nature of this integration remains to be elucidated.

Previous work showed a potential link between PA2705 and PA2707 (Snider et al., 2006; Snider & Houry., 2006) and hence it would be interesting to validate whether these proteins can interact with each other. Indeed, since vWFA domain is a metal-binding domain often participating in protein-protein interactions and in multiprotein complexes (Whittaker & Hynes., 2002), it would be expected that not only PA2705 and PA2707, if they do, are the only participants required to finally interact with the P_{pqsA}. Based on this, whether other candidate partners are required for this interaction needs elucidation. One mechanism to achieve that is by protein crosslinking. In this set up, the use of n-lauryl sarcosine for the solubilization of PA2705 complexes can still be used however, an extra step would be required for the complete removal of this agent from the samples, which could be achieved through the use of a cation exchange resin (Burgess. 1996). The protein-protein interaction (PPI) STRING database (https://string-db.org) integrates both known and predicted PPIs and can be applied to predict functional interactions of proteins. With the highest level of confidence (Interaction score >0.9), PA2707 is the only predicted functional partner of PA2705, this was because its putative homologous genes have not only been reported to be close in the genome, but have also shown to co-occur, interact and be co-expressed. In addition, with high confidence (Interaction score >0.7) and based on curated database, other candidates

were *murB* and *murD*, that encode for a UDP-N-acetylpyruvoylglucosamine reductase and a UDP-N-acetylmuramoylalanine-D-glutamate ligase, respectively. Both enzymes are reported to participate in the first stage of the formation of peptidoglycan, event that occurs in the cytoplasm and that consist on the formation of N-acetylglucosamine-N-acetylmuramyl pentapeptide (El Zoeiby et al., 2001). Interestingly, with medium level of confidence (Interaction score >0.4) PA2705 and PA2707 had a predicted functional link with PA3286, a beta-ketodecanoyl-[acyl-carrier-protein] synthase that participates in the shunt for de novo fatty acid biosynthesis (Yuan, Leeds, & Meredith., 2012). This could reflect a relation with the biosynthesis of pyoverdine as well as AHL and therefore the *las-rhl* system (Nadal et al., 2012). Finally, albeit PA2706 is the closest gene to PA2705 in the operon, the fact that PA2706 is only present in some *Pseudomonas* strains indicates that was acquired later in the evolution of *Pseudomonas* and that its role may be specifically related to PA2705 and PA2707. Therefore, it is paramount to work on elucidating the interactive partners of PA2705 in a manner that unveil its molecular relationships with QS that could more accurately place PA2705 within in the QS regulatory cascade as well as validate its role in the regulation of *pqsA.*

4 Chapter Four: *nirQ*, the denitrification regulatory protein as a potential repressor of the *pqsA* promoter during late growth

4.1 Introduction

As seen in **Chapter 3**, PqsE was found to repress the *pqsA* promoter even in the absence of PA2705, the hypothetical protein that became a potential candidate to mediate the action of this effector at the early stage of growth of *P. aeruginosa*. These observations led to conclude that PA2705 was dispensable for this regulation and that it was not required for the impact of PqsE on *pqsA* expression early in growth. In parallel, a second promoter pull down of P_{pqsA} had been performed by Dr. Rampioni at the University of Nottingham, aiming to elucidate candidates which mediate the action of PqsE during the late stage of growth of *P. aeruginosa.* In the following section, a summary of this work is covered to provide the appropriate background for the experimental work described in this chapter.

4.2 Promoter pull down of P_{pqsA} at a late stage of growth of *P. aeruginosa*

The promoter pull down was carried out in *P. aeruginosa PAO1*-N (*pqsE* Ind) using cytoplasmic cell extracts harvested from stationary cultures at OD_{600nm} 1.5 in combination with the same *pqsA* promoter region as in **Figure 3.2**. In this work, a few proteins were found binding the *pqsA* promoter. Among them, six proteins were chosen by Dr. Rampioni for further investigation to assess whether they were activators or repressors of *pqsA* and whether there was a link between those proteins and PqsE that allows the final repression of *pqsA*. The six candidate genes PA0459 (*clpC*), PA0779, PA3831 (*pepA*), PA5060 (*phaF*), PA0520 (*nirQ*) and PA4843(*gcbA*) were selected for further analysis due to their role in *P. aeruginosa* and their presence or absence when *pqsE* was uninduced or induced (**Figure 4.1**), which encouraged a link to the regulation of *pqsA* as well as to PqsE.

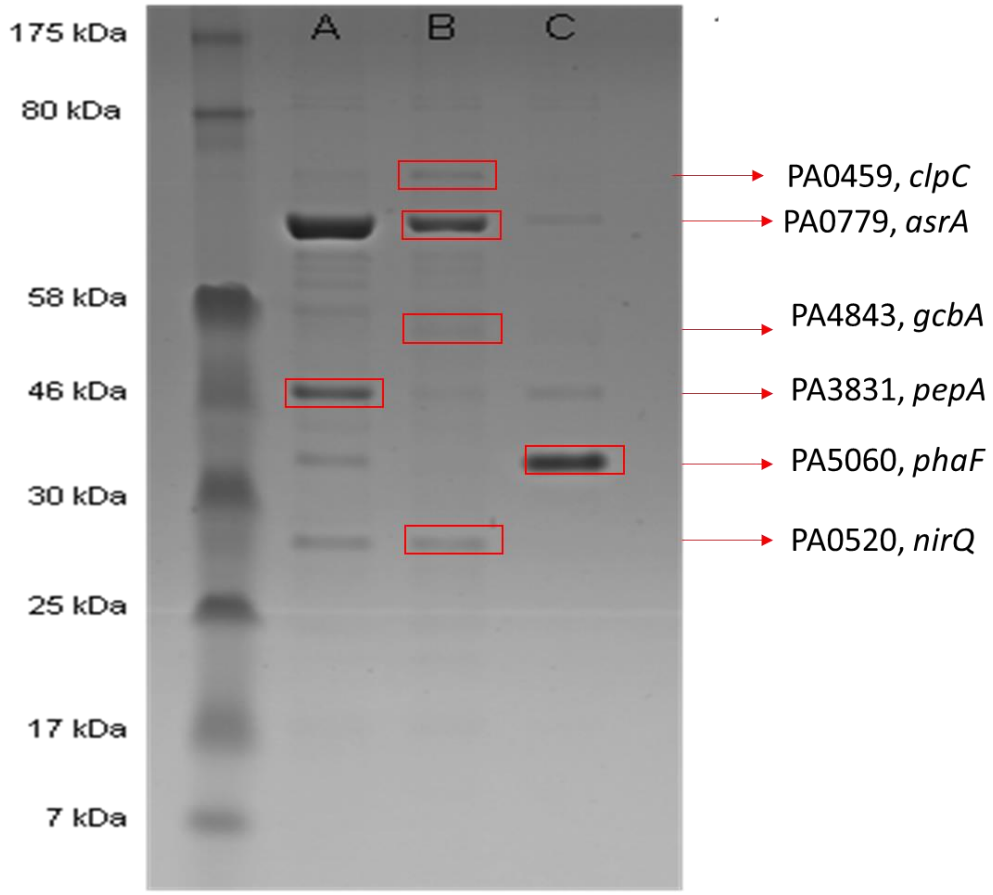


Figure 4.1 The effect of PqsE on the protein profile at the *pqsA* promoter (G. Rampioni).

Promoter pulldowns were performed using the pqsA promoter region in combination with cell extracts harvested from PAO1-N wild type, PAO1-N *pqsE* Ind not expressing *pqsE* (- IPTG) or overexpressing *pqsE* (+ IPTG). Cell extracts were harvested at OD_{600nm} 1.5. Protein bands analysed by MALDI-TOF, or LCMS-MS are enumerated. 1: PAO1-N bands; 2: PAO1-N *pqsE* Ind – IPTG; 3: PAO1-N *pqsE* Ind + IPTG.

Table 4.1. MALDI-TOF/LCMS-MS identified proteins binding to P_{pqsA}

Strain Label	Strain description	Subcellular Localization	Function	Reference
PAGR31	ΔPA0459 (*clpC*)	Cytoplasmic *	Probable ClpA/B protease ATP binding subunit	Winsor et al.2016
PAGR32	ΔPA0779 (*asrA*)	Cytoplasmic *	Cellular/stress response/adaptation to antibiotics	Winsor et al.2016, Kindrachuk et al,2011
PAGR33	ΔPA3831 (*pepA*)	Cytoplasmic*/Outer Membrane Vesicle/Extracellular	Leucine aminopeptidase/Epithelial and cell cytoxicity	Winsor et al.2016, Hauser at al, 1998
PAGR34	ΔPA0520 (*nirQ*)	Cytoplasmic *	Regulatory protein/Anaerobic respiration /Denitrification	Winsor et al.2016, Arai et al, 1994
PAGR35	ΔPA4843 (*gcbA*)	Cytoplasmic *	Two component system. Promotes cell adhesion	Winsor et al.2016, Petrova et al,2014
PAGR36	ΔPA5060 (*phaF*)	Outer Membrane Vesicle/ Cytoplasmic	Poly(hydroxyalcanoate) granule associated protein (phasin)/Biofilm formation	Winsor et al.2016, Huse et al, 2013

* Computationally predicted by PSORTb V3.0

A brief description of these candidates is presented below:

The first candidate PA0459 (*clpC*) is a predicted chaperone protein of 94.1 kDa and encodes a probable ClpA/B protease (**Table 4.1**). This family belongs to the AAA+ (ATPase associated with diverse cellular activities) superfamily and form hexametric rings that utilize energy from ATP hydrolysis to unfold substrates, translocate them through a central pore, and deliver them to an associated peptidase, ClpP (Hanson & Whiteheart., 2005; Baker & Sauer., 2012). Caseinolytic peptidases (ClpPs) proteolytic enzymes are conserved in bacteria and eukaryotes (Gottesman et.al, 2014). ClpC from *P. aeruginosa* has 70% similarity to a homolog of *Bacillus subtilis clpC.* Insertional mutation in *clpC* resulted in impaired tolerance to salt and heat shock (Kruger, Volker, & Hecker., 1994), suggesting that these proteins participate in heat shock tolerance, a role that has also been reported for *clpB* in *E. coli* (Squires et al., 1991). No apparent role has been reported for *clpC* upon the regulation of *pqsA*, albeit transcriptome analysis in PAO1 *pqsE* Ind strain performed previously in our group (unpublished data) showed that *clpC* was downregulated at 8 hours of growth when *pqsE* was overexpressed. This observation may explain why in the *pqsA* promoter pull down the gel band containing ClpC was not present when *pqsE* was overexpressed (**Figure 4.1**).

The second candidate PA0779 (*asrA*) encodes an alternate Lon protease belonging to the ATPase family associated with various cellular activities (AAA) (**Table 4.1**). It has been reported to participate in the protection from nitric oxides (NO) by regulating the *fhp* promoter, which responds to reactive nitrogen species and can protect cells against nitrosative stress. (Koskenkorva et al., 2008). In addition, *asrA* has been shown to participate in the resistance of *P. aeruginosa* to aminoglycosides by inducing the expression of the heat shock genes *htpG, ibpA, groES, clpB, dnaJ and hsIV*, and confer short-term protection towards lethal concentrations of tobramycin (Kindrachuk et al., 2011). The impact of *asrA* towards heat shock genes like *clpB* a as well as the potential role of *clpC* in the heat shock response cannot discard a plausible link between *asrA* and *clpC* in *P. aeruginosa.* Indeed, protein-protein interaction between ClpP and Lon ATPase protein has been reported in *E. coli* (Butland et al., 2005). Similar to that reported for *clpC*, transcriptome analysis in the PAO1 *pqsE* Ind strain showed that *asrA* was downregulated when *pqsE* was overexpressed (unpublished data), which can explain why the gel band containing AsrA in the *pqsA* promoter pull down is not present when pqsE was overexpressed (**Figure 4.1**).

The third candidate PA4843 (*gcaB*) corresponds to a two-component system response regulator (**Table 4.1**). It acts as a diguanylate cyclases (DGCs) and albeit it is highly similar (74.12%) to GcbA from *P. fluorescens*, GcbA does not promote biofilm formation in *P. aeruginosa*, and it is rather involved in the regulation of the initial stages of *P. aeruginosa* attachment to surfaces. This is because it participates in the switch from polar attachment to longitudinal attachment by modulating c-di-GMP levels during planktonic growth. In other words, it participates in the transition from reversible to irreversible attachment (Petrova, Cherny, & Sauer., 2014). SigX, an extra cytoplasmic sigma factor induced by sucrose, showed to increase c-di-GMP levels by targeting *gcbA* (Bouffartigues et al., 2014) and therefore increase biofilm formation in *P. aeruginosa* H103. Interestingly, mutation of *sigX* exhibit increased transcription of *asrA* (Gicquel et al., 2013) which, as just described above, was also binding

the *pqsA* promoter (**Table 4.1**), suggesting an interconnection between these pathways that ultimately are affecting *pqsA* expression. In fact, like with *clpC* and *asrA,* the same transcriptome analysis in PAO1-L *pqsE* Ind showed that *gcaB* was also downregulated when *pqsE* was overexpressed by the addition of IPTG (Unpublished data).

The fourth candidate PA3831 (*pepA*) or *phpA* (a *P. aeruginosa* homologue of *pepA*) encodes a leucine aminopeptidase related to transcription, RNA processing and degradation (**Table 4.1**). It controls alginate production due to its inhibitory effect on *algD* expression (Woolwine, Sprinkle, & Wozniak. 2001). DNA-affinity chromatography and MALDI-TOF analysis performed in *P. aeruginosa* showed AsrA and PepA bound to the promoter of *lasR* (Longo et al., 2013), suggesting that they may interact with DNA or at least be related to DNA binding proteins that participate in the regulation of QS-controlled promoters.

The fifth candidate PA5060 (*phaF*) encodes a regulatory protein associated with polyhydroxyalkanoate (PHA) biogenesis (**Table 4.1**) PHA accumulates as a carbon and energy source and under limiting nutrient conditions in the presence of an excess carbon source (Prieto et al., 1999). In *P. aeruginosa* PAO1, PHA is not only produced from fatty acids but also from substrates such as glucose, and gluconate (Timm & Steinbüchel., 1992). A study in *P. putida* elucidated that the physiologic role of PhaF is broaden in this bacterium (Galán et al., 2011). Indeed, as reported in Longo et al., (2013) besides AsrA and PepA, PhaF was also binding the promoter of *lasR,* suggesting that these candidate genes not only share common regulatory points, but may also participate in the regulation of *pqsA* as well as other QS branches in *P. aeruginosa* in conjunction or in a similar way. Indeed, Motif Alignment and Search Tool (MAST) algorithm identified in *Pseudomonas chlororaphis* strain 23, a *phz-box* like sequence 346 bp upstream of *phaF* ATG start codon, suggesting that is regulated by the LuxR-AHL QS system Phz (Shah et al., 2020). Noticeable, *phaF* was the only protein found binding the promoter of *pqsA* when *pqsE* was overexpressed (**Figure 4.1**), nevertheless, the transcriptome analyses in PAO1 *pqsE* Ind indicated that when

PqsE was overexpressed, *phaF* was repressed after 8 hours of growth and that this repression strongly augmented after 24 hours (Unpublished data).

The last candidate gene PA0520 (*nirQ*), corresponds to a denitrification regulatory protein encoding a putative ATP-binding protein nitrite reductase (**Table 4.1**). It participates in the anaerobic reduction of nitrite and nitric oxide in order to avoid accumulation of highly cytotoxic intermediate, NO (Arai ., 2011). NirQ is regulated by the two component system that respond to nitrate NarX and the dissimilative respiration regulator DNR (Arai, Kodama, & Igarashi., 1999; Härtig et al., 1999; Schreiber et al., 2007). Different from the other 5 candidate genes, the relationship between denitrification and the *pqs* system has been widely demonstrated. It is known that the *pqsABCDE-phnAB* operon is transcriptionally repressed during anaerobic growth (Filiatrault et al., 2005; Wu et al., 2005), whereas denitrification is repressed by PQS, mainly through PqsR and PqsE (Toyofuku et al., 2008; Rampioni, et al., 2010; Rampioni et al., 2016). Hence, in the promoter pull down (**Figure 4.1**), the overexpression of *pqsE* by IPTG likely downregulated *nirQ*, which could explain why NirQ was absent when PqsE was overexpressed by the addition of IPTG.

A few additional proteins were also identified, but not studied further. Among them, LasR, RhlR and PqsR were bound to the *pqsA* promoter when *pqsE* was mutated and were absent when *pqsE* was overexpressed. This is interesting as to date, RhlR but not LasR has shown interact with the *pqsA* promoter, whereas binding of PqsR has been previously reported (Xiao et al., 2006). In frame mutation of the genes encoding for the six candidate proteins ClpC, AsrA, GcbA, PepA, PhaF and NirQ both within the wild type and the PAO1 *pqsE* Ind genetic background were previously constructed in the *P. aeruginosa* PAO1-N subline, and the P_{pqsA}-lux reporter studies were performed in these mutants to elucidate the effects of the mutations on the transcriptional activity of P_{pqsA}. Surprisingly, no significant differences in the transcriptional activity of P_{pqsA} were observed between the single mutants and the parent strain. In addition, the uninduced *pqsE* Ind mutation showed elevated levels of transcriptional activity of P_{pqsA}

when compared to that of the parental strain, whereas following induction of *pqsE* almost abrogated this activity. This result evidenced the negative role that PqsE exerts toward P_{pqsA} (Rampioni et al., 2010). Intriguingly, all the mutants created within the *pqsE* Ind condition had reduced activity of P_{pqsA} when compared to that in the PAO1 *pqsE* Ind parent strain, suggesting that the candidate genes are inducers of *pqsA* expression, and that this is only apparent when *pqsE* is not transcribed due to the absence of IPTG. The fact that this effect was not evident in the strains carrying the sole mutation of the candidate genes was attributed to a masking effect exerted by the presence of the wild type *pqsE.* In these strains, *pqsE* is still transcribed as a part of the *pqsABCDE* operon, so that in the absence of a positive regulator of *pqsA*, the PqsE-mediated repression of *pqsA* is decreased as a consequence of the initial decreased transcription of *pqsABCD*E, hence reflecting wild type homeostatic levels of P_{pqsA} even in the absence of the candidate genes.

4.3 Aims of the chapter

Previous gene expression analysis suggested that the six gene candidates participate in the regulation of *pqsA*, however, these analyses were performed with the short version of the P_{pqsA}-*lux* reporter ($P_{pqsA\ short}$-*lux*) which carries the deletion of the *rhlR-box.* In this chapter, a new analysis is performed by using the $P_{pqsA\ long}$-*lux* reporter, which incorporates the same region of P_{pqsA} used for the promoter pull down and that carries the *rhlR-box*, hence aiming to elucidate whether there is a potential candidate that could mediate the action of PqsE towards P_{pqsA}. More specifically, this chapter aims to:

* Elucidate the impact of the candidate genes towards the transcription of *pqsA*
* Select a potential candidate for in depth analyses in the PAO1-L subline

The latter statement, brings along the following sub-aims:

- Validate the regulatory link between the potential candidate and *pqsA* in PAO1-L
- Unveil whether the candidate is under the control of PqsE
- Unravel whether the candidate is essential for the PqsE-mediated repression of *pqsA*
- Validate whether the candidate binds the *pqsA* promoter.

4.4 Results

4.4.1 Impact of the candidates towards the transcription of *pqsA*

A new analysis was performed with the candidate genes *clpC, asrA, pepA, nirQ, gcbA* and *phaF* to establish the impact towards transcription of P_{pqsA} using the $P_{pqsA\text{-}long}$-*lux* in PAO1-N. Results are presented in **Figure 4.2.** To begin with, the transcriptional activity of P_{pqsA} in the wildtype condition peaked at 7 hours of growth. In addition, mutation of *clpC, pepA* and *phaF* did not have an impact on the transcriptional activity of P_{pqsA} when compared to the parent strain, whereas the mutation of *asrA* and *gcbA* increased the transcriptional activity of P_{pqsA}, suggesting a repressor role towards the expression of *pqsA.* Surprisingly, mutation of *nirQ* caused a major upregulation in the transcriptional activity of P_{pqsA} suggesting that *nirQ* is a strong repressor of P_{pqsA} and may perhaps be closely related to the regulation of this operon and importantly connected to the effector PqsE.

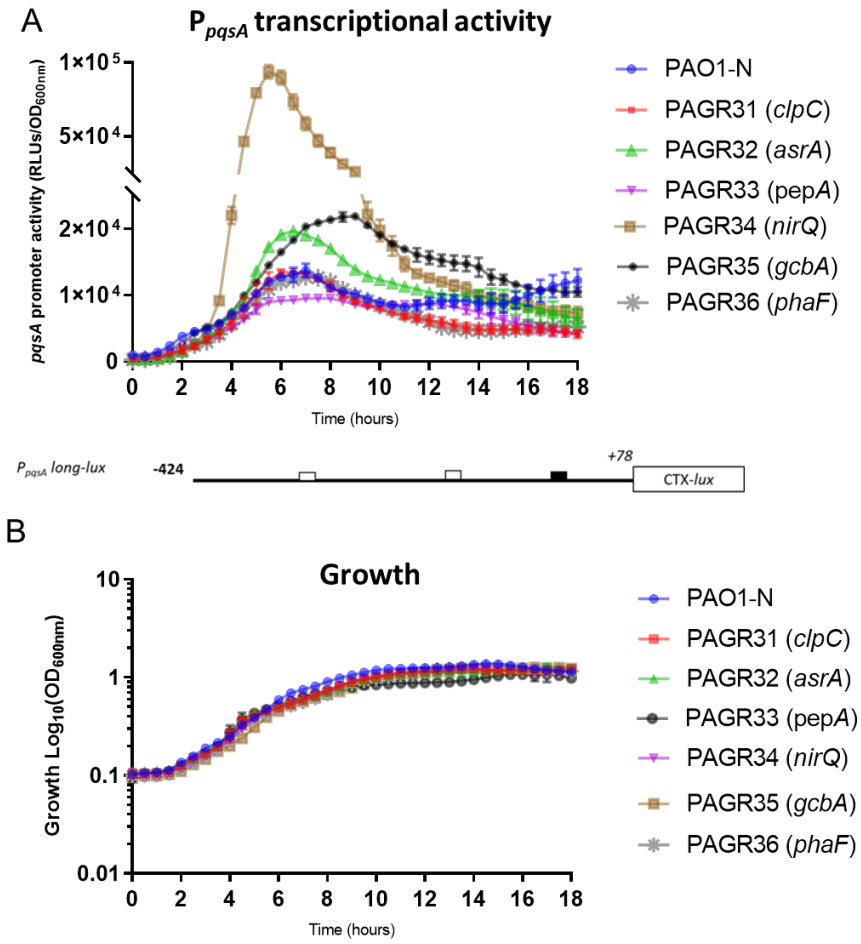


Figure 4.2. The transcriptional activity of P_{pqsA} in candidates to mediate the action of PqsE in *P. aeruginosa* PAO1-N.

A) Normalised bioluminescence production for strains PAO1-N, PAGR31 (*clpC*), PAGR32 (*asrA*), PAGR33 (*pepA*), PAGR34 (*nirQ*), PAGR35 (*gcbA*) and PAGR36 (*phaF*) mutants carrying the construct $P_{pqsA\ long}$-*lux* (Gm^R) (illustrated). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. B) The Log10 growth curve of the OD_{600nm} is shown. Error bars represent 2x standard errors calculated across three biological replicates.

4.4.2 NirQ represses the transcriptional activity of *pqsA* in *P. aeruginosa*

It was intriguing to observe that the mutation of *nirQ* within the *P. aeruginosa* PAO1-N genetic background increased the transcriptional activity of P_{pqsA} by nearly 7 times in comparison to the parent strain (**Figure 4.2**), therefore, its role in the regulation of *pqsA* and its link to PqsE was investigated in depth in this chapter. Since the work carried out in this thesis has been performed in *P. aeruginosa* PAO1-L, the impact of the mutation of *nirQ* was first verified in this

strain. To do this, the mutation of *nirQ* in *P. aeruginosa* PAO1-L was obtained as described in **chapter 2, section 2.6.2 and 2.6.3** and verified as shown in **Supplementary data, Figure S7.1.** The $P_{pqsA\text{-}long}$-*lux* reporter was then introduced into the chromosome of PAO1-L *and nirQ* mutant and the transcriptional activity of P_{pqsA} was monitored over time. The transcriptional activity of P_{pqsA} in the wildtype condition peaked at 7 hours of growth and presented slightly higher levels than those observed in the PAO1-N strain (**Figure 4.3)**. The transcriptional activity of P_{pqsA} in the *nirQ* mutant significantly increased in nearly 5 times in comparison to the wild type, reaching similar levels of expression to those observed in PAO1-N subline, but the transcriptional levels of P_{pqsA} peaked 1.5 hours later in the PAO1-L genetic background. The complementation of *nirQ* (pMENRC3 +IPTG*)* considerably decreased this activity, reaching levels even lower to those in the parent strain, hence validating that NirQ is a strong repressor of P_{pqsA}.

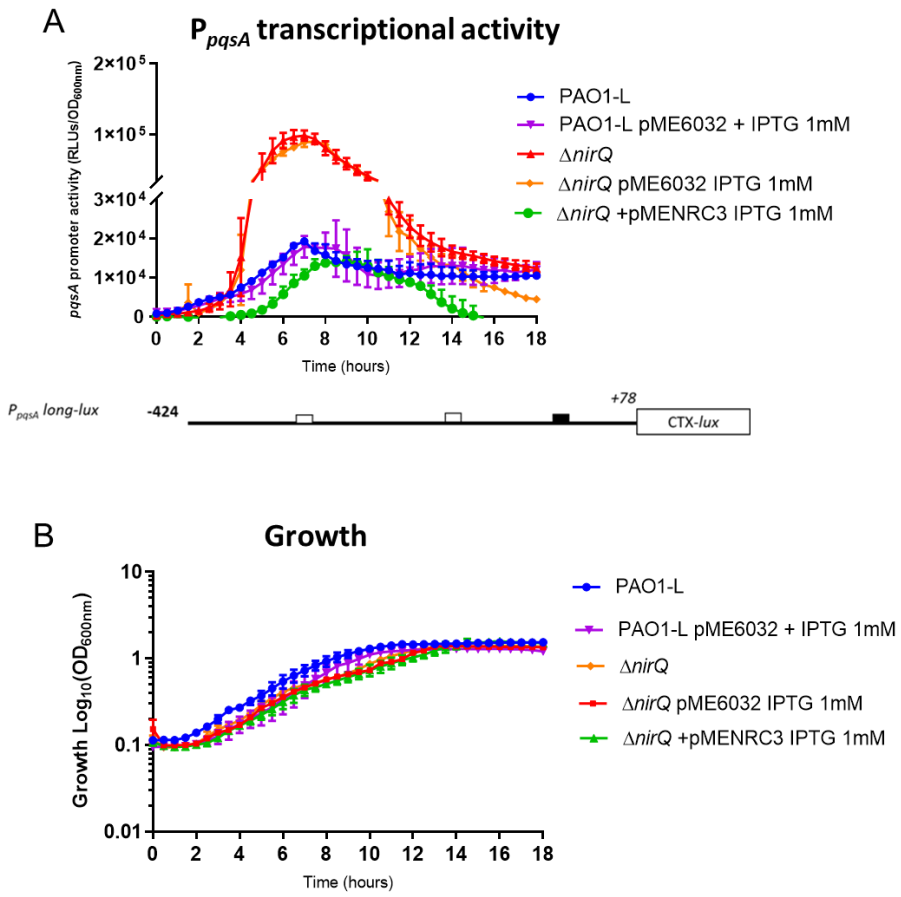


Figure 4.3. The transcriptional activity of P_{pqsA} in *P. aeruginosa* PAO1-L *nirQ* mutant.

A) Normalised bioluminescence production for strains PAO1-L and Δ*nirQ* carrying the construct $P_{pqsA\ long}$-lux (Gm^R) (illustrated). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. B) The Log10 growth curve of the OD_{600nm} is shown. Error bars represent 2x standard errors calculated across three biological replicates.

4.4.3 NirQ is a repressor of *pqsA* in an anaerobic environment

NirQ is a denitrification regulatory protein that allows growth of *P. aeruginosa* in the presence of nitrogen when oxygen is limited. Based on this, the role of *nirQ* towards the transcriptional activity of P_{pqsA} was validated further under anaerobic conditions. It has been reported, however, that *nirQ* is essential for the growth of *P. aeruginosa* under anaerobic respiration conditions as mutants in this gene failed to grow in the presence of nitrate or nitrite as a sole electron acceptor, but they did in the presence of arginine (Wauven et al., 1984; De Boer et al., 1996; Arai, Kodama & Igarashi, 1998). Based on this, *P. aeruginosa* wildtype and *nirQ* mutant were grown under anaerobic conditions as described

in **Chapter 2, section 2.1.2,** and the transcriptional expression of *pqsA* was measured by qRT-PCR.

The integrity of the purified RNA was verified in an agarose gel, whereas the purity was absorbance ratios at 260 nm versus 280 nm. Synthesis of cDNA was performed with RT Primer Mix (QIAGEN), which contains a specially optimized mix of oligo-dT and random primers that enables cDNA synthesis from all regions of RNA transcripts. For the qRT-PCR analysis, *pqsA* was amplified with primers pqsA RT-F/R as described in **Chapter 2, table 2.2.** The *16S* rRNA gene was used as the internal control. After confirmation of PCR efficiency (**Supplementary data, Figure S7.7**), the relative transcript abundance was calculated according to the 2−ΔΔCt method (Livak & Schmittgen., 2001). The relative abundance of the *pqsA* transcripts in the absence of *nirQ* significantly increased by 14-fold when compared to the wild type, validating that NirQ is a repressor of P_{pqsA} (**Figure 4.4**).

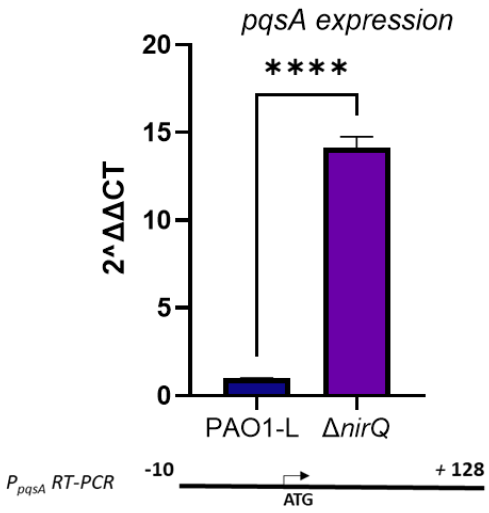


Figure 4.4. Differential expression of *pqsA* in *P. aeruginosa nirQ* mutant under anaerobic environment.

Y axis shows the relative expression of *pqsA* calculated from the cycle threshold Ct from 3 repeats ± standard deviation. Pure cDNA samples from PAO1-L and Δ*nirQ* were amplified in a 96 well-plate using 7500 applied biosystem qRT-PCR machine. The relative abundance was calculated using the ΔΔCT method. T-tests were used to assess for statistical significance**.** The amplified *pqsA* region is illustrated.

4.4.4 NirQ is regulated by PqsE

The relationship between *nirQ* and *pqsA* led us to analyse a plausible link between the regulation of *nirQ* and PqsE. This step was required as *nirQ* could act as the putative regulator that transduces the action of PqsE towards *pqsA.* Albeit it was previously elucidated that PqsE acts in a post-transcriptional manner, it was still interesting to characterize the regulation of *nirQ* at the transcriptional level to observe the degree of regulation by PqsE at both levels. To analyse this, the P_{nirQ} transcriptional and P_{nirQ} '-'-*luxCDABE* translational fusions were created in a pMiniCTX-*lux* (Gm^R) reporter as described in **Chapter 2, section 2.6.1** and verified as shown in **Supplementary data, Figure S7.4**. Afterwards, the impact of *pqsE* towards the regulation of *nirQ* was performed in a PAO1-L *pqsE* Ind strain. Results showed that compared to the parent strain, the peak of the transcriptional activity of P_{nirQ} did not significantly change in the condition with an uninduced *pqsE*, but it increased temporarily in late exponential phase (**Figure 4.5A**). IPTG-induced expression of *pqsE* (*pqsE* Ind + IPTG) decreased these levels to lower than the wildtype when stationary phase was reached, suggesting that PqsE is a negative regulator of *nirQ* and that this regulation is time-dependent. Somewhat a similar outcome was observed at the translational level. When compared to the parent strain, uninduced *pqsE* caused a slight increment in the translation of *nirQ* once its maximum levels of expression were reached and remained higher thereafter. Subsequent IPTG-induced expression of *pqsE* (*pqsE* Ind + IPTG) decreased the translation of *nirQ* to wild type levels (**Figure 4.5B**), hence, suggesting that PqsE has a repressor activity over *nirQ*.

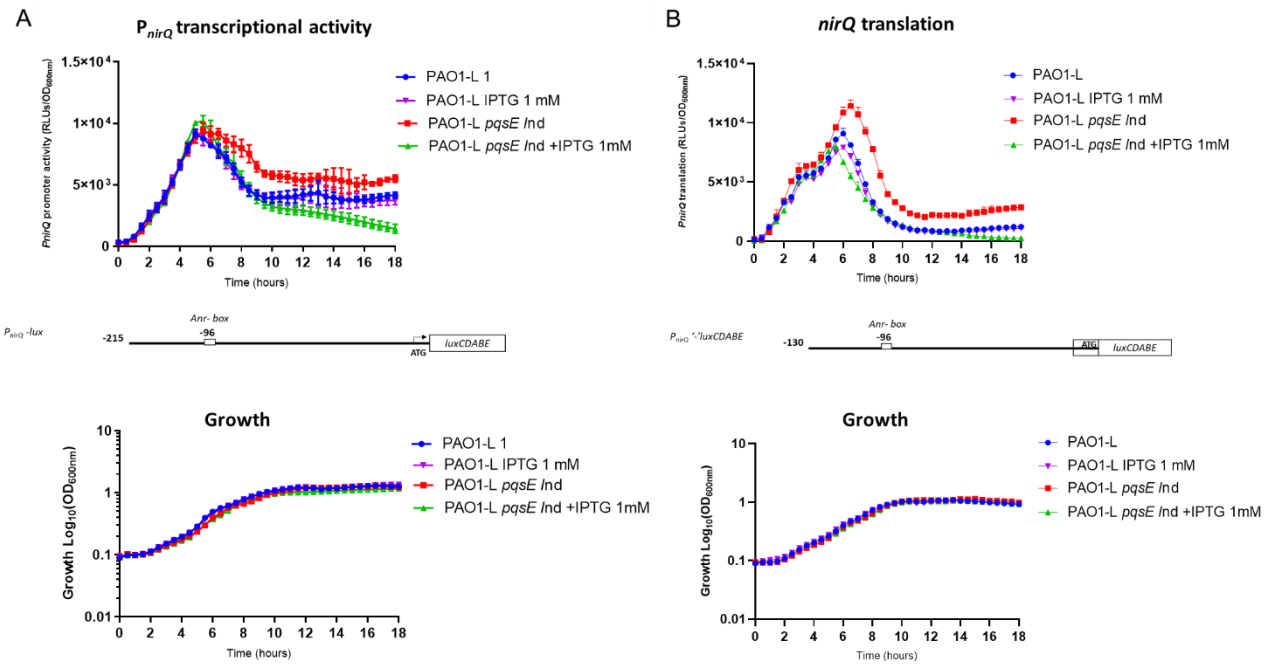


Figure 4.5. The transcription and translation of *nirQ* under induction of *pqsE*.

Top: Normalised bioluminescence production for PAO1-L and PAO1-L *pqsE* Ind carrying the A) P_{nirQ}-*lux* (Gm^R) and B) $P_{nirQ'-'}$*luxCDABE* bioreporter (illustrated) in LB broth. IPTG alone did not cause a significant impact on the transcription or translation of nirQ in PAO-L. Bottom: All strains in A) and B) were grown at 37°C for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. The Log10 growth curve shows that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

4.4.5 NirQ is dispensable for the PqsE-mediated repression of *pqsA*

This work has evidenced that *nirQ* participates in the regulation of *pqsA* and that is also under the control of *PqsE*. These observations placed *nirQ* as a strong candidate in the PqsE-mediated repression of *pqsA* expression. To validate whether *nirQ* is essential for this repression, the translation of *pqsA* was monitored in PAO1- L, PAO1-L *pqsE* Ind, *nirQ* and *nirQ pqsE* Ind mutant strains.

As seen in **Figure 4.6**, the mutation of *pqsE* in the *pqsE* Ind condition increased the translation of *pqsA*, whereas the IPTG-induced expression of *pqsE* decreased these levels, further supporting its repressor role towards *pqsA*. Next, the sole mutation of *nirQ* caused an induction of the translation of *pqsA* when compared to that of the wild type, confirming its repressor role towards *pqsA*. Mutation of *nirQ* in the uninduced *pqsE* Ind condition significantly increased the translation of *pqsA* when compared to the parent strain, whereas the IPTG-induced expression of *pqsE* (*pqsE* Ind + IPTG) completely abrogated the translation of *pqsA*. This result indicated that albeit NirQ participates in the *pqsA* regulation, is not essential for the PqsE-mediated repression of *pqsA.*

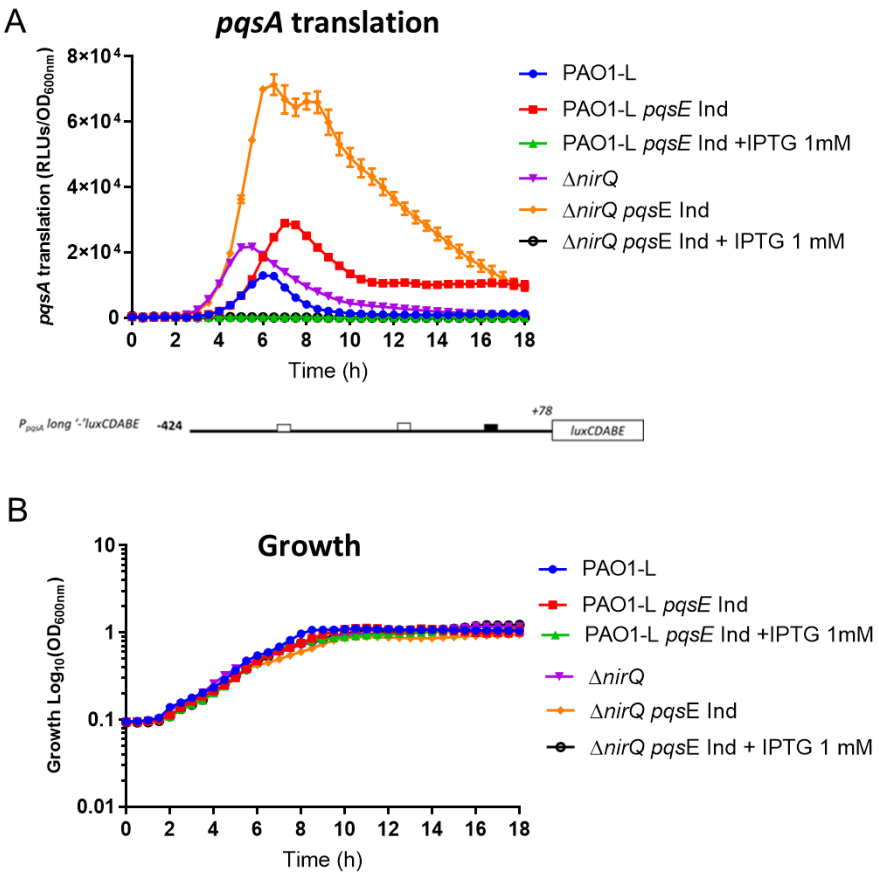


Figure 4.6. The translation of *pqsA* in *P. aeruginosa nirQ* mutant and inducing *pqsE*.

A) Normalised bioluminescence production for strains PAO1-L, PAO1-L *pqsE* Ind, Δ*nirQ* and Δ*nirQ pqsE* Ind carrying the construct $P_{pqsA\ long\ '-'}$-*luxCDABE* (Gm^R) (illustrated). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. B) The Log10 growth curve of the OD_{600nm} is shown. Error bars represent 2x standard errors calculated across three biological replicates. (*) Note that IPTG alone did not cause a significant impact on the translation of *pqsA* in PAO-L or Δ*nirQ* (**Supplementary data, Figure S7.6.1**)

4.4.6 Effect of the mutation *nirQ* on AQs production of *P. aeruginosa*

Albeit not essential for the PqsE-mediated repression of *pqsA*, it was evidenced that *nirQ* plays a role in the regulation in this regulation. To characterize the impact of the mutation of *nirQ* toward the *pqsA* related phenotype, the levels of AQs production in this mutant were determined at 16 hours of growth. Unexpectedly, mutation of *nirQ* resulted in a decrease PQS levels when compared to the wildtype (**Figure 4.7**), suggesting that somehow *nirQ* has a positive impact on the production of PQS, positioning it also as a positive regulator of *pqsA* under the conditions tested. Important to consider is that this assay was performed at 16 hours of growth when P_{pqsA} activity had reached wild type levels (**Figure 4.3**) and hence it may not reveal the AQs levels when the P_{pqsA} was significantly increased. Interesting to note was that albeit the production of HHQ remained unaltered, the levels of HQNO in the absence and after induction of *nirQ* (pMENRC3 + IPTG) were higher than those in the wild type, suggesting that AQs are still being produced, but the biosynthetic pathway seems to be displaced towards the formation of HQNO, which suggest that *nirQ* may have an impact on *pqsL* (Drees et al., 2018).

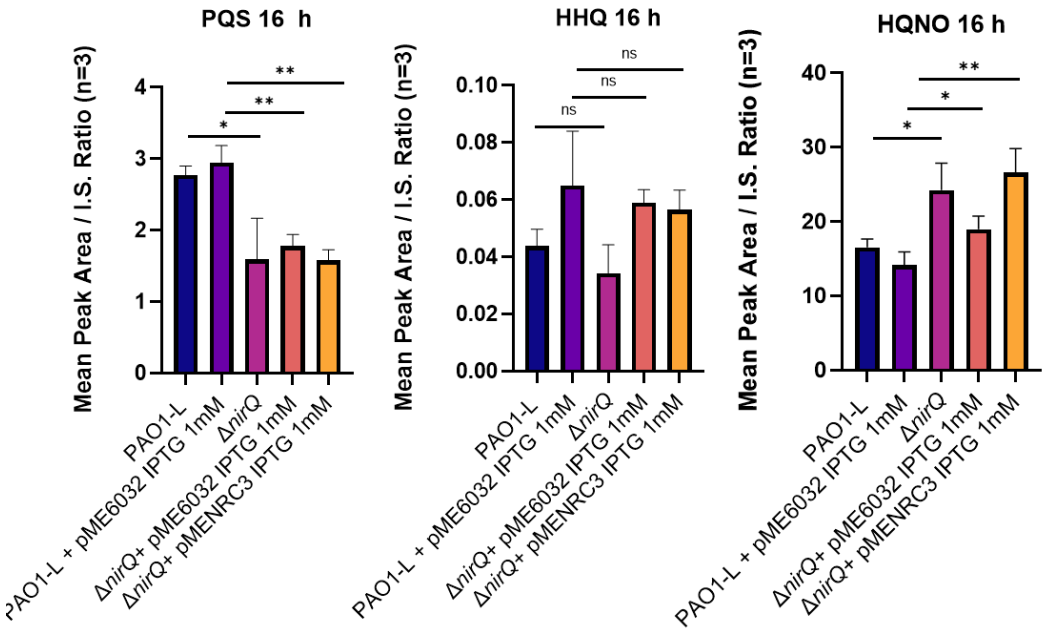


Figure 4.7. PQS quantification in P. aeruginosa nirQ mutant at 16 hours of growth.

Bacteria were grown in flasks for 16 hours at 37° C in LB media. Quantification of PQS was performed from sterile supernatants extracted with ethyl acetate. IPTG 1 mM was added to the cultures for the expression of *nirQ* (pMENRC3). Error bars represent standard deviation of three biological triplicates. T-tests were used to assess for statistical significance.

4.4.7 Elucidating whether NirQ interacts with P_{pqsA} in isolation

Previous work found NirQ binding the P_{pqsA} when *pqsE* was absent, moreover, this work evidenced a regulatory link between NirQ and *pqsA*. Since *nirQ* is transcriptional regulator, a physical interaction between NirQ and the P_{pqsA} would be expected. To corroborate this hypothesis, NirQ was purified for EMSA studies using the P_{pqsA} promoter region.

4.4.7.1 NirQ protein expression and purification

In the denitrification gene cluster from *P. aeruginosa*, *nirQ* is part of an operon encoding three open reading frames (*nirQOP*) (Arai, Igarashi, & Kodama., 1994). NirQ encodes a protein of 28.9 kDa that is 76% identical to *nirQ* of P. *stutzeri*, which is responsible for the activation of nitrite and nitrate reductases (Arai et al., 1994). It is part of the NirQ/NorQ type proteins that belong to the CGN (CbbQ/GvpN/NorQ) subafimiy and subsequent MoxR ATPases family (Snider & Houry, 2006). The crystal structure of NirQ has not been described, however it has been reported to be highly similar to CbbQ from *Pseudomonas hydrogenothermophila*; in fact, NirQ from *P. aeruginosa* could activate the RubisCO from *P. hydrogenothermophila* almost equally to that of CbbQ, albeit the *cbbq* gene did not complement neither the anaerobic growth nor the NOR activity in the absence of *nirQ*, indicating that *cbbQ* from *P. hydrogenothermophila* is unable to supplement *nirQ* in *P. aeruginosa* (Hayashi et al., 1998).

To validate wether NirQ interacts with the P_{pqsA} promoter, the purification of NirQ (28,9kDa) was carried out as described in **Chapter 2, section 2.9.** Fresh electroporated *E. coli* BL21(DE3) competent cells carrying the expression vector pNR1pet, expressing NirQ his-tagged at the C-terminus, were expressed at 37^{o}C as described in **Chapter 2, section 2.9.1**. Whole pellet cells expressing NirQ are shown in **Figure 4.8**. Polyacrylamide gel electrophoresis revealed a band below 35 kDa, which was absent in the cell expressing the empty vector (**Figure 4.8A**), suggesting that the expressed protein corresponded to NirQ. Like PA2705, n-

lauryl-sarcosine 0.2% was able to solubilize NirQ, however, the pre-purification step corresponding to imidazole titration with HisPur™ Ni-NTA nickel resin, using increasing concentrations of this agent, allowed less elution of this protein when compared to PA2705 (**Chapter 3, Figure 3.20**), with maximum elution efficiency at concentrations that ranged between 50 and 70 mM of imidazole (**Figure 4.8B**). The purification of NirQ was then carried out from its soluble fractions using metal affinity chromatography (IMAC) (Porath et al., 1975). The chromatogram showed only a small peak during imidazole elution (**Figure 4.8C top**). Polyacrylamide gel electrophoresis of these samples showed no eluted NirQ under these conditions (**Figure 4.8C bottom**), suggesting that *nirQ* did not bind the nickel column probably due to a hidden his-tag.

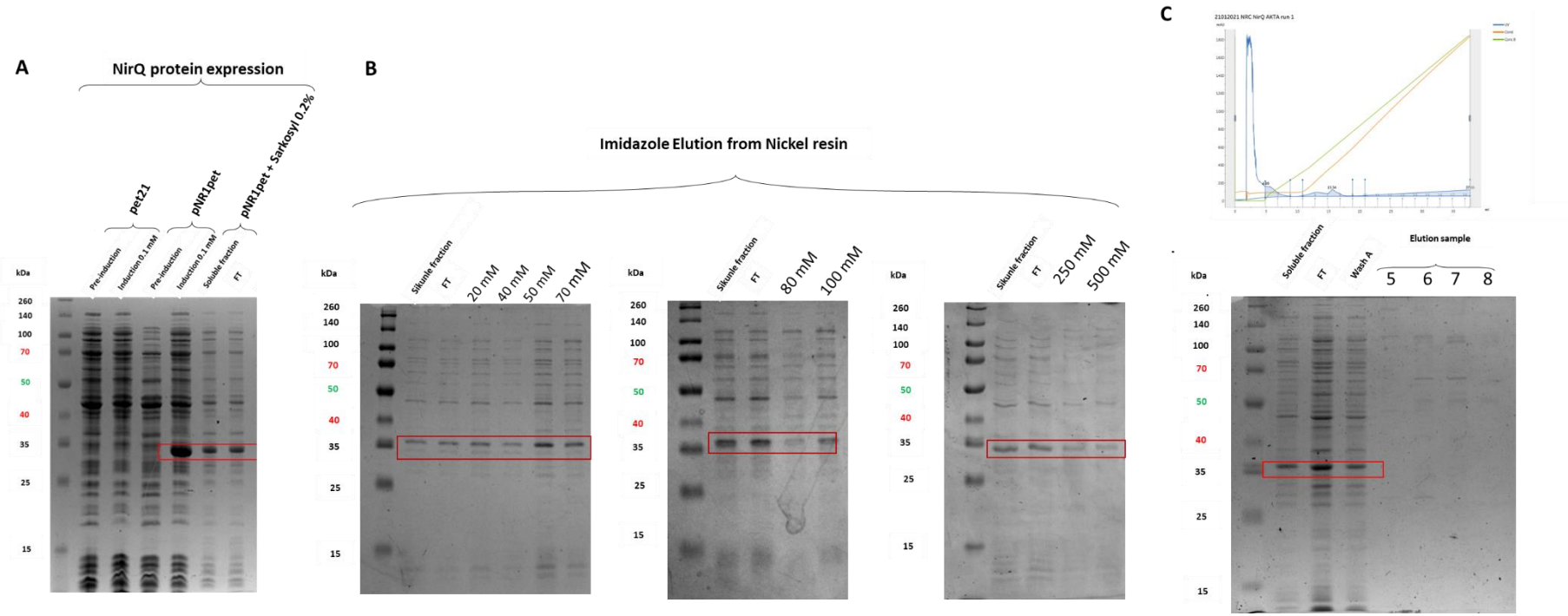


Figure 4.8. SDS-PAGE of the protein expression of NirQ, imidazole titration elution and ÄKTA purification.

A) *E. coli* BL21 (DE3) carrying the vector pNR1pet *w*as expressed at 37°C with 0,1 mM IPTG for 3 hours. The resulting pellets were adjusted to an OD_{600nm}= 0.6 and resuspended in Tris HCL buffer pH 8, treated with 1XSDS, boiled at 100°C for 10 min and then separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Pellets treated with n-lauryl sarcosine (sarkosyl) at 0.2% partially solubilized NirQ. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).

B) *E. coli* BL21 (DE3) carrying the vector pNR1pet was expressed as above. The resulting pellets were adjusted to an OD_{600nm}= 0.6 and resuspended in Tris HCL buffer pH 8. Cells were disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ

water and incubated in 40 mM Tris HCL 0,2% n-lauryl sarcosine overnight at 4°C in a rocker platform. Soluble fractions were collected and incubated with HisPur™ Ni-NTA overnight at 4°C. NirQ was eluted using increasing concentrations of imidazole (20-500 mM). Samples were treated with 2XSDS loading buffer and separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Soluble fraction corresponds to the sample containing soluble protein prior imidazole elution. FT denotes flow through and correspond to the unbound fractions of protein after incubation with Ni-NTA. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).

C) *E. coli* BL21 (DE3) carrying the vector pNR1pet was expressed as above. The resulting pellet was resuspended in Tris HCL buffer pH 8, disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in 40 mM Tris HCL 0,2% n-lauryl sarcosine overnight at 4°C in a rocker platform. The soluble fraction was collected and loaded in a Nickel Column HisTrap™. NirQ was eluted in fractions of 2 mL in an ÄKTA pure purification system. Peaked area in the chromatogram was analysed in a 12% polyacrylamide gel run at 100 Volts for 90 min. FT: Flow through after soluble fraction loaded into Nickel column. Wash A: Ni-NTA Column wash with Buffer A prior ÄKTA purification. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).

To test whether an unexposed poly his-tag at the C terminus was responsible for this outcome, *E. coli* BL21 (DE3) competent cells carrying the expression vector pNR2Cold1, his-tagged at the N-terminus, were expressed at 16 ^{o}C as described in **Chapter 2, section 2.9. 1.** Extracts from whole cell pellets showed the presence of a protein of ~29 kDa that was absent from induced cells with the plasmid alone, hence suggesting that it corresponded to expressed NirQ (**Figure 4.9A**). Sonication of the whole pellet samples indicated that NirQ remained in the insoluble fraction (**Figure 4.9B**). Moreover, like with PA2705 (**Chapter 3, Figure 3.19B Lane 6),** the solubilization of NirQ with mild solubilizing agents was carried out as described in **Chapter2, section 2.9.1**. Albeit not as effective as with PA2705, soluble NirQ was best obtained by using 0.2% n-lauryl sarcosine (**Figure 4.10 Lane 6)**.

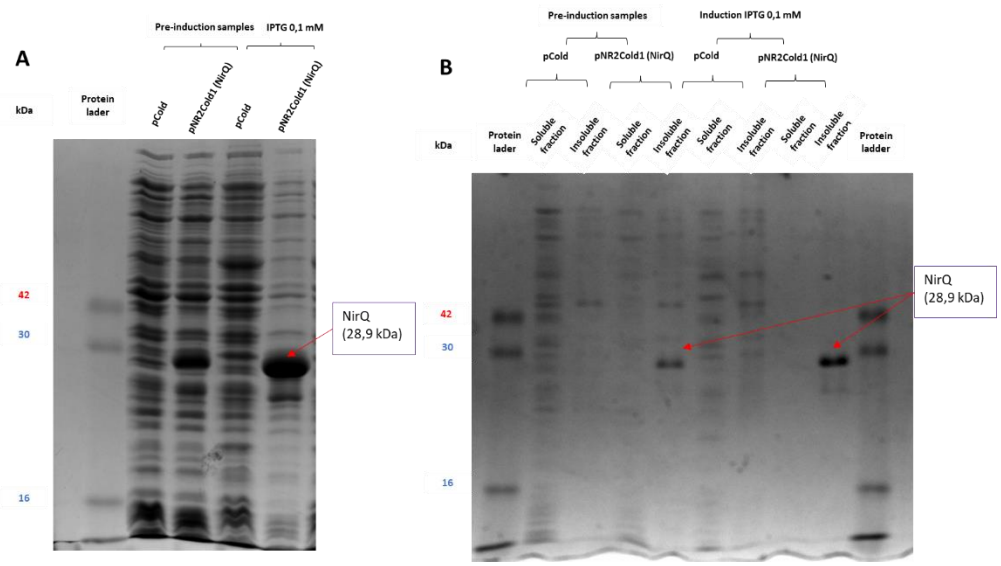


Figure 4.9. SDS-PAGE of the protein expression and solubility of NirQ.

A) *E. coli* BL21 (DE3) carrying the vector pNR2Cold1 was expressed at 16°C with 0,1 mM IPTG for 16 hours. The pellets were adjusted to an OD_{600nm}= 0.6, treated with 1XSDS, boiled at 100°C for 10 min and then separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Samples were run in reference to the Colour Prestained Protein Standard Low Range (1.7-42kDa).

B) *E. coli* BL21 (DE3) carrying the vector pNR2Cold1 was expressed as above. The resulting pellets were adjusted to an OD_{600nm}= 0.6 and resuspended in Tris HCL buffer pH 8. Cells were disrupted by sonication with an amplitude of 15*%* pulsing 3 times for 30 sec each and the soluble and insoluble fractions were collected after centrifugation at 12.000rpm for 10 min. Samples were treated with 2XSDS loading buffer and separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Samples were run in reference to the Colour Prestained Protein Standard Low Range (1.7-42kDa)

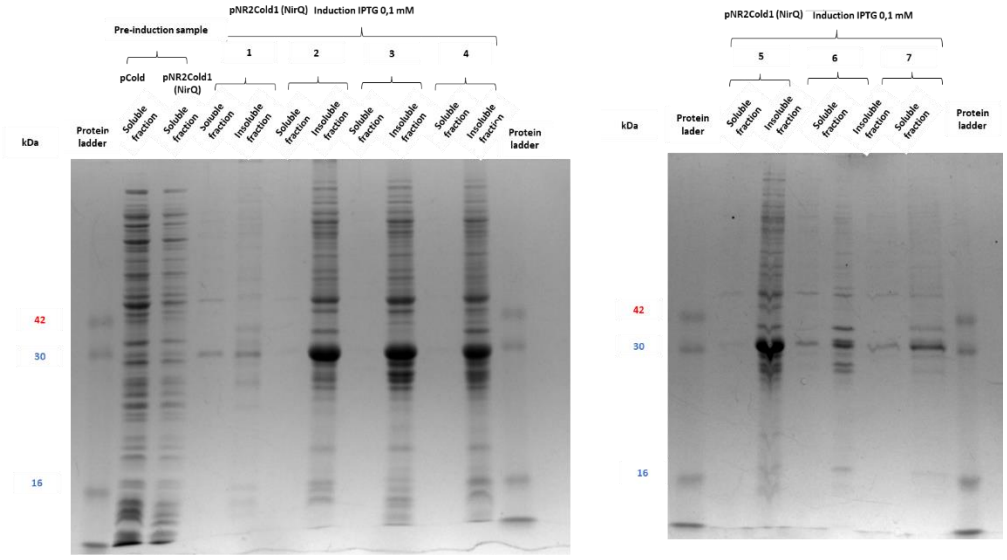


Figure 4.10. SDS-PAGE of the solubility analysis for NirQ using mild solubilizing agents.

E. coli BL21 (DE3) carrying the vector pNR2Cold1 was expressed at 16°C with 0,1 mM IPTG for 16 hours. The resulting pellets were adjusted to an OD_{600nm}= 0.6 and resuspended in Tris HCL buffer pH 8. Cells were disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in different solubilizing agents overnight at 4°C in a rocker platform. Soluble and insoluble fractions were obtained after centrifugation at 12.000 rpm for 10 min, treated with 2XSDS loading buffer, boiled at 100°C for 10 min and separated on a 12% polyacrylamide gel at 100 Volts for 90 min. Samples were run in reference to the Colour Prestained Protein Standard Low Range (1.7-42kDa). **Lane 1:** 50 mM Tris Urea 8 M, 5% Glycerol pH 8. **Lane 2**: 40 mM Tris HCL 5% propanol, 2 M Urea pH 8. **Lane 3**: 40 mM Tris HCL 0,5% Triton X 100 pH 8. **Lane 4**: 40 mM Tris HCL 5% DMSO pH 8. **Lane 5**: 40 mM Tris HCL 2M Urea pH 8. **Lane 6**: 40 mM Tris HCL 0.2% n-lauryl sarcosine. **Lane 7:** 40 mM Tris Urea 2M, pH 12.

After solubility analysis, NirQ was expressed as described in **Chapter 2, section 2.9.2** and subsequently purified in an ÄKTA pure system. To achieve this, the supernatants containing soluble NirQ were pre-loaded into a Nickel immobilized nitrilotriacetic acid (NTA) column. After loading, a low concentration of imidazole (5 mM) was added in the washing buffer A and passed through the column with bound NirQ to lessen nonspecific binding to nickel. NirQ was then eluted using an imidazole gradient as described in **Chapter 2., 2.9.3**. IMAC chromatogram showed a peak between the fractionation samples numbers 6 and 12, that corresponded to the elution of NirQ as evidenced followed polyacrylamide gel electrophoresis (**Figure 4.11A/B**).

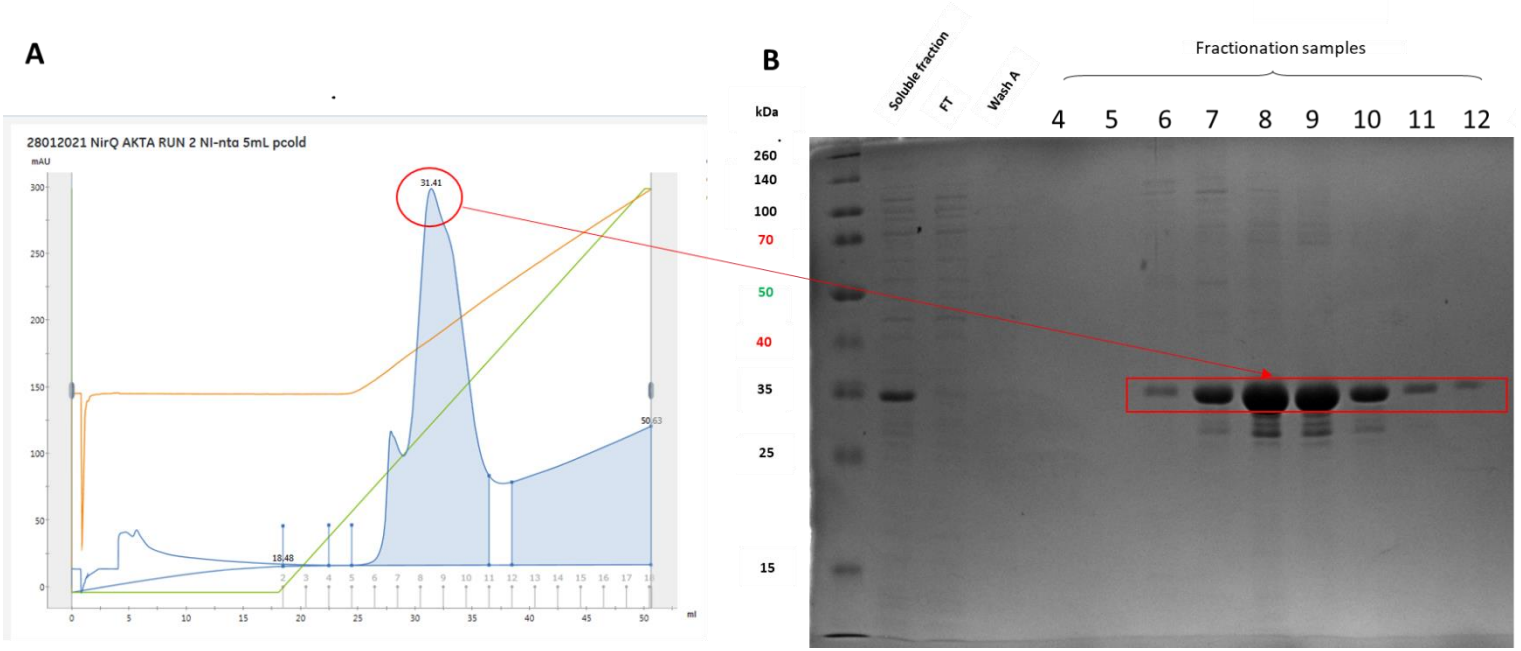


Figure 4.11. NirQ ÄKTA purification.

E. coli BL21 (DE3) carrying the vector pNR2Cold1 was expressed at 16°C with 0,1 mM IPTG for 16 hours. The resulting pellet was resuspended in Tris HCL buffer pH 8, disrupted by sonication with an amplitude of 15% pulsing 3 times for 30 sec each, washed twice with chilly milliQ water and incubated in 40 mM Tris HCL 0,2% n-lauryl sarcosine overnight at 4°C in a rocker platform. The soluble fraction was collected and loaded in a Nickel Column - HisTrap™. NirQ was eluted in fractions of 2 mL in an ÄKTA pure purification system. A) Chromatogram shows a sharp peak between fractionation samples 6-12. B) Visualization of fractionation samples 4-12 after ÄKTA purification in a 12% polyacrylamide gel. Soluble fraction: Supernatant containing soluble NirQ prior Ni-NTA column. FT: Flow through after soluble fraction loaded into Ni-NTA column. Wash A: Ni-NTA column wash with Buffer A prior ÄKTA purification. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).

4.4.7.2 His-tag detection of NirQ

After protein purification, the fractionation samples contained a purified protein of the size of NirQ. To confirm this, the immunodetection of the target protein was carried out by western blotting as described in **Chapter 2. Section 2.9.5**.

Wet-transfer of the fractionated sample containing the purified protein (**Figure 4.11**) to a nitrocellulose membrane was performed followed by the detection of the his-tagged protein on X-Ray film. As a positive control, the pNR2Cold1 expressed from whole pellet was included. As seen in **figure 4.12**, NirQ was observed in the cell extracts as well as in the fractionation sample containing pure protein. In addition, this protein was not observed in the cell extracts expressing pCold alone, hence confirming the accurate purification of NirQ.

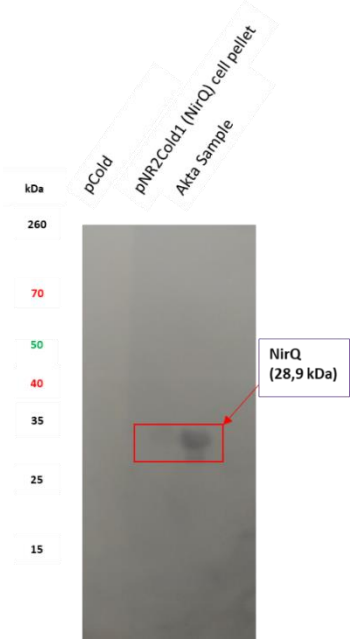


Figure 4.12. Western blot of NirQ.

His-tag detection using Penta-His HRP conjugate detected the presence of his-tag in NirQ after ÄKTA purification. Negative Control: whole lysate carrying the pCold empty plasmid induced with IPTG 0.1 mM. Positive control: whole lysate carrying the pNR2Cold1 expressed with 0.1 mM IPTG.

4.4.7.3 Size exclusion chromatography/Gel filtration

Followed purification and subsequent confirmation of the presence of NirQ by wester-blot, size exclusion chromatography (SEC) was performed as described in **Chapter 2, section 2.9.4**. A Superdex 200 10/300 GL column was used, hence allowing a separation range for molecules with molecular weights between 10,000 and 600,000 Da. As seen in **Figure 4.13A**, two major peaks were observed in the chromatogram in which NirQ was present as evidenced in the polyacrylamide gel electrophoresis (**Figure 4.13B**). Based on a typical chromatogram from a function test of Superdex 200 10/300 GL (**Figure 4.13C**) it is suggested that NirQ is present in different states of oligomerization, though its first peak indicates that NirQ is found in multimeric states as its molecular weight is at 440.000 Da, whereas its second peak indicated that NirQ is forming dimers as its molecular weight is at 67.000 Da. Elution samples containing pure NirQ were subjected to dialysis as described in **Chapter 2, section 2.9.4.**

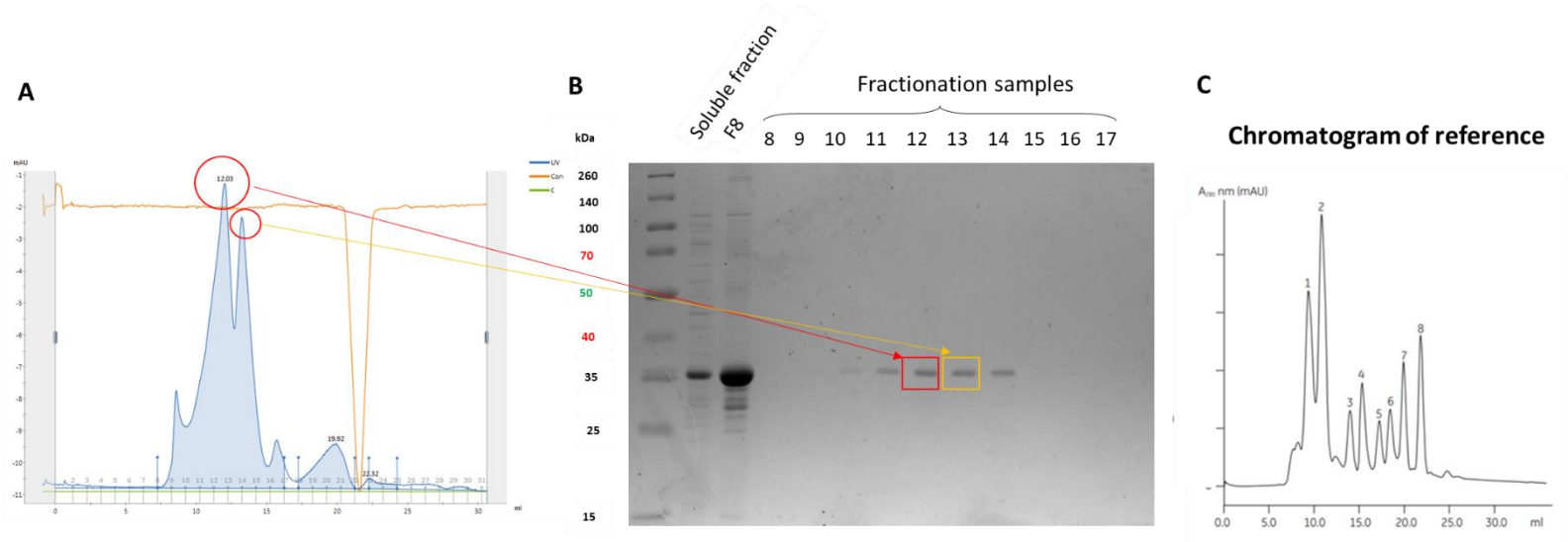
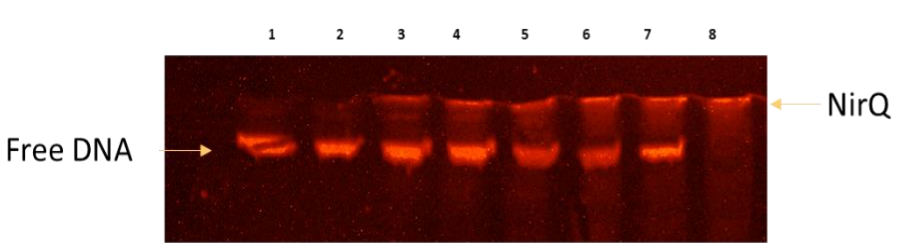


Figure 4.13. Size exclusion chromatography of NirQ.

NirQ sample after ÄKTA purification was subjected to size exclusion chromatography in 25 mM Tris-HCl, NaCl 150 mM, 0,2% n-lauryl sarcosine pH 8.0. Chromatography was carried out on a Superdex 200 10/300 GL at a flow rate of 0.7 mL/min. The volume of protein injected was 50 µL with a fractionation volume of 1 mL. A) Chromatogram shows a series of peaks between fractionation samples 8-17. B) Visualization of fractionation samples 8-17 after size exclusion in a 12% polyacrylamide gel. Soluble fraction: Supernatant containing soluble NirQ prior ÄKTA purification. F8: Purified NirQ from fractionation sample 8 after ÄKTA purification. Samples were run in reference to the Spectra Multicolour range Protein ladder (10–260 kDa).C) Typical chromatogram from a function test of Superdex 200 10/300 GL. 1) Thyroglobulin (M, 669,000) 5 mg/ml. 2) Ferritin (M, 440,000) 0.4 mg/ml. 3) BSA (M, 67,000) 8 mg/ml. 4) ß-lactoglobulin (M, 35,000) 2.5 mg/ml. 5) Ribonuclease A (M, 13,000) 5 mg/ml. 6) Cytochrome C (M, 13,600) 1.5 mg/ml. 7) Aprotinin (M, 6,512) 2 mg/ml. 8) Vitamin B12 (M, 1,355) 0.1 mg/ml. Peak 5 and 6 are separated from each other only for difference in shape.

4.4.7.4 Electrophoretic mobility shift assay (EMSA)

During this chapter, gene expression analyses demonstrated that *nirQ* is a negative regulator of P_{pqsA} and that is also under the negative control of PqsE. In addition, despite not being essential for the PqsE-mediated repression of P_{pqsA}, initial pull-down experiments identified NirQ bound to the P_{pqsA} promoter when PqsE was absent, suggesting that they interact. To validate this binding, protein-DNA interaction (EMSA) experiments were performed as described in **Chapter 2, section 2.9.7**. NirQ was quantified using a BCA assay as described in **Chapter 2, section 2.9.6**. The NirQ stock concentration was at 150 µg/mL. The promoter region of *pqsA* corresponding to that in Bates , PhD Thesis (2013) (**Chapter 3, Figure 3.1**) and 500 fmol of DNA from this promoter were used. Increasing concentrations of NirQ alone were added to the binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM Tris, pH 7.4) containing a fixed concentration of the DNA fragment and including the addition of ATP 2 mM. Like with PA2705, the loading of the sample was performed quickly to avoid loss of chemical equilibrium. Unfortunately, no conclusion could be inferred from this attempt (**Figure 4.14**). The free DNA migrated poorly albeit showed no degradation. NirQ was not observed in the conditions with DNA alone and DNA in the presence of ATP, discarding any cross contamination. NirQ was detected at the top of the gel in all the other conditions, suggesting that it remained within the wells. Due time limitations caused by COVID restrictions, this experiment could not be repeated nor optimized, hence, whether NirQ binds P_{pqsA} remains to be elucidated.



Lane 1= DNA 500 fmol
Lane 2 = DNA 500 fmol + ATP 2 mM
Lane 3 = DNA 500 fmol + ATP 2 Mm + NirQ 65ng
Lane 4 = DNA 500 fmol + ATP 2 Mm + NirQ 195 ng
Lane 5 = DNA 500 fmol + ATP 2 Mm + NirQ 390 ng
Lane 6 = DNA 500 fmol + ATP 2 Mm + NirQ 780 ng
Lane 7 = DNA 500 fmol + ATP 2 Mm + NirQ 1040 ng
Lane 8 = NirQ 1040 ng

Figure 4.14. Titration of the P_{pqsA} DNA with NirQ protein.

Increasing amounts of NirQ were mixed with 500 fmol of the *pqsA* promoter DNA, incubated for 40 min in binding buffer, and then separated on a 7.5% nondenaturing polyacrylamide gel at 70 Volts in 0.5X TBE for 3 hours at 4^{o}C. The gel was stained with SYBR® Green EMSA stain followed by SYPRO® Ruby EMSA stain components of the Electrophoretic Mobility-Shift Assay Kit (Thermofisher). After each staining, the image was documented using a Biorad Gel Doc XR Imaging and the digital images were overlaid.

4.5 Discussion

In this chapter, DNA promoter pull-down and gene expression assays were used as reference in search for a protein candidate which could mediate the action of PqsE. Among six candidates, *nirQ* had a great impact towards the transcriptional activity of *pqsA* (**Figure 4.2**), hence it was selected for further studies. The mutation of *nirQ* in PAO1-L subline drastically increased the transcriptional activity of P_{pqsA}, suggesting that this regulator is a strong repressor of this promoter, observation that was validated when complementation of *nirQ* restored wild type levels of P_{pqsA} expression in this mutant (**Figure 4.3**). NirQ is a regulator that participates in denitrification; therefore, it was paramount to validate this regulation in anaerobic conditions. In the absence of oxygen, alternative external electron acceptors like nitrate, nitrite, or nitrous oxide, can be utilized by *P. aeruginosa* (Filiatrault et al., 2006). Alternatively, arginine can be catabolized by substrate level phosphorylation and be used as an energy source for anaerobic growth (Mercenier et al., 1980). Since mutants of *nirQ* are unable to grow in anaerobic conditions, not even in the presence of nitrite or nitrate, arginine was used as an alternative external electron acceptors (Wauven et al., 1984). qRT-PCR analysis was chosen to validate this regulation due to the inability of the *lux* reporters to work under anaerobic environments as a consequence of the lack of oxygen required for the luminescence reaction (Meighen, 1993). Gene expression analysis confirmed that NirQ acts as a repressor in this regulation, as the relative expression of *pqsA* was significantly increased in the absence of *nirQ* (**Figure 4.4**). This observation was encouraging and led to analyze whether *nirQ* is controlled by PqsE. As demonstrated in **Chapter 3**, **Figure 3.10**, PqsE is suggested to act post-transcriptionally. In this scenario, the construction of the translational reporter denominated $P_{nirQ\ '-'\ -luxCDABE}$, indicated that (i) PqsE participates in the regulation of *nirQ*, (ii) PqsE acts as a repressor in this regulation and that (iii) this repression is time-dependent (**Figure 4.5**). This is in line with the promoter pull down of P_{pqsA} (**Figure 4.1**) and it can explain why the gel band containing NirQ was not present once *pqsE* was overexpressed *(pqsE*

Ind + IPTG) (**Figure 4.1**). These observations also reinforce that the *pqs* system, mainly through PqsR and PqsE, is a repressor of denitrification (Rampioni et al., 2016). The establishment of a regulatory link between *nirQ* and *pqsA* as well as *nirQ* and PqsE, led to interrogate whether *nirQ* may be the mediator of the action of PqsE towards *pqsA*. To perform this analysis the translational version of the $P_{pqsA\ long}$-*lux* reporter, denominated $P_{pqsA\ long}$ '-' -*luxCDABE* was used, as it was previously demonstrated that PqsE represses *pqsA* in a post-transcriptional event (**Chapter 3, Figure 3.10**). Even though *nirQ* played a role in the regulation of *pqsA* and was under the control of PqsE, the IPTG-induced expression of *pqsE* in the absence of *nirQ* still downregulated the translation of *pqsA* (**Figure 4.6**) indicating that *nirQ* is not the regulator that transduces the action of PqsE to *pqsA.*

Besides, it was puzzling to observe that the mutation of *nirQ* caused a decrease in the production of PQS (**Figure 4.7)**, which does not correlate to the observation of NirQ being a repressor of *pqsA* (**Figure 4.3** and **Figure 4.6**). One hypothesis that could explain this outcome is an effect exerted by PqsE and that is reflected at the time point of 16 hours. This is because the mutation of *nirQ* caused a significant increase in the expression of *pqsA* in early stages of growth, meaning that the expression of the whole operon *pqsABCDE* is also increased, hence, the elevated expression of *pqsE* is expected to cause a repressive impact towards *pqsA*, which could be a reflection of that observed at 16 hours in gene expression assays (**Figure 4.3** and **Figure 4.6**) and therefore in the production of PQS at the same time point. Another observation was that the production of HQNO was increased under these conditions (**Figure 4.7**), suggesting that the reaction is displaced towards the production of this metabolite instead of PQS, which raises the question of whether *nirQ* may impact the expression of *pqsL*, the putative flavin-dependent monooxygenase responsible for the formation of HQNO in the AQs pathway in *P. aeruginosa* (Drees et al., 2018). To clarify these observations, it is necessary to quantify AQs at ~6 hours, as at this point the transcription and translation of *pqsA* were at their maximum levels (**Figure 4.3** and **Figure 4.6**).

Since NirQ was found binding the P_{pqsA}, it was paramount to validate this interaction *in vitro*. For the expression of NirQ (28,9 kDa), similar conditions were used as those described in Hayashi & Igarashi, 2002. This was because NirQ has not been purified in the past, but has been reported to be highly similar to CbbQ from strain *Pseudomonas hydrogenothermophila* (Yokoyama et al., 1995; Hayashi et al., 1998). NirQ his-tagged at its C-terminus in a pET21 vector did not elute after ÄKTA purification (**Figure 4.8C**), this result suggested that the his-tag at the C-terminus could be hidden. It is possible that by using n-lauryl sarcosine as solubilizing agent and, considering that it has been shown to retain the native structure of proteins (Tao et al., 2010), NirQ was folded into its tertiary structure, meaning that the his-tag at this terminus may have not been exposed enough to interact with the nickel column. This was corroborated later when NirQ was expressed in a pCold expression vector, which brings a his-tag sequence that is incorporated at the N-terminus of the protein sequence. NirQ was successfully purified after ÄKTA purification (**Figure 4.11)**. Pure NirQ was then subjected to size exclusion using a Superdex 200 10/300 GL column to increase the purity of the sample and to elucidate the molecular weight at which this recombinant protein is found under these conditions, which ultimately could provide information of its conformational state (Hong, Koza, & Bouvier, 2012). Size exclusion buffer contained Tris-Cl 25 mM, 150 mM NaCl and 0.2% n-lauryl sarcosine to maintain to some extent the physiological conditions at which NirQ is found in the cell, as well as maintain its soluble state. Size exclusion chromatogram analysis showed different peaks trough the ÄKTA run (**Figure 4.13A**). The first peak was low and corresponded to the fractionation sample 9 (**Figure 4.13A**), based on the typical chromatogram from a function test of Superdex 200 10/300 GL (**Figure 4.13C**), its predicted high molecular weight may indicate the presence of aggregated protein, however SDS-PAGE did not show any visible band in this sample (**Figure 4.13B**), which could be due to a very low amount of protein content present in the sample. A major peak was observed at the fractionation sample 12 (**Figure 4.13A**), which had an estimated molecular weight of 440.000 Da (**Figure 4.13C**), and a third peak was observed immediately after at the fractionation sample 14, that

estimates a molecular weight of 67.000 Da. SDS-PAGE corroborated the presence of NirQ in samples 10 to 14 (**Figure 4.13B**) suggesting that this protein in the size exclusion elution buffer is present in different molecular states, ranging from dimers at ~67.000 Da to oligomeric conformations of ~ 440.000 Da. Transcription regulators achieve specific binding normally as dimers or further multimer forms (Browning and Busby. 2016), however, most CbbQ native proteins have shown to form hexamers (~180.000 Da)(Tsai et al., 2015), therefore it is possible that this oligomeric state is present in these samples and that it may ultimately allow the binding of NirQ to DNA.

The last aim of this chapter was to validate whether NirQ binds the promoter of *pqsA*. Due to time limitations caused by COVID, only one attempt could be carried out. For this analysis the same P_{pqsA} region as that used in the promoter pull down was used in an EMSA assay. Based on this attempt however, no apparent protein-DNA binding was observed (**Figure 4.14**). It is well known that EMSA experiments require several optimizations to finally provide the required equilibrium between the amount of protein and DNA, therefore a few factors need further optimizations such as the pH of the binding and running buffer, temperature, salt content and time of electrophoresis, among others (Hellman & Fried., 2007). In addition, it cannot be discarded that NirQ in the oligomer state found in the size exclusion sample after dialysis may not be able to interact with DNA, this is because neither enzymatic assays nor studies to corroborate its native structure could be carried out, therefore these steps are required prior continuing with EMSA optimization.

4.6 Conclusion and future directions

This chapter demonstrated that NirQ participates in the regulation of *pqsA*, but it is not the effector responsible for the PqsE-mediated repression. Fortunately, many other proteins were binding the promoter of *pqsA* depending on the presence or absence of PqsE. Since *nirQ* altered the P_{pqsA} expression levels drastically, it was interesting to perform deeper studies with this gene, nevertheless, other candidates also showed to alter the transcriptional activity

of P_{pqsA} and those ones deserve further study. In addition, albeit the mutation of *phaF* did not show significant changes in the transcriptional activity of P_{pqsA}, in the promoter pull-down, it was the only protein bound to *pqsA* when PqsE was overexpressed, and it was absent when *pqsE* was mutated, suggesting that PqsE is a positive regulator of *phaF* and that it could mediate its action towards *pqsA*. However, it could also be that this is not the case, as the same rationale was undertaken for the selection of the first candidate to mediate the action of PqsE, PA2705, which resulted to be dispensable for the PqsE-mediated repression. Important to consider is that the main regulators of the three QS systems RhlR, LasR and PqsR were also binding the promoter of *pqsA*. Since PqsE has shown to be essential for the regulation of RhlR depended genes such as *phz* and *hcn* operons and *lecA* (Farrow et al., 2008; Hazan et al., 2010; Rampioni et al., 2016; Mukherjee et al., 2018; Groleau and Pereira., 2020), and that their physical protein-protein interaction has been recently demonstrated (Taylor et al., 2021), this regulator cannot be discarded as a mediator of PqsE activity. Similarly, several regulatory events occurring in *P. aeruginosa* have shown to be dependent or independent of PqsR (Farrow et al., 2008; Rampioni et al., 2016; García-Reyes et al., 2021) and because PqsE and PqsR are part of the same system and both regulate the *pqsA* promoter, it becomes interesting to analyse their interdependency in this regulation.

5 Chapter Five: New insights into the regulation of the *pqsABCDE* operon

5.1 Introduction

This thesis has shown that the PqsE-mediated regulation of *pqsA* occurs at the post-transcriptional level and that neither *PA2705* nor *nirQ* are essential for this mediated regulation. Nevertheless, these findings contribute to increase our understanding of this regulation, revealing the existence of a higher level of complexity. In the present chapter, a broader approach is undertaken to help characterizing the post-transcriptional regulation of *pqsA*, followed by a detailed examination of the regulatory components surrounding the *pqs* operon that could play a key role on finding the accurate path towards the elucidation of the PqsE-mediated repression of P_{pqA}.

5.2 Analysis of the regulation of *pqsA* at the post-transcriptional level

The present work has demonstrated that the -311 *rhlR-box* within the P_{pqsA} is a pivotal element for the accurate understanding of the regulation of *pqsA* and that the PqsE-mediated repression of *pqsA* occurs at the post-transcriptional level. Like PqsE, RhlR is an important player within the regulation of *pqsA,* in fact, the link between these two regulators has been reported (Groleau & Pereira., 2020) suggesting that these regulators may also contribute to each other's role in the regulation of this promoter. As described in **Chapter 1**, the P_{pqsA} promoter region holds a putative LysR box, centred at -45 bp relative to the *pqsA* transcriptional start site, in which PqsR binds to promote the expression of the *pqsABCDE* operon. In the same DNA region, two putative *las/rhl* boxes centred at -151 bp and -311 bp have also been identified (Xiao et al., 2006).The analysis of these two putative boxes indicated that whilst the -311 *las/rhl* box holds a very conserved sequence elements, the -151 *las/rhl box* does not. In fact, deletion of the -311 *las/rhl* box significantly increased the transcription of *pqsA* in the *P. aeruginosa* wild type but did not in the absence of *rhlR*, suggesting that RhlR binds to the -311 l*as/rhl box* to cause a final repression of the transcription of *pqsA* (Xiao et al., 2006) . On the contrary, Brouwer et al., (2014) stated that not only PqsR but also RhlR is an activator of the expression of *pqsA*. The authors suggested the latter uses the alternative transcriptional start site centred at -339 bp upstream of the ATG codon encoding for the *pqsA* gene (Eckweiler et al., 2012). This alternative transcriptional start site is closely located downstream of the *las/rhl box* reported by Xiao *et al*., (2006), which led to hypothesise that the final repression upon *pqsA* observed by these authors occurred via a post-transcriptional mechanism. By using secondary structure prediction analysis, the authors showed that albeit RhlR promotes the transcription of the *pqsABCDE* operon **(Figure 5.1),** the resulting transcript creates a folding structure that mask the translation initiation site of the operon, specifically by sequestering the Shine Dalgarno (SD) sequence and blocking access to the ribosomal subunit 30S promoting this way a final decrease of the translation

efficiency (Brouwer et al., 2014). The translational levels of *pqsA* using a *lux* fusion indicated that PqsR can induce the translational expression of *pqsA* when RhlR is absent, due to the abolishment of the RhlR-induced transcript. Likewise, no translational expression of *pqsA* was observed in a *pqsR* mutant due to the masking effect of the RhlR-induced transcript. Despite of the fact that the presented data is compatible with the post-transcriptional mechanisms proposed by the authors, the conflict arises as the creation of a translational fusion nominated 'plong', which held both *pqsA-339* and *pqsA-71* promoter regions and the additional 70 bp downstream *pqsA-71* was not fused to the CTX-*lux* LuxC protein, which induction would lead to the formation of an hybrid protein and that characterizes the translational fusions. It is suggested, therefore that the conclusions made for this regulation were based on a transcriptional reporter, as the promoter region of *pqsA* was placed in the MCS of the CTX-*lux* reporter and far from the translational start codon and Ribosome binding site (RBS) of the *lux* protein, hence, generating a single transcriptional unit with no hybrid proteins (Hand & Silhavy, 2000). Consequently, the results presented by the authors may not reflect exactly how RhlR downregulates *pqsA* and the accurate mechanism needs further examination as it could be pivotal for the understanding of the PqsE-mediated repression.

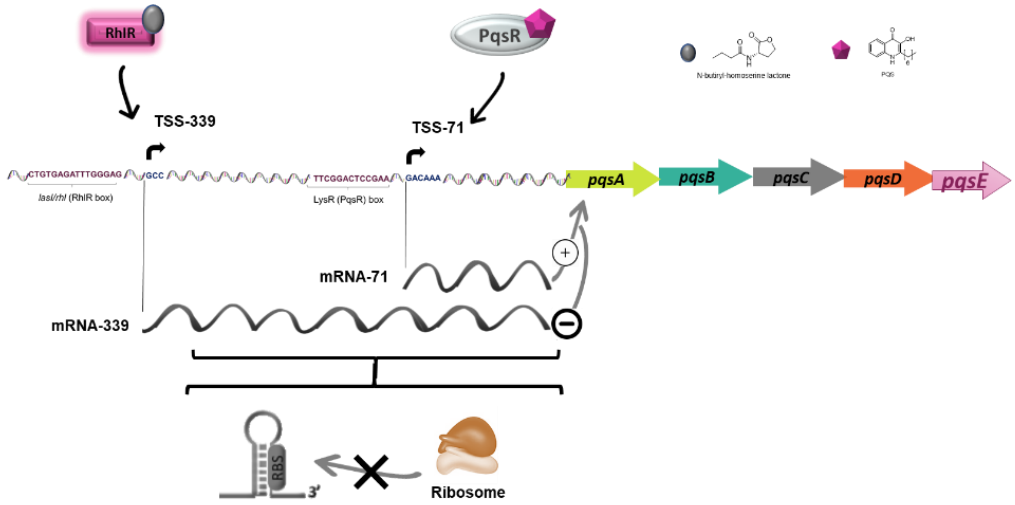


Figure 5.1. Currently accepted model of transcriptional and translational regulation of the *pqsABCDE* operon by RhlR.

The (C4-HSL)-RhlR complex and the PqsR-PQS induce transcription of the *pqs*ABCDE operon. The Transcriptional start site (TSS) -339 and (TSS) -71 are governed by RhlR and PqsR, respectively. The -339 induced mRNA is proposed to form a secondary structure that masks the translation initiation site of *pqsA*, specifically by blocking the access of the ribosome to the RBS sequence, resulting in a post-transcriptional repression of *pqsA*, balancing the production of AQs in *P. aeruginosa*. Modified from Brouwer et al., (2014).

5.3 The mystery of *pqsE* upstream region

To date, the regulation of the *pqsABCDE* operon has been studied as a whole, however, some evidence has revealed regulatory events occurring within the *pqs* operon under specific environments (Eckweiler et al., 2012; Knoten et al., 2014). These findings evidence the dynamics and plasticity of how an operon can be regulated and encourage a more detailed examination of these regions. Considering that PqsE plays independent roles *in P. aeruginosa* pathogenesis (Rampioni et al., 2010) it could be speculated that this effector is regulated independently from the *pqsABDC* operon. Analysis of the 500 bp region upstream of *pqsE* using BPROM database (Solovyev, 2016) revealed new insights into the existence of an additional layer of regulation occurring within this region **(Figure 5.2).** First, a Shine- Dalgarno (SD) like motif 'AGGAGG' (Shine and Dalgarno, 1975) placed 10 bp upstream from the ATG start codon of *pqsE* was identified. **(Figure 5.2).** A potential -10 and -35 promoter sequences were also found, suggesting a probable initiation of mRNA transcription. Furthermore, a cAMP receptor protein (CRP) like box 'TGTGATCT' overlapping the -35 site in the *pqsE* promoter was identified, suggesting that the *P. aeruginosa* CRP homologue virulence factor regulator (Vfr) may regulate *pqsE*.

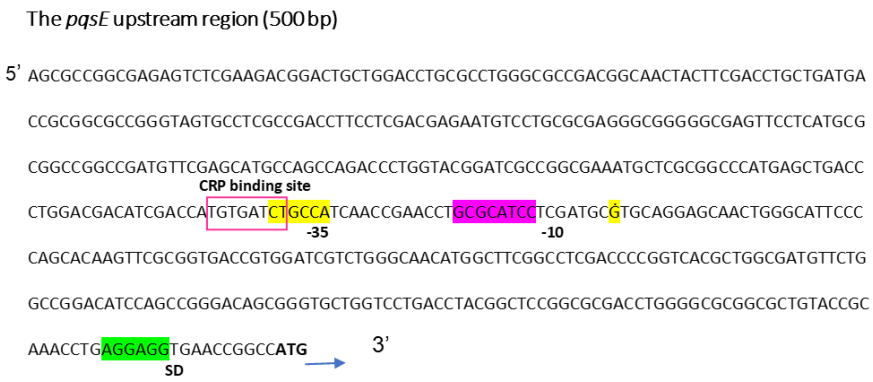


Figure 5.2. The *pqsE* upstream region.

DNA sequence of 500 bp directly upstream of the *pqsE* translational start codon is represented. Shine-Dalgarno sequence is highlighted in green (SD). Predicted -10 and -35 regulatory elements are shown in purple and yellow, respectively. A CRP like box (squared in red) is found overlapping the -35 sequence. For CRP-like proteins to modulate gene expression, the CRP-cAMP complex binds to a specific sequence within the promoter region. This sequence is usually found upstream of core promoter elements (Class I), overlapping the −35 element (Class II), or indirectly via interactions with alternative co-regulators (Class III) (Busby & Ebright., 1999; Lawson et al., 2004)

5.4 The Vfr regulator in *P. aeruginosa*

The ability of *P. aeruginosa* to cause damage to a specific host mostly depends on the production of virulence factors (Sadikot et al., 2005). In this pathogen, the production of many of these is directly or indirectly controlled by the transcriptional regulator Vfr. This regulator positively regulates *toxA*, regA, a type III secretion system (T3SS) as well as the *las* and the *rhl* quorum sensing systems (Albus et al., 1997; Croda-García et al., 2011; Fuchs et al., 2010; Medina et al., 2003; West et al., 1994). In *E. coli* the Vfr homologue CRP, is tightly linked to the levels of the second messenger signal adenosine 3,5-cyclic monophosphate (cAMP). The cAMP-dependent signaling system, controls metabolism in response to available carbon sources. Here, cAMP operates as a positive regulator of the cAMP receptor protein CRP (Rickenberg, 1974). In *E. coli,* the formation of the cAMP-CRP complex results in the regulation of hundreds of genes and operons, most of which participate in catabolic repression (Gosset et al., 2004). In *P. aeruginosa*, *vfr* encodes a 24.225 Da protein 67% identical and 91% similar to *E. coli* CRP (West et al., 1994). In

contrast to the metabolic role of CRP in *E. coli*, in *P. aeruginosa*, cAMP plays a key role in controlling virulence.

As previously reported, the CRP/FNR superfamily of transcription factors, require cAMP for their activation, however, some reports indicate that the ligand sensing and subsequent response of Vfr differs biochemically from that of CRP where Vfr has a significantly higher affinity for cAMP and it can also be activated by cGMP. This distinct properties of Vfr have been related to the specific differences in their ligand pocket region, where Vfr has a threonine at position 133 whilst CRP possesses a serine at the analogous position 128. This was further validated when mutation of threonine at the position 133 showed poor cAMP affinity and it was no longer responsive to cGMP, displaying a slight reduction in DNA-protein interaction (Serate et al., 2011). These observations suggest tha although the majority of the amino acid residues associated with cAMP and DNA binding in both bacteria are conserved, small amino acid substitutions can have a significant impact on the functionality of these proteins. This was illustrated by West et al., (1994), who showed that expression of *vfr* in *E. coli* was able to complement a *crp* deficient mutant and mediate cAMP-modulated catabolic repression. In contrast, *crp* was unable to completement a *vfr* mutant in the regulation of exotoxin A or protease production in *P. aeruginosa.* In the same fashion, *vfr* partially restored the β-galactosidase activity in a *E. coli crp cya* mutant (which does not produce cAMP), but it failed to complement the mutation of *crc* in *P. aeruginosa*, which encodes to the global regulator of catabolite repression control (CRC), further evidencing that Vfr and CRC and not exchangeable between these two organisms despite their structural similarities (Suh et al., 2002)

Vfr autoregulates its own expression in a cAMP-dependent manner (Fuchs et al., 2010). It also impacts on the regulation of the *las/rhl* systems as Vfr positively regulates the transcription of *lasR* (**Figure 1.3**) although in a non cAMP-dependent manner (Fuchs et al., 2010). Based on this data, Vfr has been suggested to act as a regulator placed at the top of the QS cascade, as there is not yet another known regulator that directly mediate the activation of *lasR* (Albus et al., 1997). Vfr also regulates *rhlR* (Medina et al., 2003) binding

several *vfr* boxes located within the *rhlR* promoter region. Binding to one of these boxes has been shown to have a negative impact on *rhlR* transcription (Croda-García et al., 2011). Considering that Vfr has an important impact on QS and virulence factor production, a link between Vfr and the *pqs* regulatory system cannot be discarded.

5.5 Aims of the chapter

This chapter focuses on the study of the *pqsA* repression at the post-transcriptional level, aiming to gain further insights into the mechanism by which QS regulators and PqsE drive the repression of *pqsA*. In more detail, this work aims to:

* Characterize the *pqsA* regulation at the post-transcriptional level and establish the impact of the main QS regulators on this regulation.
* Validate the mechanism by which RhlR represses *pqsA*
* Unveil the potential role of Vfr towards the *pqs*E regulation

5.6 Results

5.6.1 The PqsE-mediated repression towards *pqsA* is dependent on PqsR.

To date, the regulation of *pqsA* has been studied at the transcriptional level (Xiao et al., 2006; Diggle et al., 2007; Hazan et al., 2010; Rampioni et al., 2010), however, previously in this work, it was demonstrated that PqsE is a final repressor of the expression of *pqsA* and that this regulation occurs at the post-transcriptional level (**Chapter 3, Figure 3.11).** In addition, considering that LasR, RhlR and PqsR were binding the P_{pqsA} in the pull down carried out at the late stage of growth of *P. aeruginosa* (**Chapter 4, section 4.2**), led to interrogate how *pqsA* is post-transcriptionally regulated by other key QS regulators known to participate in the regulation of *pqsA* and whether PqsE is still able to repress the *pqsA* promoter in their absence. For consistency in the PAO1-L background, these experiments were also performed at the transcriptional level to establish a robust comparison between the regulation at both transcriptional and post-transcriptional levels in this strain.

To achieve this, in-frame deletions of *pqsR*, *lasR* and r*hlR*, and their possible combinations **(Table 5.1)** were obtained within the PAO1-L and PAO1-L *pqsE* Ind genetic background as described in **Chapter 2, section 2.6.3 and 2.6.4** and subsequently verified as shown in **Supplementary data, Figure S7.3**.

Table 5.1. In-frame deletion mutants in PAO1-L and the PAO1-L *pqsE* Ind strains.

Strain	Description
pqsE Ind	PAO1 derivative in which *pqsE* expression is under the control of a P*tac* promoter.
Δ*rhlR pqsE* Ind	PAO1 *rhlR* mutant derivative in which *pqsE* expression is under the control of a P*tac* promoter
*Δrhl*R Δ*pqsR*	PAO1 derivative carrying in-frame deletions of *rhlR* and *pqsR*
Δ*rhR* Δ*pqsR pqsE* Ind	PAO1 *rhlR pqsR* double mutant derivative in which *pqsE* expression is under the control of a P*tac* promoter
Δ*lasR pqsE* Ind	PAO1 *lasR* mutant derivative in which *pqsE* expression is under the control of a P*tac* promoter
Δ*lasR* Δ*pqsR pqsE* Ind	PAO1 *lasR pqsR* double mutant derivative in which *pqsE* expression is under the control of a P*tac* promoter
Δ*rhR* Δ*lasR pqsE* Ind	PAO1 *rhlR lasR* double mutant derivative in which *pqsE* expression is under the control of a P*tac* promoter
Δ*pqsR*	PAO1 derivative carrying in-frame deletion of *pqsR*
Δ*pqsR pqsE* Ind	PAO1 *pqsR* mutant derivative in which *pqsE* expression is under the control of a P*tac* promoter
Δ*rhlR* Δ*lasR* Δ*pqsR*	PAO1 derivative carrying in-frame deletions of *rhlR*, *lasR* and *pqsR*
Δ*rhlR* Δ*lasR* Δ*pqsR pqs* Ind	PAO1 *rhlR lasR pqsR* triple mutant derivative in which *pqsE* expression is under the control of a P*tac* promoter
Δ*lasR* Δ*pqsR*	PAO1 derivative carrying in-frame deletions of *lasR* and *pqsR*

In the following section, the impact of the mutation of the main QS regulators LasR, RhlR and PqsR towards the transcription and translation of *pqsA* will be described. To perform these experiments, the transcriptional $P_{pqsA\ long}$-lux and the translational $P_{pqsA\ long}$'-'-*luxCDABE* were used to follow the expression patterns in these *P. aeruginosa* strains *(*)*. The results are shown below in separated graphs (**).

(*) Note that IPTG alone did not significantly impact the transcription or translation of *pqsA* in PAO1-L, *rhlR, lasR, pqsR, rhlR lasR*, *lasR pqsR*, *rhlR pqsR* or *rhlR lasR pqsR* mutants (**Supplementary data, Figure S7.6.1-2**).
(**) Growth curves can be found in **Supplementary data, Figure S7.8**.

5.6.1.1 Impact of RhlR on *pqsA* transcription and translation

RhlR represses the *pqsA* promoter by directly binding to the *las/rhl* box, 5'CTGTGAGATTTGGGAG3', centred at -311 bp upstream of the *pqsA* TSS (Xiao et al., 2006). as well as directly repressing *pqsR* (Wade, et al., 2005), hence it is expected that its mutation affects the homeostasis of the levels of *pqsA*. Briefly, when compared to the wild type parent strain, mutation of *rhlR* slightly increased the transcription and translation of *pqsA* (**Figure 5.3**) although in the latter it showed a biphasic activation, indicative of post-transcriptional events that led to increase the translation of *pqsA* in the absence of *rhlR* and that were not evidenced using a transcriptional reporter. Based on this, it can be concluded that RhlR is a repressor of the *pqsA* expression.

Interestingly, in the uninduced PAO1-L *pqsE* Ind the transcription of P_{pqsA} decreased when compared to the wild-type and the IPTG-induced expression of *pqsE* did not change this reduction in the transcription of P_{pqsA} (**Figure 5.3A**). In contrast, the same experiment performed with the *pqsA* translational fusion showed that, in the condition with uninduced *pqsE*, the translation of *pqsA* was highly increased but addition of IPTG (*pqsE* Ind + IPTG) resulted in a strong repressor activity of PqsE over the translation of *pqsA.* (**Figure 5.3B**). Furthermore, in the absence of RhlR and PqsE (uninduced *pqsE* Ind), the transcriptional activity of P_{pqsA} increased, but unexpectedly the IPTG-induced expression of *pqsE* in this double mutant resulted in a further increase in *pqsA* transcription. In contrast, when using the *pqsA* translational fusion in this double mutant there was a vast increase in the translation of *pqsA* which was strongly repressed upon IPTG-induced expression of *pqsE*. Therefore, PqsE is only able to exert a repressor activity over *pqsA* expression in a post-transcriptional event both in the presence and absence of RhlR.

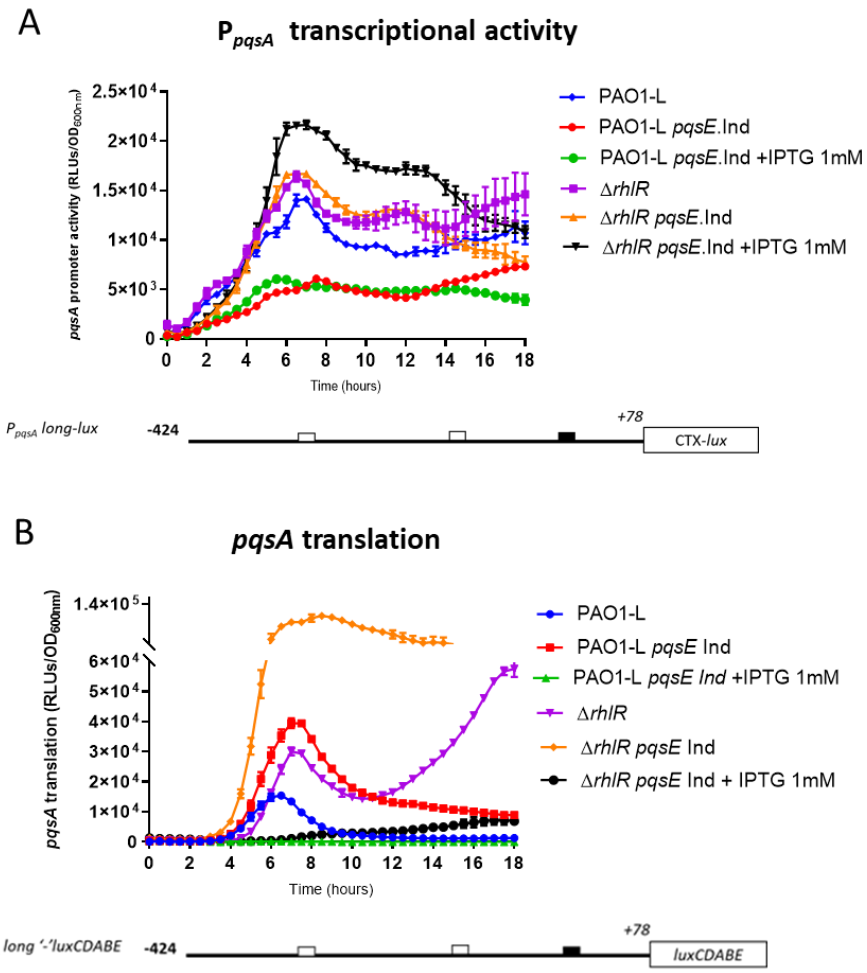


Figure 5.3. The transcription and translation of *pqsA* in the absence of *rhlR* in *P. aeruginosa*.

A) The transcriptional activity and B) The translation of *pqsA* is shown. Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*rhlR* and Δ*rhlR pqsE* Ind strains harboring the $P_{pqsA\ long}$-*lux* (Gm^R) and the $P_{pqsA\ long\text{-}'\text{-}'}$-*luxCDABE* ($Gm^R$) bioreporter (illustrated) in LB broth. Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.1.2 Impact of LasR on *pqsA* transcription and translation

LasR is a positive regulator of *pqsR*, which ultimately causes an induction of *pqsA*, hence its mutation is also expected to alter the P_{pqsA} homeostasis.

When compared to the wild-type parent strain, mutation of *lasR* slightly decreased the transcription of P_{pqsA} (Figure 5.4B) whilst it abrogated the

translation of *pqsA* until late stationary phase (Figure 5.4B) validating that LasR is an activator of *pqsA.*

In the absence of LasR and PqsE (uninduced *pqsE* Ind), the transcription (**Figure 5.4A**) and translation (**Figure 5.4B**) of *pqsA* notably increased, but it was severely advanced at the transcriptional level whereas considerably delayed at the translational level. Interestingly, the transcription of P_{pqsA} remained unalterable after IPTG-induced expression of *pqsE* in this double mutant, whereas the translation of *pqsA* was completely abrogated in the same condition, suggesting that *lasR* is not essential for the PqsE-mediated repression at the translational level.

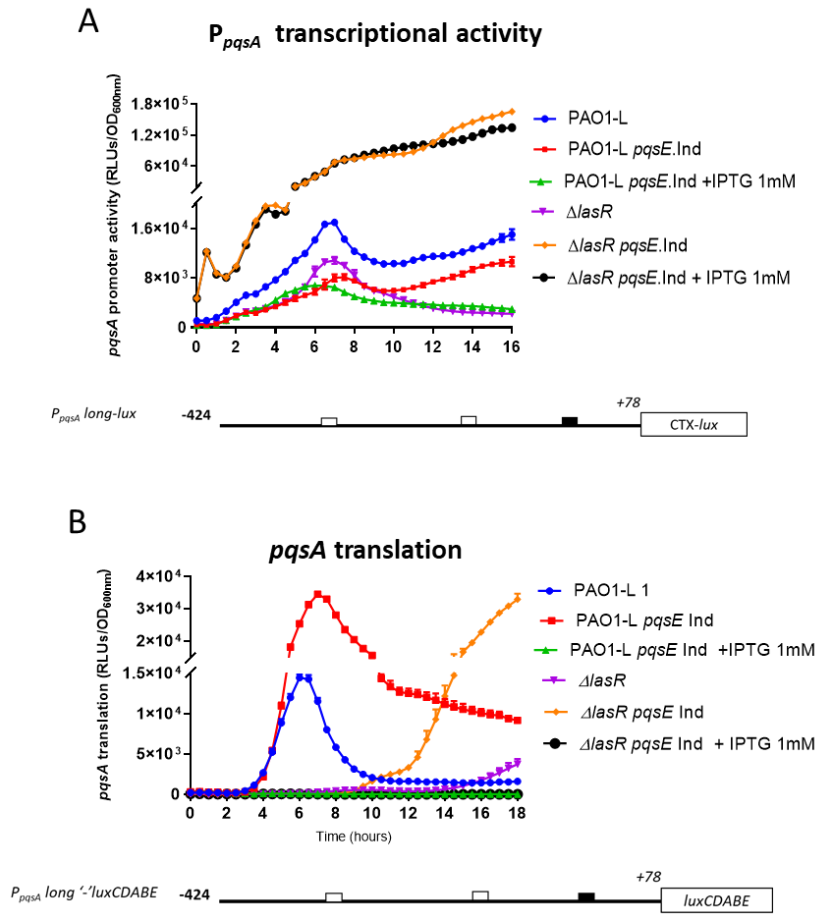


Figure 5.4. The transcription and translation of *pqsA* in the absence of *lasR* in *P. aeruginosa*.

A) The transcriptional activity and B) The translation of *pqsA* is represented. Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*lasR* and Δ*lasR pqsE* Ind strains harbouring the $P_{pqsA\ long}$-*lux* (Gm^R) and the $P_{pqsA\ long}$-*luxCDABE* (Gm^R) bioreporter (illustrated) in LB broth. Strains were grown at 37 °C for 18 hours in a 96-well TECAN plate. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.1.3 Impact of LasR and RhlR on *pqsA* transcription and translation

When compared to the wild-type parent strain, a double mutation of *lasR rhlR* slightly decreased the transcription of P_{pqsA} (**Figure 5.5A**) whilst increased the translation of *pqsA* in a biphasic way (Figure 5.5B), following a pattern similar to that observed in the presence of the single *rhlR* mutant (**Figure 5.3B**). When the same mutations are present within the uninduced *pqsE* Ind condition, a significant increase in the transcription of P_{pqsA} was observed whereas the translation of *pqsA* was not only reduced but also delayed in a similar way to *lasR* single mutant (**Figure 5.4B**). Finally, IPTG-induced expression of *pqsE* reduced the transcription of P_{pqsA} whilst abrogated the translation of *pqsA* when compared to the same condition with an uninduced *pqsE*, suggesting that the LasR/RhlR regulators are not essential for the PqsE-mediated translational repression.

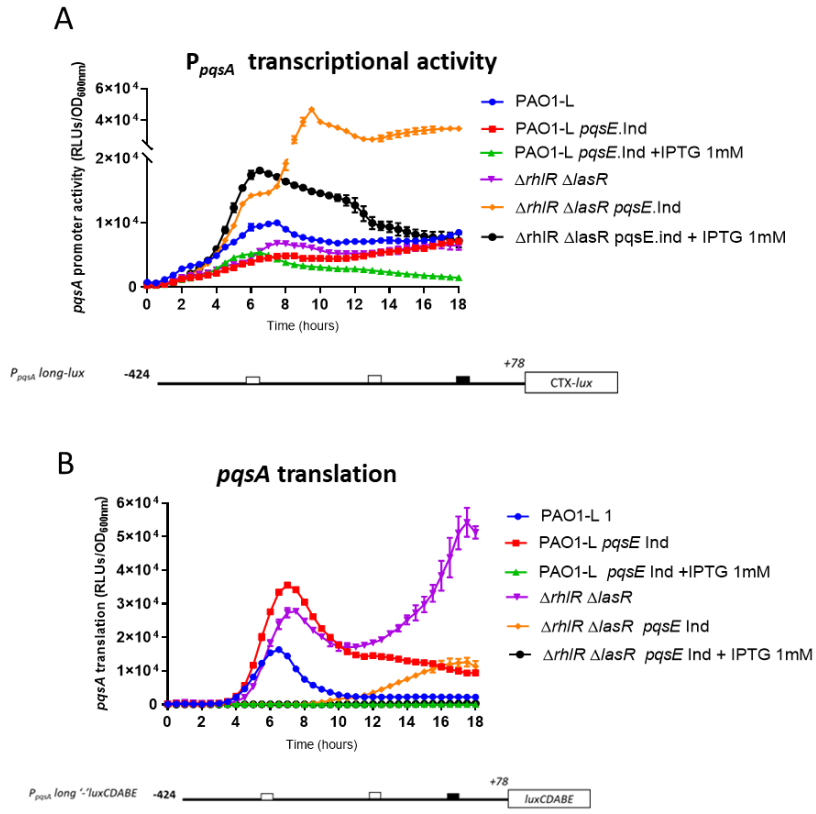


Figure 5.5. The transcription and translation of *pqsA* in the absence of *rhlR* and *lasR* in *P. aeruginosa*.

A) The transcriptional activity and B) The translation of *pqsA* is displayed. Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*rhlR* Δ*lasR* and Δ*rhlR* Δ*lasR pqsE* Ind strains harbouring the $P_{pqsA\ long}$-*lux* (Gm^R) and the $P_{pqsA\ long}$ -/-*luxCDABE* (Gm^R) bioreporter

(illustrated) in LB broth. Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.1.4 Impact of PqsR on *pqsA* transcription and translation

PqsR induces P_{pqsA} by directly binding the LysR-type box 5'TTCGGACTCCGAA3' with dyad symmetry at -45 bp before the *pqsA* TSS (Xiao et al., 2006)., and its mutation is expected to abrogate the P_{pqsA} activity (Wade, et al., 2005). When compared to the wild-type, mutation of *pqsR* significantly lowered the transcription of P_{pqsA} (**Figure 5.6A**), this trend was observed from the early stages to the late exponential phase of growth however, this activity gradually increased thereafter reaching nearly wild type levels when early stationary phase was reached. On the other hand, the same mutation completely abrogated the translation of *pqsA* (**Figure 5.6B**) which suggests that post-transcriptional events occurred in the absence of this regulator that finally reflected the inductor role of PqsR in the regulation of *pqsA.*

In the absence of PqsR and PqsE (uninduced *pqsE* Ind), the transcription of P_{pqsA} was reduced, and the subsequent IPTG-induced expression of *pqsE* decreased these levels from early stationary phase. In the same double mutant, the translation of *pqsA* was not only drastically induced but also significantly advanced, which could not be restored to the levels of *pqsR* mutant upon induction of *pqsE* expression by IPTG, suggesting that the PqsE-mediated repression towards *pqsA* translation is a PqsR-dependent mechanism.

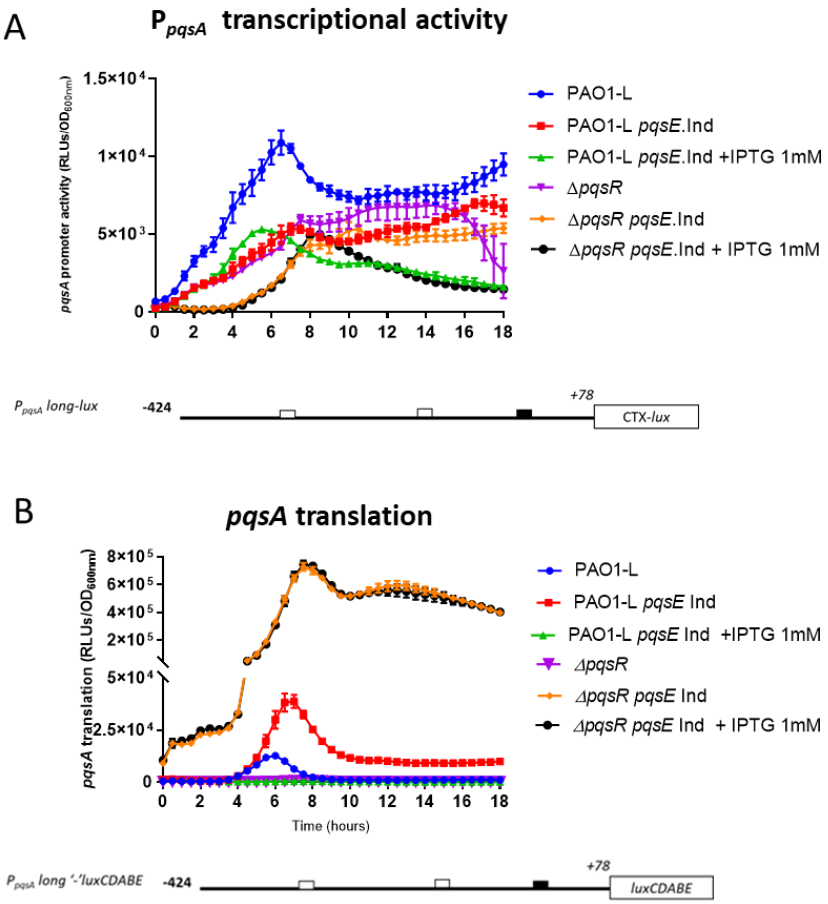


Figure 5.6. The transcription and translation of *pqsA* in the absence of *pqsR* in *P. aeruginosa*.

A) The transcriptional activity and B) The translation of *pqsA* is displayed. Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*pqsR* and Δ*pqsR pqsE* Ind strains harbouring the $P_{pqsA\ long}$-*lux* (Gm^R) and the $P_{pqsA\ long}$ -'-'-*luxCDABE* (Gm^R) bioreporter (illustrated) in LB broth. Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. Error bars represent 2x standard errors calculated across three biological replicates.

Considering that a *pqsR* mutant does not produce PQS, and that this AQs has shown to act trough PqsR dependent and independent mechanisms (Rampioni et al., 2016) as well trough post-transcriptional events (Hazan et al., 2010), it was interesting to discern whether PQS or PqsR was the key component in this regulation. To address this, PQS was added to cultures of a *pqsR pqsE* Ind mutant in the presence and absence of IPTG. Results show that the addition of PQS to this condition caused an increment in the translation of *pqsA* when compared to the same condition without PQS **(Figure 5.7)**. Addition of HHQ, which does not form iron complex, also induced the translation of *pqsA*, suggesting that it was

the regulatory role of PQS that caused this induction. To confirm this observation, the addition the non-signalling quinolone molecule ; 2-Methyl-3-Hydroxy-4-Quinolone (mPQS) was used as a control, since this molecule is capable of binding iron like PQS, but is unable to trigger gene expression *via* PqsR (Diggle et al., 2007). Addition of this molecule to the cultures did not cause any impact on the translation of *pqsA* (**Figure 5.7A**), validating that is not the iron chelating properties of PQS responsible for this phenomenon.

Furthermore, the expression of pMENR7 (pME6032::*pqsR* + IPTG) in a *pqsR pqsE* Ind strain downregulated *pqsA* when *pqsE* was expressed (**Figure 5.7B**,**bottom**), effect that was enhanced when PQS was added externally, evidencing that this molecule is an enhancer of PqsR activity (Wade et al., 2005)

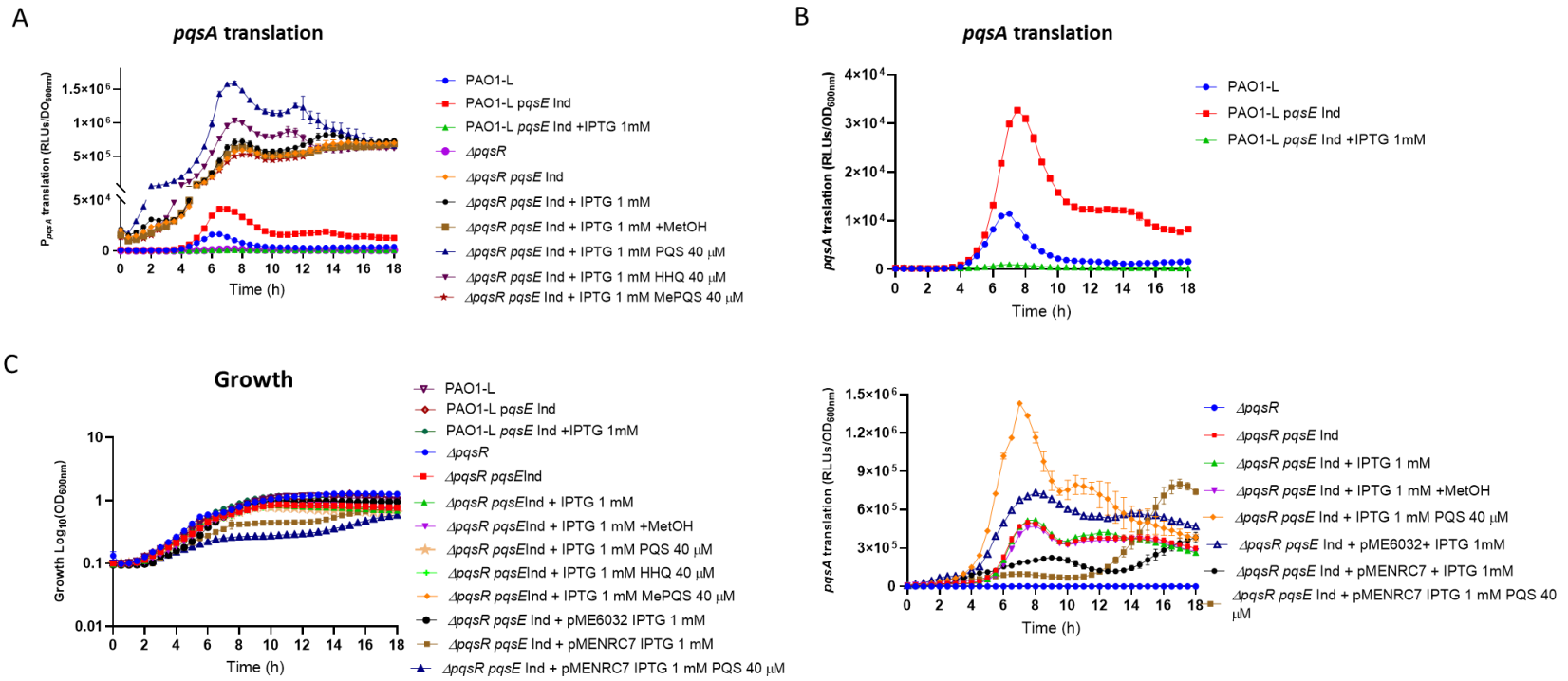


Figure 5.7. The PqsE-mediated repression of *pqsA* in *P. aeruginosa*.

Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*pqsR* and Δ*pqsR pqsE* Ind strains harbouring the $P_{pqsA\ long\ '-'}$-*luxCDABE* (Gm^R) bioreporter in LB broth. Strains were grown at 37°C for 18 hours in a 96-well TECAN plate A) The translation of *pqsA* in the presence of PQS, HHQ or mPQS at 40 uM B) The translation of *pqsA* is displayed in separated graphs (Top and Bottom). PQS was added to the cultures at 40 uM. IPTG at 1 mM was added to express *pqsR* from a plasmid (pMENRC7). C) The Log10 growth curve are shown. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.1.5 Impact of a *rhlR /pqsR* double mutant on *pqsA* transcription and translation

When compared to the wild-type condition, the double mutation of *rhlR* and *pqsR* presented slight but not significant changes in the transcription of P_{pqsA} (**Figure 5.8A**), whereas the translation of *pqsA* was not only advanced but greatly increased (**Figure 5.8B**). The latter mirrors the translation of *pqsA* when *pqsR* was mutated on its own (**Figure 5.6B**). The double *pqsR/rhlR* mutation within the uninduced *pqsE* Ind condition increased the P_{pqsA} transcription when compared to that in the PAO1-L *pqsE* Ind strain, an effect attributed to the lack of *pqsE*. IPTG-induced expression of *pqsE* within this double mutant increased the transcription of P_{pqsA}, reaching higher levels than the uninduced condition. The latter activity pattern follows the trend observed previously in **Figure 5.3A**, in which overexpression of *pqsE* within the *ΔrhlR pqsE* Ind condition induced the P_{pqsA} transcriptional activity, once more suggesting that PqsE behaves as an inductor of *pqsA* in the absence of RhlR; a trend that remained consistent regardless of the presence/absence of PqsR

The translation of *pqsA* in the double mutant *rhlR pqsR*, remained unchanged in the uninduced *pqsE* Ind condition even when *pqsE* was overexpressed (*pqsE* Ind + IPTG), suggesting that *pqsR* has a dominant role in controlling *pqsA* translation due to in its absence, neither the *las/rhl* system nor *pqsE* were able to alter the final translational expression of *pqsA*.

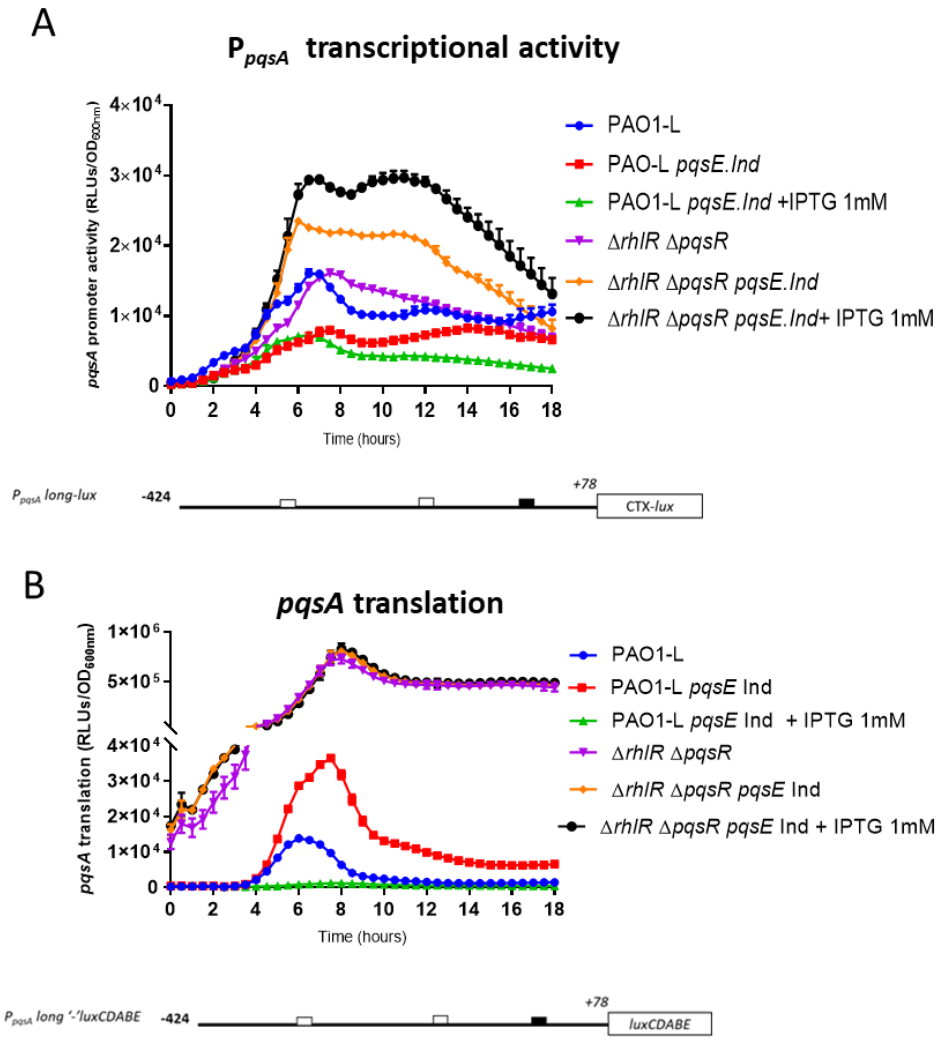


Figure 5.8. The transcription and translation of *pqsA* in the absence of *rhlR* and *pqsR* in *P. aeruginosa*.

A) The transcriptional activity and B) The translation of *pqsA* is displayed. Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*rhlR* Δ*pqsR* and Δ*rhlR* Δ*pqsR pqsE* Ind strains harbouring the $P_{pqsA\ long}$-*lux* (Gm^R) and the $P_{pqsA\ long}$ '-'-*luxCDABE* (Gm^R) bioreporter (illustrated) in LB broth. Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.1.6 Impact of a *lasR*/*pqsR* double mutant on *pqsA* transcription and translation

At the transcriptional level, mutation of *lasR* and *pqsR* reduced the transcription of P_{pqsA} when compared to the wild type (**Figure 5.9A**). The P_{pqsA} transcriptional activity pattern followed a similar trend than that from a *lasR* single mutant (**Figure 5.4A**). In addition, the transcription of P_{pqsA} remained unaltered in this

double mutant within the uninduced *pqsE* Ind condition and the IPTG-induced expression of *pqsE* did not impact on the transcriptional levels of P_{pqsA} although a slightly advancement was observed.

At the translational level, the double *lasR*/*pqsR* mutant dramatically increased the translation of *pqsA* (**Figure 5.9B**), following an expression pattern similar to that observed in a *pqsR* mutant (**Figure 5.6B**)**.** As observed above in the *rhlR pqsR pqsE* Ind mutant, mutation of *lasR* and *pqsR* within the uninduced *pqsE* Ind condition did not show any significant change in translation of *pqsA* when compared to the *lasR pqsR* double mutant or this mutant overexpressing *pqsE* (*pqsE* Ind + IPTG).

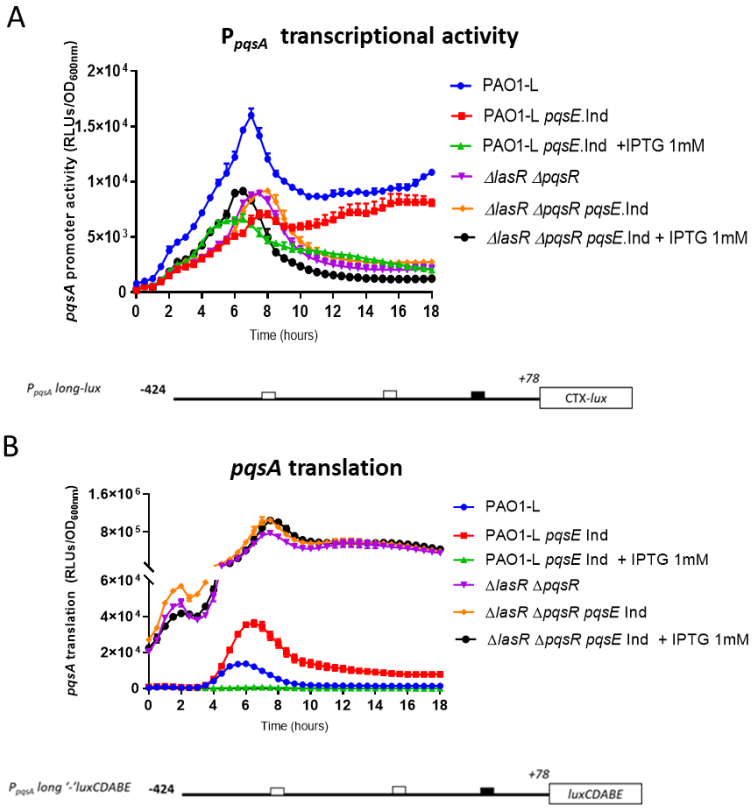


Figure 5.9. The transcription and translation of *pqsA* in the absence of *lasR* and *pqsR* in *P. aeruginosa*.

A) The transcriptional activity and B) The translation of *pqsA* is presented. Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*lasR* Δ*pqsR* and Δ*lasR* Δ*pqsR pqsE* Ind strains harbouring the $P_{pqsA\ long}$-*lux* (Gm^R) and the $P_{pqsA\ long}$ '-'~*luxCDABE* (Gm^R) bioreporter (illustrated) in LB broth. Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.1.7 Impact of a *lasR/rhlR/ pqsR* triple mutant on *pqsA* transcription and translation

When compared to the wild type, the triple *rhlR*/*lasR*/*pqsR* mutant showed a reduction in the transcription of P_{pqsA} (**Figure 5.10A**), which presented a similar activity pattern to that observed in a single *lasR* mutant (**Figure 5.4A**). The triple mutation within the uninduced *pqsE* Ind condition however, increased the transcription of P_{pqsA} when compared to that in the PAO1-L *pqsE* Ind, which reached temporal P_{pqsA} wild type levels between 5 and 7 hours of growth, followed by a constant decline in transcription of P_{pqsA} until late stationary phase. The IPTG-induced expression of *pqsE* in the absence of *rhlR*, *lasR* and *pqsR* showed no major differences when compared to that in the uninduced *pqsE* Ind condition, except for the slight, although not significant, induction of the transcription of P_{pqsA} observed between 6 and 9 h of growth. The latter result suggests once more, that *pqsE* behaves as an inductor of the transcription of P_{pqsA} in the absence of these regulators but the latter effect is only temporary.

At the translational level, mutation of *rhlR*, *lasR* and *pqsR* significantly increased the P_{pqsA} translational activity when compared the wild type (**Figure 5.10B**). No major changes were found in this triple mutant within the *pqsE* Ind condition in the absence or presence of IPTG, further supporting the key role of PqsR on the PqsE-mediated repression of *pqsA*.

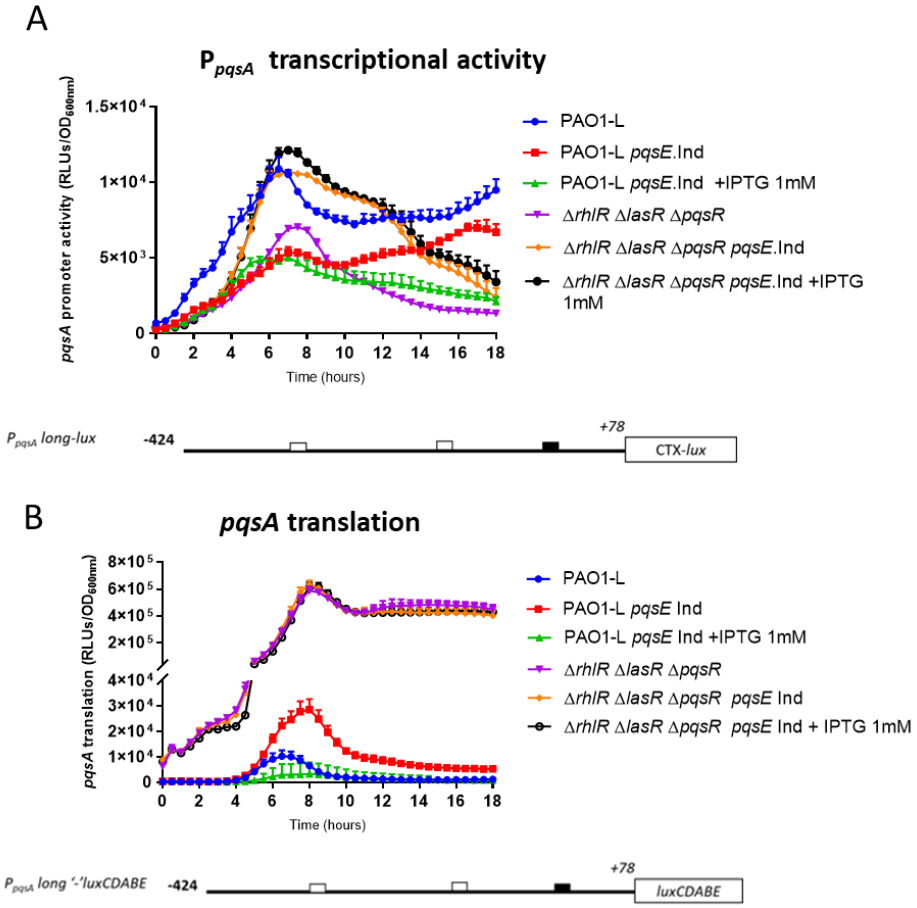


Figure 5.10. The transcription and translation of *pqsA* in the absence of *rhlR*, *lasR* and *pqsR* in *P. aeruginosa*.

A) The transcriptional activity and B) The translation of *pqsA* is presented. Normalised bioluminescence production for PAO1-L, PAO1-L *pqsE* Ind, Δ*rhlR* Δ*lasR* Δ*pqsR* and Δ*rhlR* Δ*lasR* Δ*pqsR pqsE* Ind strains harbouring the $P_{pqsA\ long}$-*lux* (Gm^R) and the $P_{pqsA\ long}$ '-'*luxCDABE* (Gm^R) bioreporter (illustrated) in LB broth. Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.2 New insights into the RhlR regulation towards *pqsA*: Analysis of two different transcripts and its repressive role towards this operon.

As previously indicated in Brouwer et al., (2014) the repression of *pqsA* under the control of RhlR was proposed to occur through a post-transcriptional event, in which RhlR, by inducing the expression of *pqsA*, creates a long mRNA transcript that forms a secondary structure in the 5' untranslated leader region, thereby masking the access to the RBS within the *pqsA* and subsequently, preventing the

translation initiation site of *pqsA*. In addition, in the same work, the authors validated this finding by analyzing the *pqsA* expression at the translational level, using a translational *lux* reporter named 'plong' which was incorrectly designed and hence behaved as a transcriptional reporter, questioning the accuracy of the proposed mechanism (**Figure 5.1**).

Analysis of the *pqsA* promoter region suggested the probable formation of two alternative transcripts formed within the *pqsA* promoter: a 258 bp length transcript (Term1) and 337bp length transcript (Term2). Both transcripts begin from the -339 TSS of *pqsA* and end prior the ATG start codon of *pqsA*. Term1 and Term2 were cloned into the shuttle vector pME6032 as described in **Chapter 2, section 2.6.6**, hence, in a manner that allows monitoring the native activity of these transcripts. The construction of these vectors is shown in **Supplementary data, Figure S7.9.**

5.6.2.1 Term1 and Term2 are repressors of *pqsA*

The constructed transcripts Term1 and Term2 were introduced into the PAO1-L wild type by conjugation and the impact on the transcription and translation of *pqsA* was recorder over time.

When compared to parent strain, the addition of the pME6032 empty vector caused a modest increase in the transcriptional activity of P_{pqsA} (**Figure 5.11A**), which was observed specifically at the stationary phase. Considering this, PAO1-L pME6032 was used as reference control for this analysis. As seen in **Figure 5.11A,** the transcripts Term1 and Term2 significantly and equally decreased the transcriptional activity of P_{pqsA} during the whole growth of *P. aeruginosa*, suggesting that these transcripts held the required elements to cause a repression of P_{pqsA}. On the other hand, Term1 and Term2 also decreased the translation of *pqsA* (**Figure 5.11B**), supporting the previous finding. It was intriguing that the induction of the pME6032 empty vector in this set up dramatically impacted the translation of *pqsA*, which requires further analysis and due to time constrains could not be completed. All in all, since Term1 and Term2 effectively repressed the *pqsA* promoter, from now and onwards these transcripts are referred as *pqsX-T1* and *pqsX-T2*.

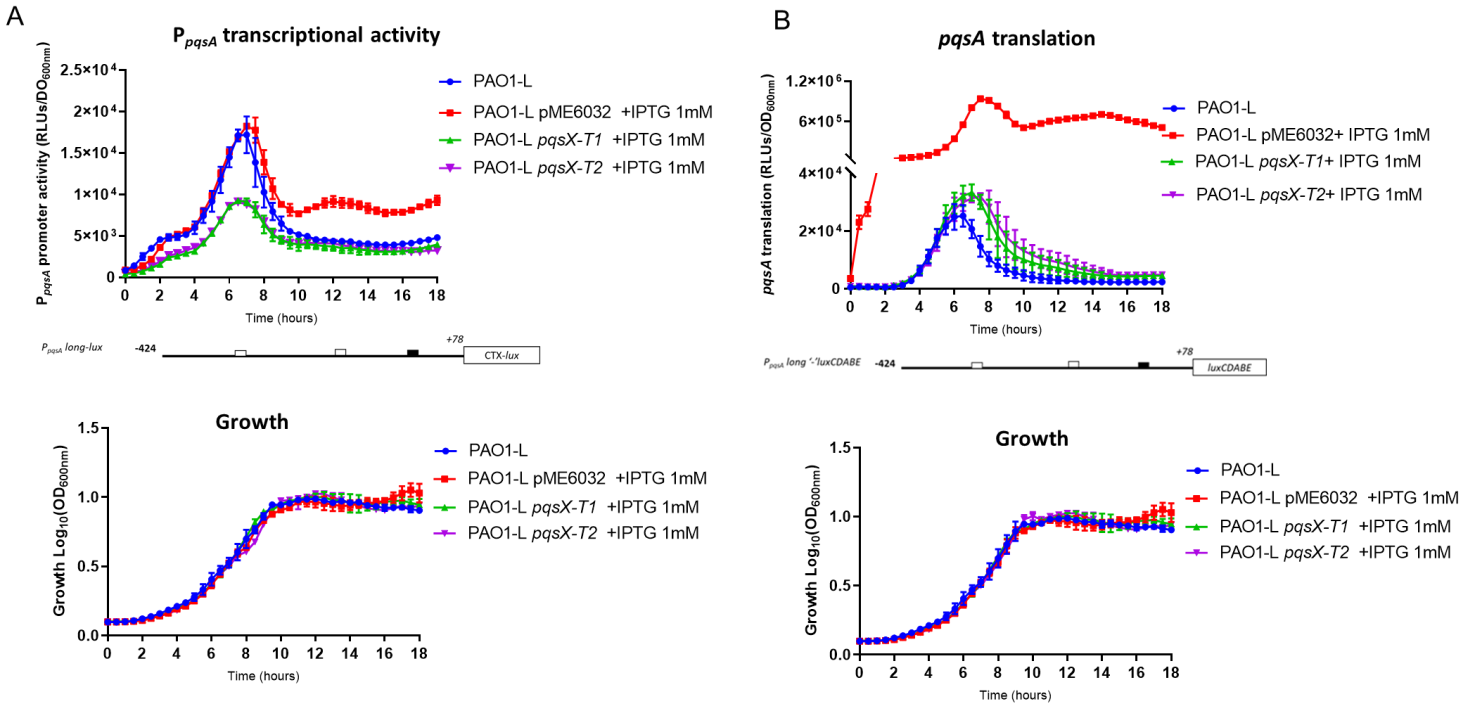


Figure 5.11. Impact of the transcripts Term1 (*pqsX-T1*) and Term2 (*pqsX-T2*) towards *pqsA* in *P. aeruginosa*.

Top: The A) transcriptional activity and the B) translation of *pqsA* is presented. Normalised bioluminescence production for strains PAO1-L, PAO1-L *pqsX-T1* and PAO1-L *pqsX-T2* carrying the transcriptional reporter $P_{pqsA\ long}$*-lux* (Gm^R) or translational version $P_{pqsA\ long'-'}$*-luxCDABE* (Gm^R) (illustrated) . Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. Bottom: The Log10 growth curve shows that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.2.2 Impact of *pqsX-T1* and *pqsX-T2* on *pqsE* expression

The previous work suggested that both alternative transcripts were able to downregulate the transcription and translation of *pqsA* which, therefore, should result in the downregulation of the *pqsABCD* operon, including *pqsE*. Since PqsE has dual roles as thioesterase and regulator of virulence in *P. aeruginosa* (Farrow et al, 2008), it cannot be discarded that this effector protein may be independently controlled by other regulatory elements, which could ultimately influence the P_{pqsE} activity in a separate manner. Considering the latter observation, it was interesting to study the impact of these alternative transcripts towards the P_{pqsE} activity in this pathogen. To address this, the reporter pMiniCTX::P_{pqsE}-lux was constructed as described in **Chapter 2, section2.6.1**. It is important to note is that since no research has been performed around the *pqsE* upstream region and aiming to avoid the omission of any regulatory element present in it, a total of 500 bp upstream of the *pqsE* gene were included for the construction of this reporter. The obtention of this reporter was validated as shown in **Supplementary data, Figure S7.4** and was named P_{pqsE}-lux.

The P_{pqsE}-lux reporter was then introduced in the chromosome of PAO1-L and the impact of *pqsX-T1* and *pqsX-T2* towards the activity of P_{pqsE} was recorded over time.

The activity of the upstream region of *pqsE* (P_{pqsE}) in the wild type condition presented a plateau level of maximum activity that peaked at the 7 hours of growth (**Figure 5.12**). A slight, not significant increase in P_{pqsE} activity was observed upon induction of the control pME6032 empty plasmid, which presented a similar pattern of P_{pqsE} activity when compared to the wild-type, trend that remained uniform during the whole growth of *P. aeruginosa*. In the same strain, the transcripts *pqsX-T1* and *pqsX-T2* strongly incremented the P_{pqsE} activity, impact that was equally observed in both conditions. The latter result clearly shows that *pqsX-T1* and *pqsX-T2* have a positive impact on the expression of *pqsE*.

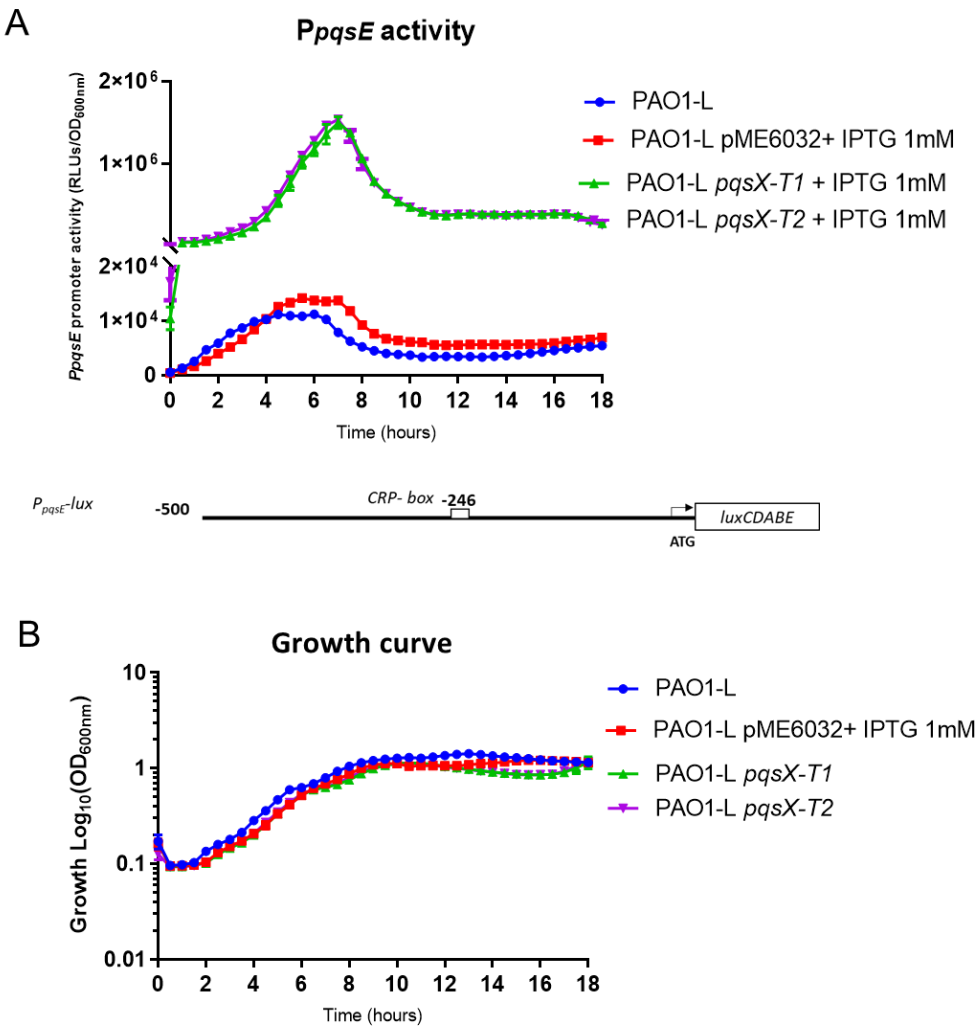


Figure 5.12. Impact of *pqsX-T1* and *pqsX-T2* towards the activity of P_{pqsE} in *P. aeruginosa*.

A) Normalised bioluminescence production for strains PAO1-L, PAO1-L *pqsX-T1* and PAO1-L *pqsX-T2* carrying the reporter P_{pqsE}-*lux* (Gm^R) (illustrated). Strains were grown at 37°C for 18 hours in a 96-well TECAN plate. B) The Log10 growth curve shows that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.2.3 Impact of *pqsX-T1* and *pqsX-T2* on the production of virulence traits

As presented above, the alternative transcripts downregulate P_{pqsA} whilst upregulate P_{pqsE}. Effective repression and induction of P_{pqsA} and P_{pqsE} respectively, caused by *pqsX-T1* and *pqsX-T2* is expected to have an impact on the production of virulence-related traits controlled by the *pqs* QS system. To investigate this, phenotypic assays including

the quantification of the AQs PQS, HHQ and HQNO as well as pyocyanin were carried out in *P. aeruginosa*.

5.6.2.3.1 Impact of *pqsX-T1* and *pqsX-T2* on alkyl quinolone (AQs) production in *P. aeruginosa*.

Quantification of AQs was performed at 7 hours of growth as at this time the P_{pqsA} activity peaked at both transcriptional and translational level.

The results are shown in **Figure 5.13A**. When compared to the wild-type carrying the empty pME6032 plasmid control, a modest reduction in PQS, HHQ and HQNO was caused by *pqsX-T1* and *pqsX-T2*, nevertheless this effect was not statistically significant.

Since no clear effect towards the AQs biosynthesis was observed at 7 hours, a second quantification at 12 hours of growth was performed as it should be a delay in their regulatory process. AQs quantification at 12 hours is shown in **Figure 5.13B**. The control pME6032 empty plasmid showed an impact on AQ production at this time point, nevertheless, *pqsX-T1* and *pqsX-T2* did not show any significant differences in PQS, HHQ or HQNO production. Strikingly, these results do not correlate with the impact towards the P_{pqsA} activity observed in **Figure 5.11**. which suggest the existence of post-transcriptional events or another level of regulation which may be involved in maintaining AQs homeostasis.

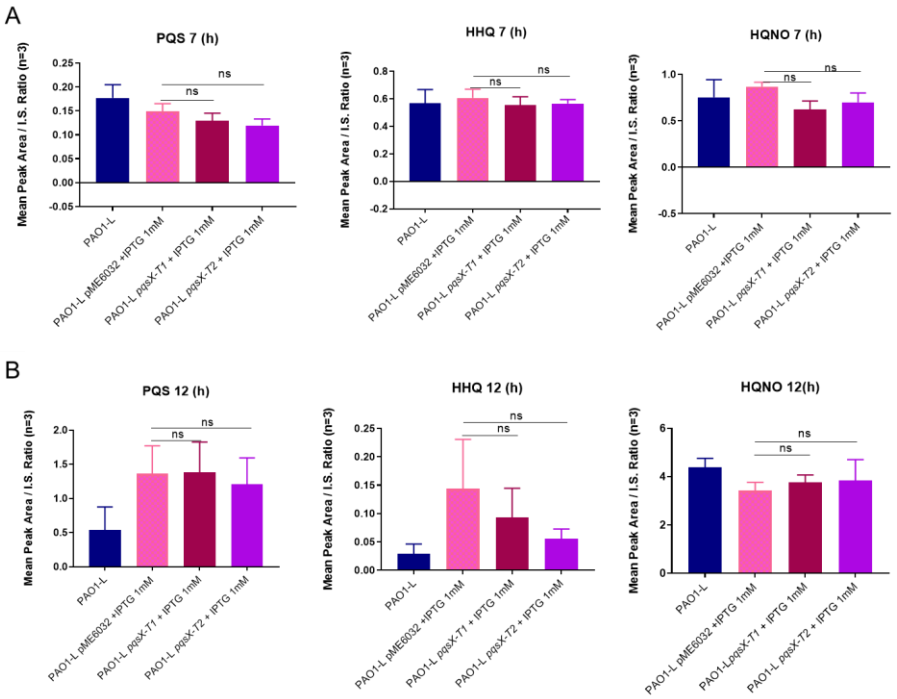


Figure 5.13. Impact of the *pqsX-T1* and *pqsX-T2* towards Alkyl-quinolone production in *P. aeruginosa*.

Bacteria were grown in flasks for A) 7 and B) 12 hours at 37° C in LB media. Quantification of PQS and HHQ was performed from sterile supernatants extracted with ethyl acetate. Error bars represent standard deviation of three biological triplicates. T-tests were used to assess for statistical significance.

5.6.2.3.2 Effect of *pqsX-T1* and *pqsX-T2* in pyocyanin production in *P. aeruginosa*

Pyocyanin production is known to be widely controlled by the *las/rhl* system, PqsR, PQS and PqsE, among others (Gallagher et al., 2002; Diggle et al., 2003; Farrow et al., 2008; Rampioni et al., 2010). Considering that the the transcripts *pqsX-T1* and *pqsX-T2* dramatically induced P_{pqsE} activity, it would be expected that these transcripts increase the levels of pyocyanin. Pyocyanin was quantified at 7 and 12 hours of growth. The results are shown in **Figure 5.14.** In contrast to what was anticipated, at 7 hours pyocyaning production decreased with respect to the empty vector controls whereas at 12 hours it reached similar levels to the control.

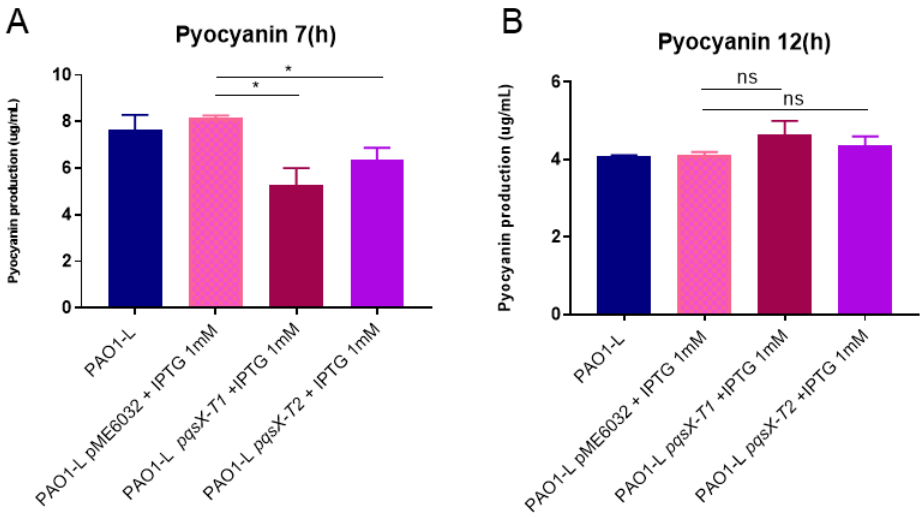


Figure 5.14. Impact of *pqsX-T1* and *pqsX-T2* towards pyocyanin production in *P. aeruginosa*.

Bacteria were grown in flasks for A) 7 and B) 12 hours at 37°C in LB Pyocyanin quantification was performed from sterile supernatants extracted with chloroform and measured at OD_{520nm}. Error bars represent standard deviation of three biological triplicates. T-tests were used to assess for statistical significance.

5.6.2.3.3 The transcript *pqsX-T2* restores pyocyanin production in a *P. aeruginosa pqsA* mutant

Pyocyanin is responsible for the blue-green color characteristic of Pseudomonas. Mutation of *pqsA* abolishes PQS and pyocyanin production (Rampioni et al., 2010). In order to investigate whether the alternative transcripts may be able to restore pyocyanin production in a *pqsA* mutant, the plasmid pME6032 harboring the transcripts *pqsX-T1* or *pqsX-T2* was conjugated into PAO1-L and PAO1-L *pqsA* mutant strains. In the wild type, the transcripts *pqsX-T1* and *pqsX-T2* enhanced the green pigmentation corresponding to the production of pyocyanin (**Figure 5.15**). When compared to the *pqsA* mutant condition, the initial expression of *pqsX-T1* or *pqsX-T2* were sufficient to restore pyocyanin production in the absence of *pqsA*, suggesting a probable role in virulence in *P. aeruginosa* in a PQS-independent manner. Nevertheless, after repetitive use of the strain, the pyocyanin phenotype was lost in the strain Δ*pqsA* expressing *pqsX-T1* (**Figure 5.15**). Intriguingly, this phenomenon was also observed after in the same strain expressing *pqsX-T2* (data not shown).

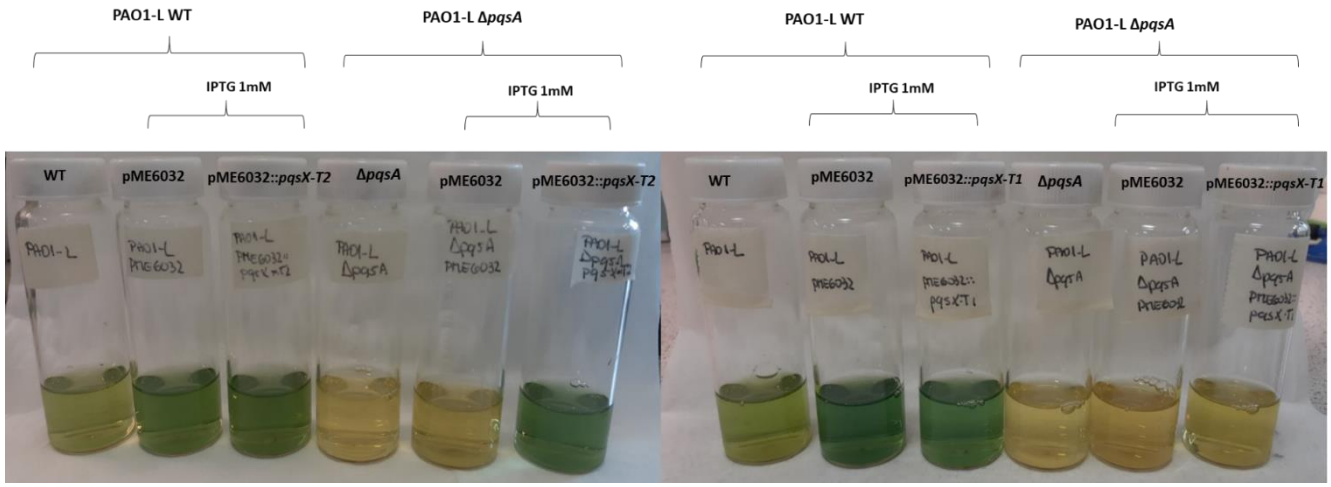


Figure 5.15. Pyocyanin production in *P. aeruginosa* PAO1- L and *pqsA* mutant expressing the transcripts *pqsX-T1* and *pqsX-T2*.

The pyocyanin production is abolished in a pqsA mutant. Initial expression of *pqsX-T1* and *pqsX-T2* restored the production of pyocyanin in the absence of *pqsA*, albeit it could only be recorded for strain Δ*pqsA pqsX-T2*. Strains were grown in 100 mL flasks in LB media for 16 hours at 37°C with agitation at 200 rpm.

5.6.2.3.4 The *pqsX*-T1 and *pqsX*-T2 transcripts failed to restore pyocyanin production in the absence of *pqsE*.

The *pqsX* transcripts considerable induced the P_{pqsE} activity in a wild type condition and specifically, the transcript *pqsX-T2* restored pyocyanin production in a *pqsA* mutant strain. The latter observation led to question whether this regulation is dependent on PqsE. To investigate this, transcript *pqsX-T2* and *pqsX-T1* were overexpressed in the absence of *pqsE*. Due to time limitations, this experiment was performed using the available *pqsE* mutant in the PAO1-N subline, hence, it is important to note the existence of a 58,569 bp deletion in the genome of this subline when compared to the PAO1 Washington strain (PAO1-W) (Stover et al., 2000) and PAO1-L (unpublished).

In a *pqsE* mutant, none of the transcripts were able to restore pyocyanin production (**Figure 5.16**) suggesting that the regulation of pyocyanin by these transcripts relies on PqsE.

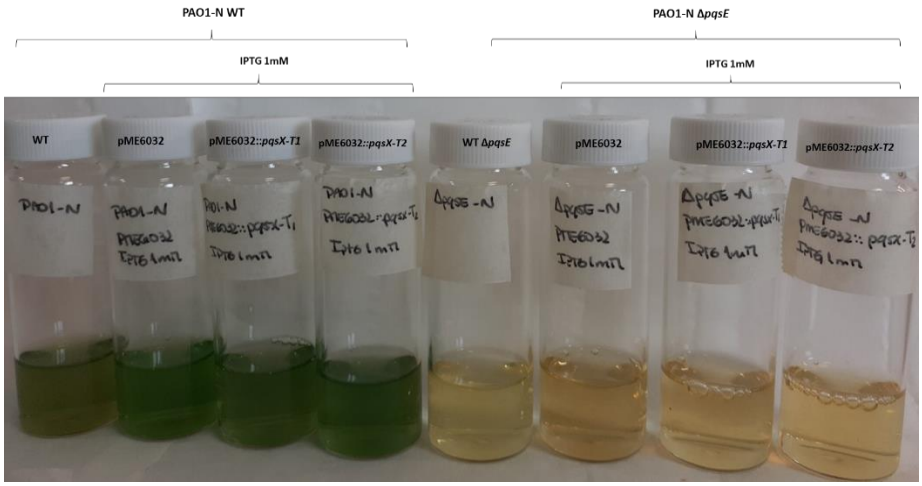


Figure 5.16. Pyocyanin production in *P. aeruginosa* PAO1-N *pqsE* mutant expressing the transcripts *pqsX-T1* and *pqsX-T2*.

The pyocyanin production is abolished in the absence of *pqsE*. Neither *pqsX-T1* nor *pqsX-T2* restored the production of pyocyanin. Strains were grown in 100 mL flasks in LB media for 16 hours at 37°C with agitation at 200 rpm.

5.6.3 Vfr as a potential regulator of *pqsE* expression

As described in **Chapter 1**, the *pqsABCDE* operon possesses two transcriptional start sites placed upstream and downstream of the start codon of *pqsA,* respectively. The latter observation suggests that the regulatory processes taking part within this regulation are not limited to the *pqsA* promoter, and that other regulatory events may occur in downstream regions, that could eventually differentially have an impact on the expression of the *pqsABCDE* genes. Considering that PqsE has shown to be a key regulator within the QS circuitry, and that its role in the production of some virulence factors has shown to be independent from the *pqs* system (Farrow et al., 2008; Rampioni et al., 2010), it was hypothesized that the regulation of *pqsE* may be take place via additional regulators. Analysis of the *pqs* operon suggested the presence of a CRP like box, which homologue in *P. aeruginosa* correspond to Vfr.

Chatterjee et al., (2002) Indicated in that CRP and MetR proteins bind to the *lux* promoter DNA, with CRP being dependent on the presence of cAMP, moreover, the authors demonstrated that whilst MetR had a negative impact on the production of luminescence, CRP was an activator of it. Using the reporter pMiniCTX::P_{tac}-lux, which produces high levels of luminescence, it was analyzed if the presence of Vfr impact the light output caused by this reporter. To address this experiment, the pMiniCTX::P_{tac}-lux was conjugated into PAO1-L and *vfr* mutant. The light output was monitored over time and the results are shown in **Figure 5.17**. Mutation of *vfr* in the wild type condition did not show major differences in luminescence when compared to the parent strain, thereby indicating that this regulator does not interfere with the final light output caused by the l*ux* promoter.

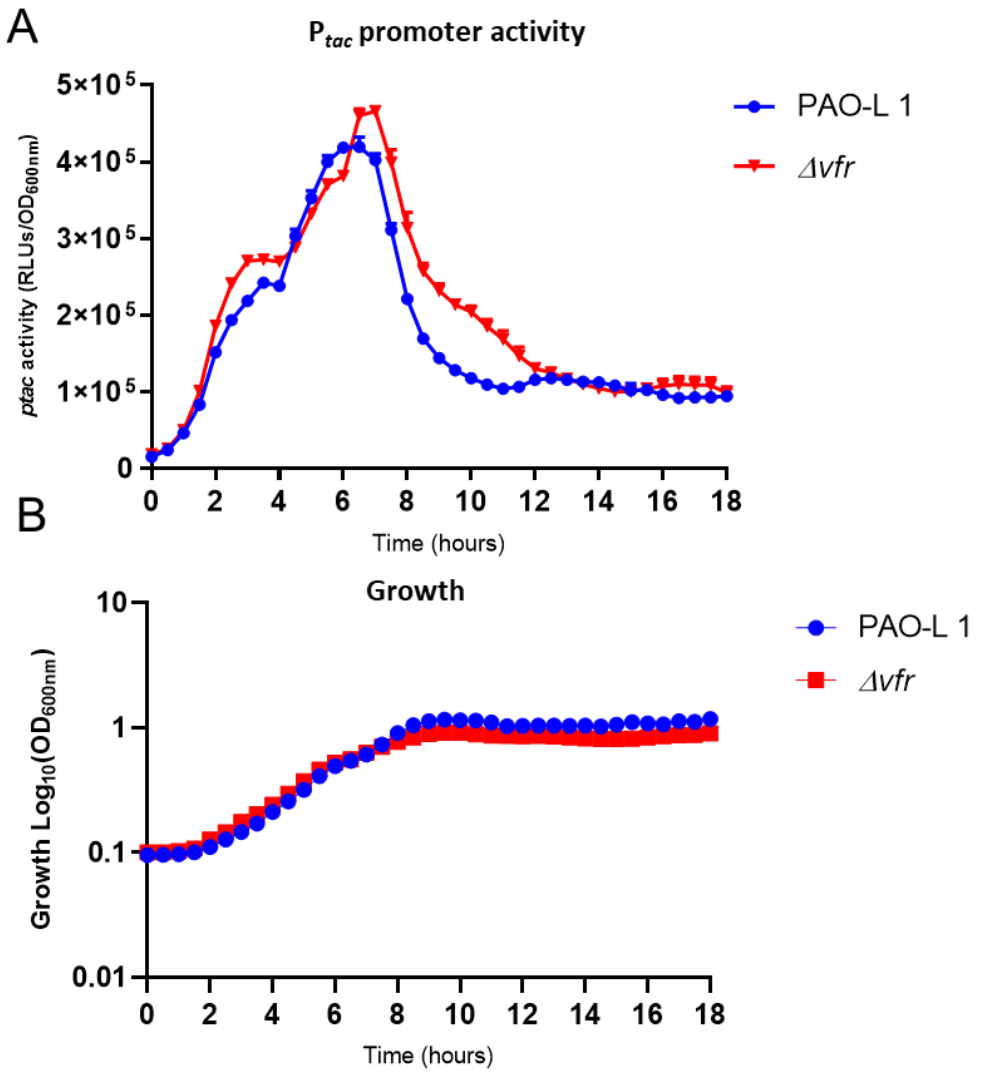


Figure 5.17. Evaluating the probable interference from the CRP like protein VFR upon CTX-*lux* bioreporter.

A) Normalised bioluminescence production for strains PAO1-L and Δ*vfr* carrying the reporter pMiniCTX::*ptac-lux* (Gm^R). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. B) The Log10 growth curve shows that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

5.6.3.1 Vfr as a repressor of *pqsE*

As described in **section 5.4**, the action of CRP like proteins depends on the presence of cAMP, however, studies have shown that this action can also be controlled in a cAMP-independent manner (Fuchs et al., 2010). *P. aeruginosa* encodes two intracellular adenylate cyclases responsible for the cAMP synthesis (Smith et al., 2004), CyaB plays a primary role whereas to a lesser extent does CyaA. In order to analyze whether Vfr controls the expression of *pqsE*, and if this regulation depends on cAMP, the maximum P_{pqsE} activity was recorded in PAO1-L, *vfr*, *cyaB* and *vfr cyaB mutants*. The obtention of the *cyaB* single and *vfr cyaB* double mutant are shown in **Supplementary data, Figure S7.1**.

When compared to the wild-type strain, deletion of *vfr* significantly increased the P_{pqsE} activity (**Figure 5.18**), suggesting that Vfr is a repressor of *pqsE*. Insertion of the empty pME3032 into PAO1 wild type had a slight but unsignificant impact towards the P_{pqsE} activity, effect that was also observed in PAO1 *vfr* mutant (**Figure 5.18**). Complementation of *vfr* (pMENRC4 + IPTG) in this strain although not comparable to wild-type levels, reduced the P_{pqsE} activity significantly, suggesting that this regulator is a repressor of *pqsE*. On the other hand, mutation of *cyaB* in the wild type condition showed a dramatic increment in P_{pqsE} activity, whereas when this mutation is present within the *vfr* mutant genetic background, the P_{pqsE} activity was still increased but considerably reduced, presenting levels similar to that observed when *vfr* was mutated alone. Followed complementation of *vfr* did not restore the P_{pqsE} activity in the absence of CyaB, suggesting that this regulation depends on cAMP.

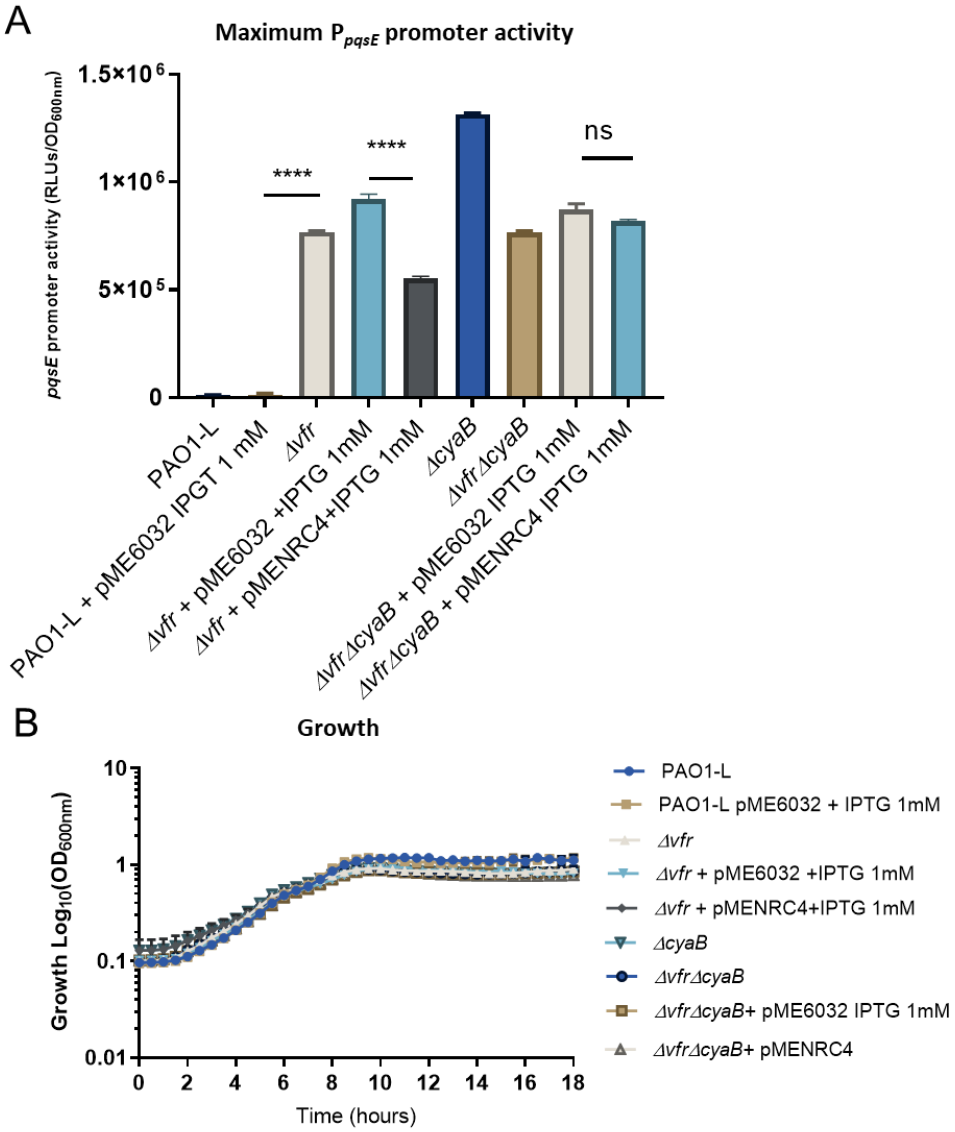


Figure 5.18. Regulation of Vfr upon P_{pqsE}.

A) Maximum values of P_{pqsE} activity in PAO1-L, Δ*vfr*, Δ*cyab* and Δ*vfr* Δ*cyab* carrying the reporter P_{pqsE}-*lux* (Gm^R). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. B) The Log10 growth curve shows that all strains grew similarly. Error bars represent 2x standard errors calculated across three biological replicates.

5.7 Discussion

This chapter shows studies carried out to further our understanding on the PqsE-mediate repression of *pqsA* expression revealing a higher level of complexity than anticipated involving RhlR, LasR and PqsR. As seen in **chapter 1**, LasR is believed to be at the top of the QS circuitry, however this accepted theory has been questioned, proposing a new circular model (Allegretta et al., 2017). Whilst LasR positively regulates *rhlR* (Pesci et al., 1997) and *pqsR* (Wade et al., 2005), RhlR is a repressor of *pqsR* (Wade et al., 2005) and *pqsA* (Xiao et al., 2006). Indeed, RhlR has also shown to activate the *las* system when LasR is absent, genotype of high frequency in CF patients infected with *P. aeruginosa* (Hoffman et al., 2009). PqsR in turn, has also been shown to regulate *rhlR* and *lasR* in a positive manner (Allegretta et al., 2017), demonstrating a high level of complexity that needs to be taken into account.

In this work, the analysis of the expression of P_{pqsA} in the absence of the main QS regulators evidenced the existence of post-transcriptional events that were reflected when using the transcriptional and translational *lux* fusions. The analysis of the regulation of *pqsA* at the translational level unveiled key contestants governing this regulation. Firstly, the mutation of RhlR, validated its role as a repressor of this promoter and suggested, to some extent, that it may be required for the full repression of *pqsA* mediated by PqsE (**Figure 5.3B**). In this experiment, the mutation of *pqsE* in the *pqsE* Ind condition caused an increase in the translation of *pqsA*, which was abrogated upon IPTG-induced expression of *pqsE*, hence, confirming its repressor role in this regulation. When *rhlR* was mutated in the wild type parent strain, two peaks in the translation of *pqsA* were observed. Intriguingly, the second peak began at the early stationary phase and continue to increase until the last time point taken (**Figure 5.3B**). This observation provides further evidence on the role of RhlR in the negative regulation of *pqsA.* Moreover, this could also reflect that RhlR is required for a full repression of this promoter because the repression of *pqsA* is lost in this condition which carries wild type *pqsE*. This observation is supported by the

fact that albeit the induction of *pqsE* in a *rhlR pqsE* Ind mutant still downregulated *pqsA*, this repression was not maintained over the time, and the levels of *pqsA* commenced to increase at the early stationary phase and more interestingly, reached *pqsA* levels like those observed in a uninduced *pqsE* Ind condition, suggesting that the mutation of *rhlR* had, to some extent, a downstream impact on the PqsE-mediated repression.

The mutation of *lasR* (**Figure 5.4B**) did not affect the PqsE-mediated repression of *pqsA* at the translational level, but instead impacted on the timing of the activation of *pqs*A, confirming that these systems are interlinked (Diggle et al., 2003). The fact that the translation of *pqsA* was abolished during most of the *P. aeruginosa* growth confirms that LasR is a positive regulator of *pqsA* (Wade et al., 2005). The delay in the activation of the translation of *pqsA* in the *lasR pqsE* Ind condition can be explained by the lack of the positive effect of *lasR* in the regulation towards *pqsR* (Xiao et al., 2006; Farrow & Pesci, 2017) placing LasR at the top of the QS cascade and questioning the circular circuit proposed by Allegretta et al., (2017).

The fact that the deletion of *pqsE* caused a significant increase in the translation of *pqsA* in the absence of *pqsR* was surprising (**Figure 5.6B**). This is because the whole *pqsABCDE* operon is expected to be inactivated in the absence of this regulator (**Figure 5.6B**) (Rampioni et al., 2010). A deletion of *pqsE* gene when *pqsR* is absent, hence, is not expected to cause a differential expression of *pqsA*. Previously, qRT-PCR analysis in PAO1 grown in LB showed the *pqsA* transcript still formed in the absence of *pqsR*, however, the *pqsE* transcript was not present in the same condition (García-Reyes et al., 2021). Therefore, it is not clear whether *pqsE* in PAO1-L subline strain is still expressed at very low levels when *pqsR* is absent or if it is independently regulated in this condition. For example, the report from García-Reyes et al., (2021) showed expression of *pqsE* transcript in PA7 strain ATTC 9027, which carries a shift-frame deletion of *pqsR*. In this scenario, it is plausible that the mutation of *pqsE* caused a derepression of *pqsA,* being PqsR then required for the PqsE-mediated downregulation of *pqsA*. Besides, important to note is that PQS can act independently of PqsR (Rampioni et al., 2016), and in a *pqsR* mutant PQS is not

produced, meaning that this AQs could also be a mediator in the PqsE-mediated repression. The addition of PQS to the PAO1-L *pqsR pqsE* Ind strain did not downregulate *pqsA* when *pqsE* was overexpressed **(Figure 5.7A**) and instead further increased the *pqsA* levels. Intriguingly, the increase in *pqsA* translation caused by PQS was due to its regulatory role rather than to its iron chelating properties, as the PQS methyl analogue, 2-methyl-3-hydroxy-4-quinolone (mPQS), which only maintains the chelating properties of PQS (Diggle et al., 2007), did not alter the *pqsA* expression levels when used under the same conditions. This observation was further supported when the addition of HHQ (which does not chelate iron), to the PAO1-L *pqsR pqsE* Ind strain also increased the translation of *pqsA* levels when *pqsE* was overexpressed. In summary, these results suggest that PQS can induce *pqsA* in the absence of *pqsR* when *pqsE* is overexpressed and this regulation is not a result of its iron chelating properties. Moreover, the presence of *pqsR* is essential for the PqsE-mediated repression of *pqsA*, as the complementation of this gene in the *pqsR pqsE* Ind strain resulted in lower levels of *pqsA* when *pqsE* was overexpressed (**Figure 5.7B**), an effect that was enhanced when PQS was added which further validates that PQS is a co-inducer of PqsR (Wade et al., 2005).

Besides PqsE, Brouwer et al.,(2014) established that not only PqsR, but also RhlR induces the *pqsA* transcription. The latter, however, is proposed to create a long transcript that restricts the access to the Shine-Dalgarno sequence, hence blocking the ribosome from initiating the translation of *pqsA*. In bacteria, studies on the role of RNA molecules in gene expression has notably increased. Particularly, small noncoding regulatory RNAs (sRNAs), have gained major attention due to their role in gene expression and pathogenesis (Storz et al., 2011). They also participate in the QS regulation (Kay et al., 2006; Sonnleitner et al., 2011; Sonnleitner & Haas, 2011; Malgaonkar & Nair, 2019), which expands the complexity of these systems, but that may help to understand regulatory mechanisms occurring after transcription.

In the present work, two alternative transcripts identified within the *pqsA* promoter named *pqsX*-T1 (258 bp) and *pqsX*-T2 (337 bp) have shown to

downregulate *pqsA* expression at the transcriptional and translational level (**Figure 5.11**), suggesting that the elements required to repress *pqsA* are present within these sequences and conflicts with the suggested mechanism proposed in Brouwer et al., (2014). Important to note, however, is that the expression of these transcripts was performed in *P. aeruginosa* genetic background, hence, the impact towards the transcription and translation of *pqsA* could also be attributed to proteins that interact with these regions. In this context, the regulatory analysis of these transcripts towards *pqsA* requires to be carried out as heterologous expression in an alternative expression system like *E. coli*. Intriguingly Wurtzel et al., (2012), using a combination of TSS mapping and whole-transcriptome data analysis in *P. aeruginosa* PA14 identified the presence of 165 transcripts lacking an ORF, hence most likely to be non-coding RNA (ncRNA), from which Lrs1 and Lrs2 were intergenic non-coding RNA, and that presented conserved binding site with affinity to LasR. Using lacZ-fusions, northern blot and EMSA analysis the authors validated the dependence of Lrs1 from LasR, and that its concentration was dependent on the presence of the RNA chaperone Hfq through direct interaction (Wurtzel et al., 2012). Surprisingly, Lrs1 (249 bp) was placed -268 bp upstream from the *pqsA* TSS in this strain (**Supplementary data, Figure S7.10**), matching the predicted *5'* end of *pqsX-T1 and pqsX-T2* (**Supplementary data, Figure S7.9**). Different from PAO1-L, however, the *lrs1* region in PA14 has 5 additional nucleotide insertions. The latter findings validate the existence of an alternative transcript that is formed within the *pqsA* promoter and that is shorter to that proposed in Brouwer et al., (2014). Remarkably, to date the only regulator known to directly regulate the *pqsA* promoter is RhlR, by binding the *rhlR-box* centred at -311 bp relative of the *pqsA* TSS (Xiao et al., 2006). The latter rationale raises the question of whether the mutation of *lasR* in the study by Wurtzel et al., (2012) lowered the subsequent expression of *rhlR* (Pesci et al., 1997), hence diminishing the levels of Lrs1 transcription in the *lasR* mutant. In this hypothetical scenario, the mutation of *hqf* could also cause a decrease in Lrs1 levels not only by direct binding and causing a probable destabilization of Lrs1 (Wurtzel et al., 2012), but also by downregulating the translation of *rhlI*

(Sonnleitner et al., 2006), lowering the levels of C4-HSL and decreasing the activation of *rhlR.* The above theory gains further support when the authors reported that the Lrs1 transcript can be detected even in the absence of *lasR*, specifically during late stationary phase, which led to hypothesise that the mutation of *lasR* delayed the induction of the *rhl* system and subsequent expression of Lrs1. Indeed, the relation between RhlR and the transcript Lrs1 was recently demonstrated in Vrla et al., (2020). In this work, the use of a mCherry reporter found that the mutation of *rhlR* resulted in a ~7-fold reduction in the expression of *lrs1*. In addition, Chuang et al., (2019) found that *lrs1* transcript is an inductor of *lasR*, as qRT-PCR revealed that the mutation of *lrs1* abrogated the *lasR* transcript levels (Chuang et al., 2019). These findings may explain why the induction of *pqsX-T1* and *pqsX-T2* in PAO1-L wild type did not significantly alter the AQs levels (**Figure 5.13**). Where the induction of *pqsX-T1* and *pqsX-T2* caused an increased activity of *lasR* expression, it is expected that at least *rhlR* and *pqsR* are also induced as result of the above event, causing an induction of the *pqsABCDE* operon by PqsR that is balanced by the negative regulation played by PqsE and RhlR, which could result in homeostatic levels of AQs in this condition (**See Figure 5.19)**. Alternatively, this outcome may be a result of a mechanism of compensation by the kynurenine pathway. This is because the overexpression of *pqsE* caused by *pqsX-T1* and *pqsX-T2 (***Figure 5.12)** is expected to cause a decrease in the production of PQS **(Figure 5.19).** PQS has a role as an extracellular iron chelator (Diggle et al., 2007) and the abrogation of this AQs is expected to increase free iron within the cell **(Figure 5.19)**. Oglesby et al., (2008) found that addition of iron to wild type PAO1 caused an increase in the production of PQS and suggested that the kynurenine pathway was providing anthranilate from tryptophan as a source for its production. This was because the genes encoding for the kynurenine enzymes *kynA* and *kynU* were induced by high iron as compared with low iron in wild-type condition, hence the increment in iron caused by the abrogation of PQS could compensate its production de novo.

In addition, *kynB* and *kynU* have shown to be under the positive control of LasR (Martin Schuster et al., 2003b), which may be another way to contribute to maintain the homeostasis of the production of PQS.

Interestingly, Knoten et al., (2014) reported the presence of an internal transcript starting within the *pqsC* gene, that was extended into *pqsD, pqsE*, and *phnA* when P. *aeruginosa* was grown in nutrient-limiting conditions, hence ensuring the production of anthranilate for PQS production under certain environmental conditions. Based on this, whether another internal promoter is present through the *pqsABDE-phnAB* operon and whether homeostatic regulatory loops take place in the regulation of *pqsA,* becomes crucial to unveil and stablish the bases of possible compensatory mechanisms that aim to maintain the state of equilibrium in *P. aeruginosa*.

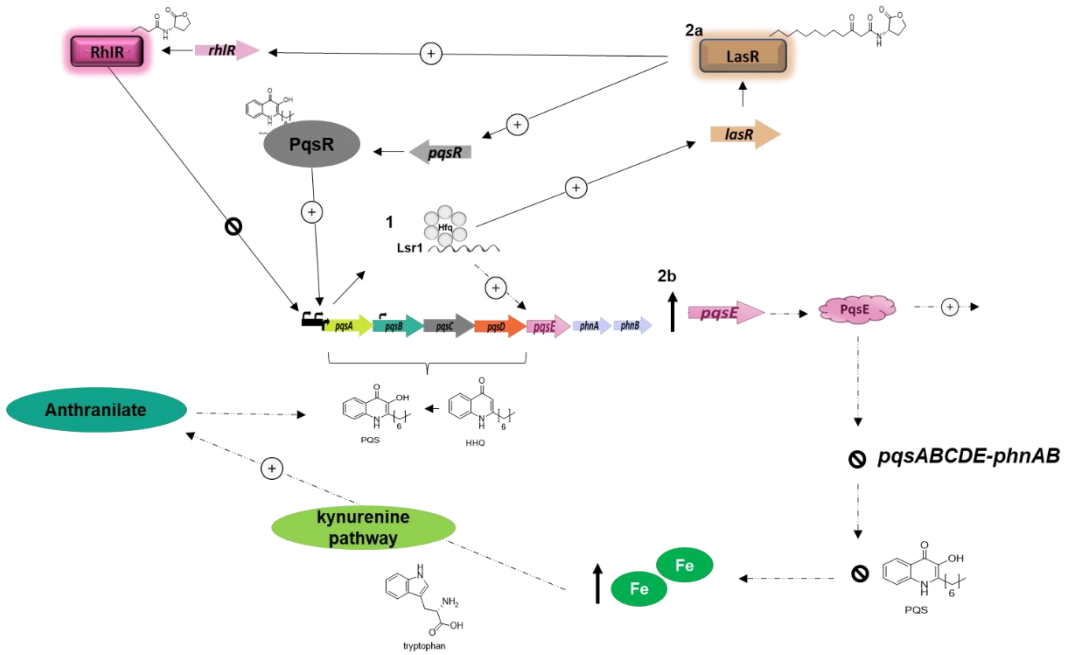


Figure 5.19. Hypothesized mechanisms of the alternative transcript Lrs1 towards the *pqs* system and the production of AQs.

In solid lines 1) Lrs1, a ncRNA that interacts with the RNA chaperone Hfq for its stability and function activates the expression of 2a) *lasR*, which in turn induces the expression of *rhlR* and *pqsR*, maintaining the homeostatic levels of AQs in *P. aeruginosa*. Alternatively, in dashed lines 1) Lrs1 promotes the activity of 2b) *pqsE*, effector that induces pyocyanin production, represses the *pqsABCDE-phnAB* cluster and subsequently abrogates the production of PQS. Increased free iron within the cell due to the lack of PQS induces the formation of anthranilate trough the kynurenine pathway, counterbalancing the initial decline in AQs and maintaining homeostatic levels of AQs in *P. aeruginosa*.

Wurtzel et al., (2012), also reported the lack of pyocyanin in a Lrs1 mutant. This is interesting as the induction of the *pqsX-T2* alternative transcript in this work restored pyocyanin production in the absence of *pqsA* (**Figure 5.15**) which does not produce PQS (Gallagher et al., 2002), hence suggesting that this regulation is PQS-independent and that may target other sections of the *P. aeruginosa* genome. In this scenario, it cannot be discarded that besides LasR, Lrs1 targets RhlR or/and PqsE, as mutation of either regulator also abrogates pyocyanin production, and RhlR is essential for PqsE to restore its production (Hazan et al., 2010). On the other hand, the authors reported that the lack of Lrs1 did not alter pyoverdine levels. Like pyocyanin, the production of pyoverdine is also RhlR-PqsE co-dependent, however mutants in *rhlR* and *pqsE* display increased and wild-type levels of pyoverdine, respectively (Hazan et al., 2010). The latter observation suggests that the alternative transcript targets PqsE and may also participate in the PqsE-mediated regulation. These considerations could explain the inability of *pqsX-T1* and *pqsX-T2* in restoring pyocyanin in the absence of this effector in a PAO1-N *pqsE* mutant (**Figure 5.16**) and questions whether in the *pqsA* mutant, which consequently does not express *pqsE*, *pqsX-T2* was able to express *pqsE* independently and restore pyocyanin production, posit that cannot be discarded, considering the significant impact that *pqsX-T2* had towards the P_{pqsE} activity**.** Based on this and considering that RhlR is responsible for the synthesis of Lrs1 (Vrla et al., 2020), it is expected that the mutation of *rhlR* and subsequently *lrs1* impacts to some extent, the action of PqsE in downregulating *pqsA*, this theory may explain why two peaks in *pqsA* translation were observed in a PAO1 *rhlR* mutant, which as discussed at the top of this section, it seemed to impact to some degree on the effect of *pqsE* in downregulating *pqsA* (**Figure 5.3B**). Hence, whether Lrs1 impacts the performance of *pqsE* in downregulating *pqsA,* becomes an attractive pathway to continue analysing. Besides, considering that PqsE presents a RNA binder KH domain (Folch et al., 2013), and that it possesses phosphodiesterase activity towards single- and double-stranded DNA as well as mRNA (Yu et al., 2009), it cannot be discarded that PqsE post-transcriptionally modifies Lrs1 in order to act together with RhlR or to trigger RhlR /PqsE interaction (Taylor et al., 2021).

Strikingly, Wurtzel et al., (2012) using RNA seq performed in isogenic Lrs1 mutant in LB at an early stationary phase reported almost identical transcriptome of the wild type strain, except for the upregulation of the anthranilate dioxygenase operon *antABC and pffr1-2*. This outcome is interesting as it suggests that the role of Lrs1 is linked to anthranilate production, however the fact that both *antABC and pffr1-2* were upregulated at the same time was unexpected. To date, overexpression of *pffr1-2* has only been observed in low iron conditions, which could suggest that the lack of Lrs1 leads to an iron starvation response. Pffr 1-2 participate in the repression of *antABC* by repressing *antR* to spare anthranilate towards PQS biosynthesis (Oglesby et al., 2008), meaning that whilst *pffr 1-2* is overexpressed, *antR* and *antABC* are expected to be downregulated, which is not the case. The upregulation of *antABC*, however, could be a result of the lack of pyocyanin reported by the authors. The lack of pyocyanin could diverge the reaction towards the accumulation of chorismate. Chorismate can be converted to anthranilate by *phnAB* (Gallagher et al., 2002) and cause the activation of *antABC* (Hazan et al., 2010).

Analysis of the Lrs1 sequence suggested, however, that the outcome observed under this condition in Wurtzel et al., (2012) may correspond to an undesirable impact of the mutation *lrs1* region (+1 to +191) towards the *pqsA* activity, as it precedes the LysR-box which is +217 bp from the 5' end of the Lrs1 transcript (**Supplementary data, Figure S7.10**). Nevertheless, the work from Chuang et al., (2019) suggested that mutation of *lrs1* does not impact this promoter activity. Alternatively, the mutation of *lrs1* is expected to abrogates *lasR* expression (Chuang et al., 2019). The absence of *lasR* in this thesis abolished the translation of *pqsA* (**Figure 5.4B)** probably due to its regulatory role towards *pqsR* (Wade et al., 2005). Abrogation of the *pqsABCDE-phnAB* activity and the subsequent production of HHQ and PQS, which are autoinducers of PqsR (Wade et al., 2005) can result in the accumulation of anthranilate and concomitant increased activity of *antABC* (Hazan et al., 2010). Moreover, the decreased activity of *pqsR* due to the lack of *lasR* and AQs can lead to a derepression of

antA, as it shown to be under the negative control of PqsR (Oglesby et al., 2008; Choi et al., 2011) hence, both mechanism may contribute to the increased levels of *antABC* in *lrs1* mutant **(Figure 5.20).**

In this context, therefore, the abrogation of pyocyanin in the *lrs1* mutant reported in Wurtzel et al., (2012) could be due to a lack of expression of *pqsE* (Rampioni et al., 2016), whereas the wild-type levels of pyoverdine may correspond a mechanism of compensation mediated trough *prrf 1-2* **(Figure 5.20).** PQS, in an PqsR-independent manner (Rampioni et al., 2016), is required for the transcription of genes coding for iron starvation response including pyoverdine (Hazan et al., 2010; Rampioni et al., 2016). The lack of PQS independent of the presence of wild type *pqsR* in a *lrs1 mutant,* therefore, is expected to cause a downregulation in pyoverdine and iron starvation response genes. A lack of PQS and pyoverdine in *P. aeruginosa* may led to the inability of this pathogen to chelate iron from the environment resulting in depletion of intracellular iron levels. The master regulator Fur (ferric uptake iron regulator), under low iron conditions derepresses the iron starvation sigma factor *pvdS* (Leoni et al., 2000) responsible for pyoverdine biosynthesis (Wilson et al.,2001). In parallel, Fur also derepresses *prrf 1-2* (Wilderman et al., 2004) , which in turns represses *antR* favouring the production of anthranilate towards the biosynthesis of PQS (Oglesby, 2008). Since PQS is not produced in a *lrs1* mutant due to the abrogation of *lasR* expression and subsequently *pqsR,* it may contribute further to the accumulation of anthranilate, causing a disbalance in this regulation, that presented a parallel overexpression of *prrf 1-2* and *antABC*, whilst the wild-type levels of pyoverdine levels are due to a compensation by the action of PvdS.

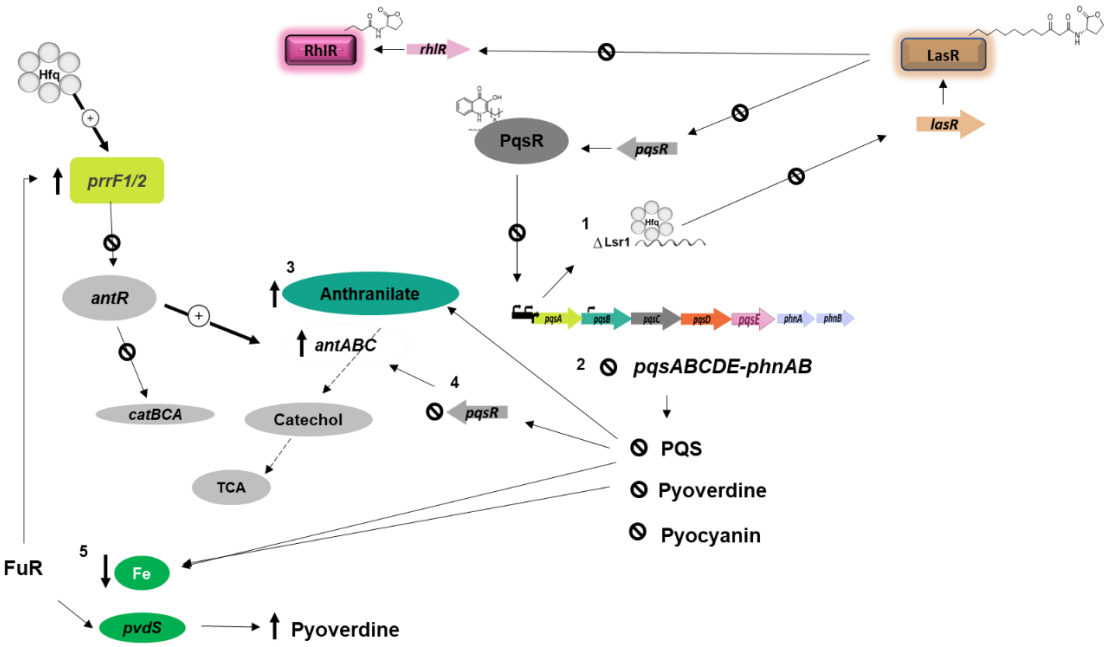


Figure 5.20. Hypothesized mechanism of the transcriptome outcome in *lrs1* mutant.

1) The mutation of *lrs1* abolishes the *lasR* expression and the subsequent activation of *rhlR* and pqsR, which in turn leads to abrogate the 2) *pqsABCDE-phnAB* cluster expression and the production of PQS, pyoverdine and pyocyanin. The lack of PQS 3) favours the accumulation of anthranilate and induces *antABC*. 4) fails to activate *pqsR* and causes a derepression of *antABC*. 5) The lack of pyoverdine causes a depletion of available iron in P. aeruginosa, cofactor required for the Fur mediated repression of *pvdS*. The subsequent derepression mediated by Fur towards *pvdS* activates the synthesis of pyoverdine, probably explaining the wild-type levels observed in lrs1 mutant (Wurtzel et al., 2012). In iron depleted conditions, Fur also causes the derepression of *prrf 1-2*, which favours the accumulation of anthranilate and, therefore, the production of PQS by repressing *antR* and *antABC*. Since PQS is unable to be synthesised, the accumulation of anthranilate continues overexpressing *antABC* whilst *pffr-1-2* is also induced.

In this work, important changes in *pqsA* expression by the addition of pME6032 as a control vector in PAO1-L were observed (**Figure 5.11**). This was unexpected and does not have an apparent explanation. This phenomenon has been previously reported in *E. coli*, particularly, the sole presence of an empty vector has affected biofilm formation (Mathlouthi, Pennacchietti & De Biase., 2018), nevertheless, the later observation varies from plasmid to plasmid and growth conditions, and yet the mechanism of this changes has not been fully studied. The fact that *pqsX*-T1 did not restore the pyocyanin production in a *pqsA* mutant is under investigation, as the initial expression of this transcript did show restoration of pyocyanin, however, the phenotype was lost after repetitive experiments. The same phenomena occurred after in the same strain expressing *pqsX-T2*. It is unclear whether the vector itself could cause this deregulation, albeit is intriguing that a similar event occurred before when pME6032 was used for gene complementation, in which the long-term usage of the strain led to the loss of pyocyanin production that was originally observed (Bretones, PhD Thesis, 2016). Sequencing of these vectors is under way and may provide some further info into the cause of this phenomenon.

In addition, important to note is that impact of the transcripts *pqsX-T1* and *pqsX-T2* in a *pqsE* mutant for the restoration of pyocyanin need further validation in *P. aeruginosa* PAO1-L, as due to time limitations were only evaluated within the *P.aeruginosa* PAO1-N subline, which carries a 58,569 bp deletion compared to the reference PAO1 Washington strain (PAO1-W) (Stover et al., 2000) and Lausanne (Unpublished data).

Finally, the creation of *vfr* and *cyab* mutants gave new insights into the regulatory role of the virulence factor regulator Vfr. Here, it has been seen that Vfr controls *pqsE* in a cAMP-dependent manner (**Figure 5.18**). The predicted CRP binding site is placed -246 placed bp upstream from the ATG start codon of *pqsE*. This prediction led to hypothesize that the role of *vfr* upon the regulation of *pqsE* is in a positive manner, as *E. coli* promoters which are positively regulated by the CRP usually have a CCS centred at the positions -41, -61, or -72 bp upstream from the transcriptional start site (TSS) (Kolb, 1993), however, the experiments presented in this study, showed the opposite, which

could indicate that the regulation patterns in *P. aeruginosa* may differ from those in *E. coli*, however this assumption need further elucidation as the exact TSS of *pqsE* has not been elucidated yet.

5.8 Conclusion and future directions

This work has contributed to the elucidation of some of the essential components that governs the *pqs* regulation, in particular the repressor role attributed to PqsE towards the *pqsA* occurred at the post-transcriptional level and it was no longer present in the absence of PqsR. In addition, the role of ncRNA has gained major attention due to their important roles in virulence that had not been previously appreciated. In this work, a mechanism of the action of the alternative transcript Lrs1 found in a previous study using *P. aeruginosa* PA14 genome is proposed. Due to the high similarities in sequences between Lrs1 and the transcript *pqsX-T1* in PAO1-L created in this work, the alternative transcript suggests playing a role in this pathogen. Based on recent data, Lrs1 plays a pivotal role in QS regulation and based on the data presented in this thesis, it is plausible that Lrs1 targets PqsE, however, albeit not as essential as PqsR, it cannot be discarded that Lrs1 may impact to some extent the performance of PqsE towards the repression *pqsA*. On the other hand, the discovery of this alternative transcript perhaps contributes to the understanding of the puzzling variation in *pqsA* expression observed at the transcriptional level in the presence or absence of the *rhlR-box*. Yet, further studies are needed to fully understand the contribution of this transcript towards the regulation of *pqsA,* including its identification and characterization in *P. aeruginosa* PAO1-L, its targets in this pathogen and the mechanism by which it represses P_{pqsA}. In addition, this work indicates that Vfr participates in the *pqs* regulation by repressing *pqsE,* which opens new directions to continue exploring the intricate regulation taking part within the *pqs* operon. All in all, the quorum sensing networks in *P. aeruginosa* present extremely complex mechanisms that drive virulence in this pathogen, and due to its highly interconnected regulatory pathways that strategically balance the homeostasis

in *P. aeruginosa,* prevents and challenges the accurate understanding of these regulations.

6 Chapter Six: Conclusions

Quorum-sensing is a widespread regulatory mechanism that in a cooperative manner synchronies the expression of an arsenal of virulence factors, many of which are tightly regulated by an intricate circuitry of three QS systems that control directly or indirectly over 10% of the genes in *P. aeruginosa* (Schuster et al., 2003; Wagner et al., 2003; McGrath et al., 2004; Wade et al., 2005; Schuster & Greenberg 2006; Allegretta et al. 2017).

In the *pqs* system, *pqsE*, encoded by the last gene of the *pqsABCDE* operon, has gained major attention due to its multiple roles in the biology of *P. aeruginosa* (Hazan et al., 2010; Drees & Fetzner, 2015; Rampioni et al., 2016). Amongst others, PqsE controls the production of AQs by repressing the *pqsA* promoter (Rampion et al., 2010). However, since PqsE does not possess a DNA binding domain, this regulation is likely to be indirect as a result of a chain of events possibly involving additional mediators that ultimately transduce the activity of PqsE to the target promoter.

The two promoter pull downs of P_{pqsA} performed at the early and late stages of growth of *P. aeruginosa*, revealed interesting outcomes. The large number of proteins found binding the *pqsA* promoter, either in the absence or presence of PqsE, suggested a regulatory branch that is highly interconnected with functionally diverse pathways. In addition, the fact that the absence or presence of PqsE changed the profile of proteins interacting with the *pqsA* promoter further supports this. It was intriguing to observe at the early stage of growth PA2705 bound to the *pqsA* promoter only when PqsE was overexpressed, which positioned this protein as a potential mediator in the PqsE mode of action. Hence it became an attractive target of study. The analysis performed in **Chapter 3** demonstrated that PA2705 is dispensable for the PqsE-mediated repression, nonetheless it was interesting to elucidate that it can still

modulates the expression of *pqsA* and that is under the control of PqsE. Further studies found a relationship between PA2705 and the AraC transcriptional regulator PA2704 as well as with other branches of *las* and *rhl* QS systems, which suggests a wider role of PA2705 in the QS regulatory network.

At the late stage of growth of *P. aeruginosa*, six main candidates were chosen by Dr. Rampioni to analyse its relation to *pqsA* as well as to PqsE. Amongst them, NirQ had a dramatic impact towards the transcriptional activity of *pqsA* and therefore it was studied further in **Chapter 4**. It could be clearly observed that NirQ is a strong repressor of *pqsA*, and like *PA2705*, is also under the control of PqsE, which further supports its participation in controlling denitrification (Toyofuku et al., 2008; Rampioni et al., 2016). The absence of *nirQ*, however, was not an impediment for PqsE to represses *pqsA*, hence **Chapter 3** and **Chapter 4** did not evidence the missing mediator of the action of PqsE. Due to time constraints, the binding of PA2705 and NirQ to the *pqsA* promoter could not be verified. This, however, opens a new line of study for both proteins, but it is highly intriguing for PA2705, as it does not possess an apparent DNA binding domain and neither its precise role nor its potential interactive partners are known. Major attention deserves its link to PA2707 and PA2704, as well as how PA2706, which is only present in some *pseudomonas* strains, is related to this regulation.

It was puzzling to observe in the second promoter pull down LasR, RhlR and PqsR bound to the *pqsA* promoter. Albeit Wade et al., 2005 found that neither LasR nor RhlR bind the *pqsA* promoter, Xiao et al., (2006) identified the -311 distal *lux-box* relative to the *pqsA* gene as the binding site by which RhlR represses *pqsA*. Moreover, no evidence has suggested yet that LasR interacts with the *pqsA* promoter, as this has only shown to regulate *pqsR* by binding a *las/rhl* box centred at 513 bp upstream of the *pqsR* translational start site (Xiao et al., 2006), hence this is an interesting finding which deserves further investigation. The binding of PqsR to the

pqsA promoter has been reported before (McGrath et al., 2004), but a cooperative role with PqsE towards the regulation of *pqsA* has not been reported yet. Albeit RhlR was suggested to be dispensable for the PqsE-mediated repression of *pqsA* expression at the transcriptional level (Rampioni et al., 2010), **Chapter 3** evidenced that RhlR behaves as an inductor of *pqsA* when *pqsE* is absent. This outcome demonstrated a key role of the *rhlR-box* for the binding of RhlR and repression of *pqsA.* In addition, these observations along with phenotypic assays contributed to elucidate that PqsE acts in a post-transcriptional manner. To this end, in **Chapter 5,** the PqsE-mediated repression of *pqsA* was characterized at this level and the impact of the main QS regulators LasR, RhlR and PqsR was analysed.

The main observations from this analysis indicated that:

(I) LasR is an inductor of *pqsA* at the translational level and it at a top of the QS regulatory cascade. This was evidenced when the single mutation of this regulator abrogated the translation of *pqsA* until the end of stationary phase, and no significant induction of *pqsA* was observed even in the presence of the wild type *pqsR* in these conditions. These observations supports a hierarchical model of QS and challenges the proposal of a circular model suggested by Allegretta et al., (2017). Moreover, it was evidenced that LasR is not required for the repression of *pqsA.*

(II) RhlR is a repressor of *pqsA* at the translational level and suggested to impact, to some extent, the performance of PqsE in downregulating *pqsA*. This is because, once the stationary phase was reached in the *rhlR* mutant, the translation of *pqsA* reached levels similar to those observed when *rhlR* was mutated along with *pqsE*. Moreover, albeit the induction of *pqsE* in the absence of *rhlR* downregulated the translation of *pqsA* at the early stage of growth, these levels slightly

increased close to those observed when *pqsE* was mutated alone.

(III) PqsR is essential for the PqsE-mediated repression. Overexpression of *pqsE* in the absence of *pqsR* failed to downregulate this *pqsA*. This indicates that the PqsE-mediated repression of *pqsA* is PqsR-dependent.

Along with this finding, it was also evidenced that the regulation of *pqsA* is more complex and involves the participation of ncRNA. This was evidenced when the creation of two alternative transcripts different to that proposed by Brouwer et al., (2014) and that are under the control of RhlR, showed to downregulate the transcription and translation of *pqsA*, whilst inducing the activity of *pqsE*. This was encouraging as a similar transcript to *pqsX-T1*, named Lrs1 has been identified in PA14, and it has been shown to be regulated by Hfq and LasR (Wurtzel et al., 2012), as well as by RhlR (Vrla et al., 2020). The latter evidence could contribute to unveil whether this ncRNA is the missing element that connects RhlR to PqsE. This is because the ligand pocket of PqsE is narrow and deep buried, suggesting that it could potentially fit a long molecule. Its structural presents a kinked α-helix located next to its active site that is similar to that of KH domains (Folch et al., 2013). KH domains are involved in nucleic acid recognition and stabilization. Indeed, in the same work, the authors predicted that PqsE could fit single-stranded DNA/RNA substrate in its active site tunnel, and revealed that mutation of two of the KH residues drastically reduced the production of pyocyanin (Folch et al., 2013). In addition, PqsE presents, albeit very weak, phosphodiesterase activity towards single- and double-stranded DNA and RNA (Yu, 2013). Hence whether the alternative transcript may be modified by PqsE remains to be elucidated. This could ultimately contribute to unravel how RhlR and PqsE are interconnected. Interestingly, it was recently reported that PqsE and RhlR can physically interact (Taylor et al., 2021). The suggested mechanism was unusual and involved allosteric interactions that were

evidenced when the substitution of tryptophan 182 from PqsE, which is deeply buried in the active site and therefore inaccessible to RhlR, disrupted their interaction (Taylor et al., 2021). Therefore, dissecting whether PqsE recognizes and stabilizes this transcript to interact with RhlR and modulate the action of RhlR towards their target genes, or whether the transcript by interacting with PqsE changes the allosteric conformation of the protein to interact with RhlR requires further investigation. In fact, if this alternative transcript is expressed only when *rhlR* is present and this is required for PqsE to mediate its action, it could explain why the ability of PqsE in downregulating the translation of *pqsA* seemed to be affected when *rhlR* was mutated (see above, point (II)).

Finally, it was evidenced that the expression of *pqsE* is more dynamic, and it is not only driven by the *pqsA* promoter. The presence of -10 and -35 regulatory elements and a predicted CRP box at -246 bp upstream of the ATG start codon of *pqsE* led to the discover that the global transcriptional regulator Vfr controls the expression of *pqsE*, which further supports that the regulation of the *pqs* system is wider and far more complex than anticipated.

All in all, this work contributed to the elucidation of new components participating in the PqsE-mediated repression of *pqsA.* It provided new insights on how this regulation takes place in *P. aeruginosa* and opened new lines for further investigation.

7 Supplementary data

S1. Construction of the in-frame deletion mutants *PA2705*, *PA2704, nirQ* and *cyab*

In frame deletion mutants were obtained as described in **Chapter 2, section 2.6.2 - 2.6.4.** To confirm the successful double crossover events, a number of potential allelic exchange knockout mutants were confirmed by colony PCR. **Figure S7.1** shows the results, which were subsequently confirmed by DNA sequencing.

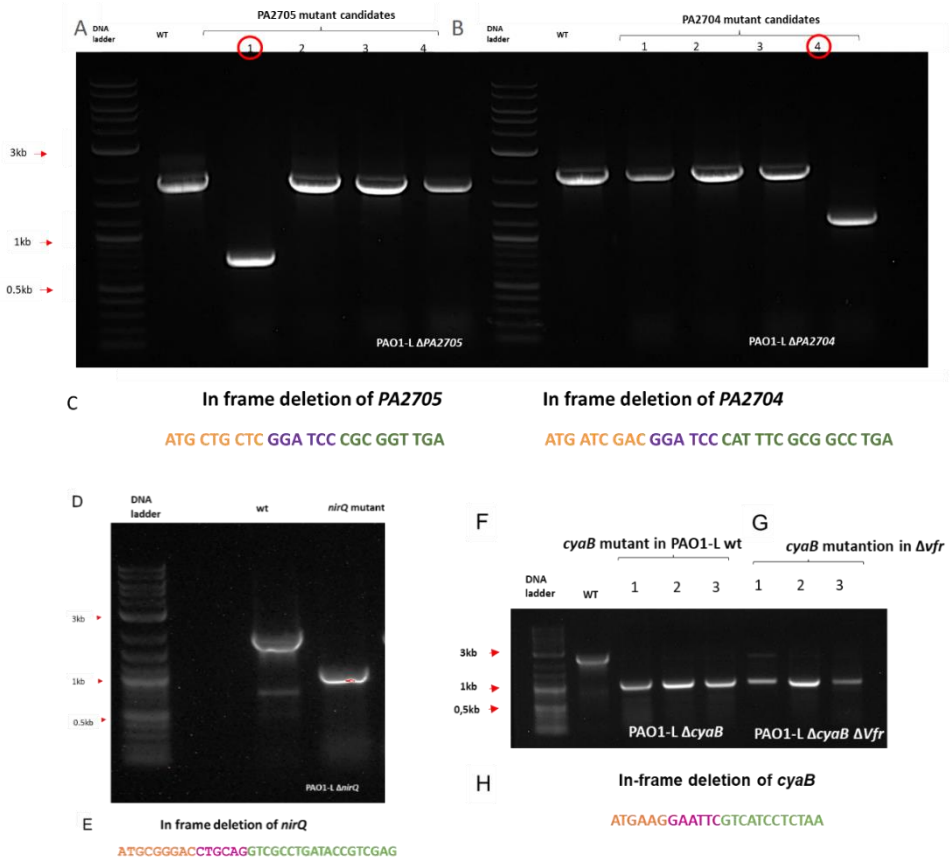


Figure S7.1. Screening of the mutants *PA2705, PA2704, nirQ* and *cyaB* in P. aeruginosa PAO1-L.

Tetracycline sensitive colonies were screened by colony PCR for the presence of the mutant gene in (A)*PA2705(1182 bp)* and (B) *PA2704 (1020)* (D) *nirQ (783 bp)* (F) *cyaB (1392)* and G) *vfr cyab*. DNA amplification for the loss of *PA2705* and *PA2704* was performed using primers pairs PA2705-Up-F/Ds-R and PA2704-Up-F/Ds-R, respectively. DNA amplification for the loss of *nirQ* was performed using primer pairs NirQ Up-F/Ds-R. DNA amplification for the loss of *cyaB* was performed using primers pair cyab Up-F/Ds-R (Table 2.2). (C), (E) and (H): DNA sequences verified that deletions were in-frame. Orange DNA sequences denote upstream coding regions, green DNA sequences denote downstream coding regions and purple DNA sequences indicate flaking regions. Quick-Load 2-Log (NEB) was used as DNA Ladder.

S2. Construction of the *pqsE Ind* in pDM4 Gm^R and obtention of *pqsE Ind* strain derivatives

Since PAO1 Lausanne subline carries the gene encoding for the chloramphenicol acetyl transferase, which confers resistance to chloramphenicol, the *pqsE* Ind genetic elements (**'**) from the pDM4::*pqsE* Ind (Cam^R) vector were cloned into a pDM4 Gm^R derivative (**Figure S7.2 A/B**). Insertion of the conditional mutation of p*qsE (pqsE* Ind*)* in the strains PAO1-L, ΔPA2705, Δ*rhlR* and Δ*nirQ* were obtained by double crossover as described in **Chapter 2, section 2.6.4. (Figure S7.2 C)**

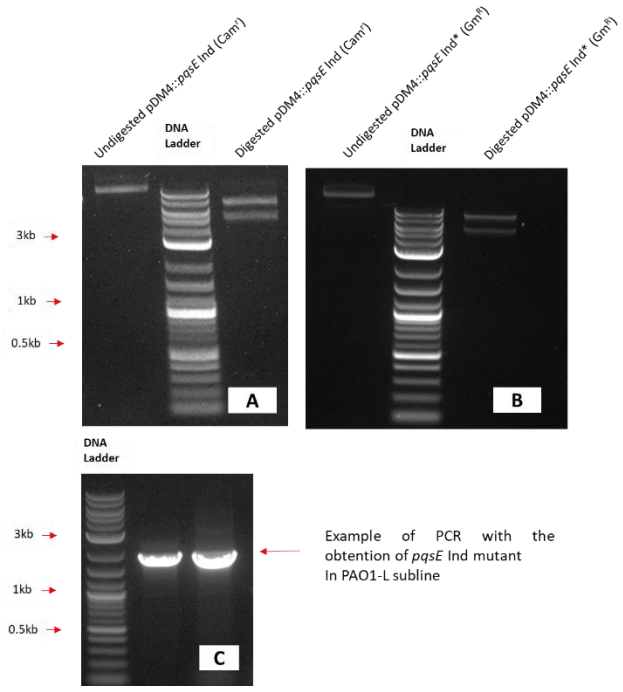


Figure S7.2. Agarose gel of the obtention of pDM4::*pqsE* Ind*(Gm^R) plasmid and conditional *pqsE* Ind mutant.

A) Suicide plasmid pDM4::*pqsE* Ind Cam^R was digested with restriction enzymes XbaI and XhoI to release the region holding the *pqsE* Ind elements(') B) Digestion of pDM4::*pqsE* Ind Gm^R with restrictions enzymes XbaI and XhoI to confirm insertion of the *pqsE* Ind elements in pDM4 Gm^R derivative C) Example illustrating a PCR with the obtention of a *pqsE* Ind conditional mutant in PAO1-L genetic background. PCR was confirmed by sequencing using primers ptac-SEQ-F/pqsE-SEQ-R (Table 2.2). Quick-Load 2-Log (NEB) was used as DNA Ladder.

(**'**) *pqsE* Ind genetic elements: (a) an XbaI-BamHI fragment of 0.5 kb of the upstream region of *pqsE* (b) the 2.0 kb BamHI Sm/Spc integron from pHP45Ω; (c) the 1.5 kb BamHI-EcoRI lacIQ Ptac inducible promoter fragment of pME6032 and (d) an 0.5 kb EcoRI-XhoI fragment carrying the *pqsE* open reading frame.

S3. Construction of the mutants containing *pqsR* and *lasR* in-frame deletions

To insert the *pqsR* mutation sucrose enrichment was performed as described in **Chapter 2, section 2.6.4,** whereas carbenicillin enrichment was for the insertion of the mutation of *lasR* as described in Chapter **2, section 2.6.3**. After double crossover events, allelic exchange knockout mutants were confirmed after screening individual colonies by PCR (**Figure S7.3**).

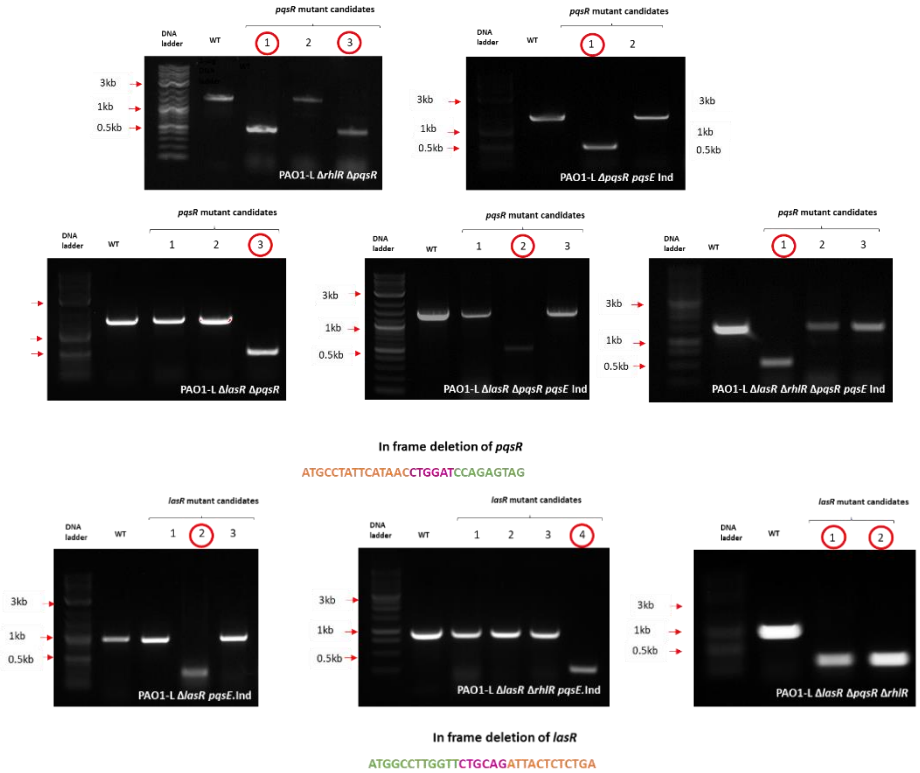


Figure S7.3. Screening for the insertion of *pqsR* and *lasR* mutations in *P. aeruginosa* PAO1-L strains.

Tetracycline sensitive colonies were screened by colony PCR for the presence of *pqsR* and *lasR* mutation using primers pairs PqsR-SEQ-F/ R and LasR-SEQ-F/R, respectively (Table 2.2). DNA sequences verified that deletions were in-frame. Orange DNA sequences denote upstream coding regions, green DNA sequences denote downstream coding regions and purple Quick-Load 2-Log (NEB) was used as DNA Ladder.

S4. Construction of the pMiniCTX-*lux* transcriptional and translational reporters

The transcriptional reporters of P_{PA2705}, P_{pqsA}, P_{nirQ} and P_{pqsE} and the translational reporters of P_{PA2705}, P_{pqsA}, P_{nirQ} in pMiniCTX-*lux* were constructed as described in **Chapter 2 section 2.6.1**. and it is illustrated in **Figure S7.4**

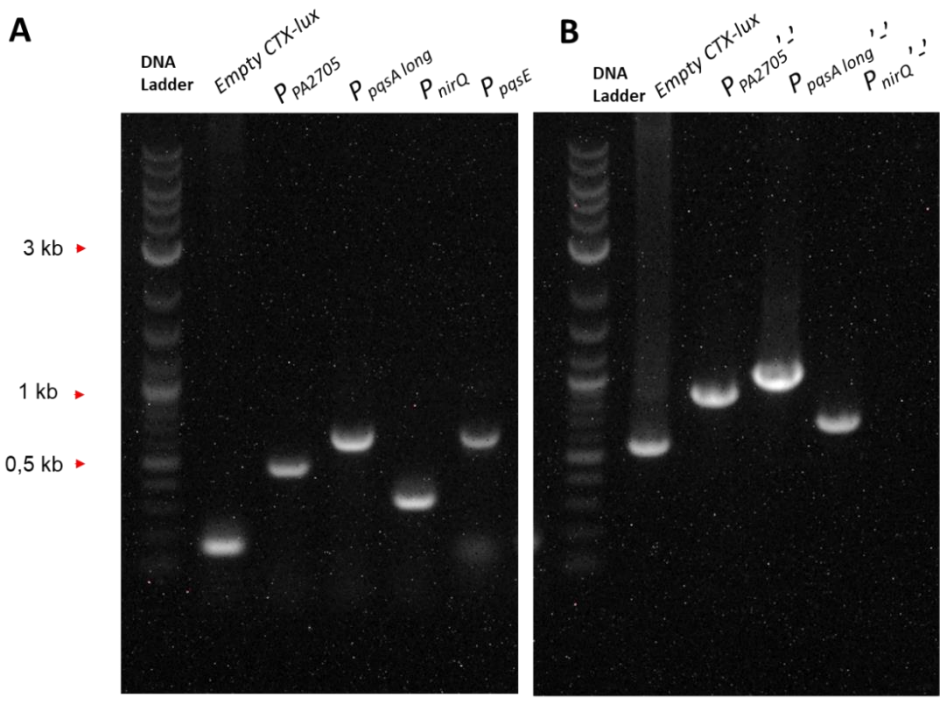


Figure S7.4. Screening for the obtention of transcriptional and translational reporter in pMiniCTX-*lux*.

A) PCR products of the transcriptional pMiniCTX carrying P_{PA2705}(366 bp) $P_{pqsA\ long}$ (502 bp), P_{nirQ} (215) and P_{pqsE} (500 bp), respectively. Amplification of the pMiniCTX-lux alone is including as a negative control (107bp). PCR amplification were performed using primer pairs MCS-SEQ-F /R (Table 2.2) B) PCR products of the translational pMiniCTX carrying P_{PA2705}(369 bp) $P_{pqsA\ long}$(502 bp) and P_{nirQ} (130bp), respectively. Amplification of the pMiniCTX-lux alone is including as a negative control (495bp). PCR amplifications were performed using primer pairs KS-CTX-F /LuxC-CTX-R and validated by DNA sequencing. Quick-Load 2-Log (NEB) was used as DNA Ladder

S5. Construction of the pMiniCTX::$P_{pqsA\ \Delta rhlr\ box}$-lux transcriptional reporter

Mutation of the -311 *rhlR-box* within the *pqsA* promoter was carried out as described in **Chapter 2, section 2.6.5** and it is illustrated in **Figure S7.5.**

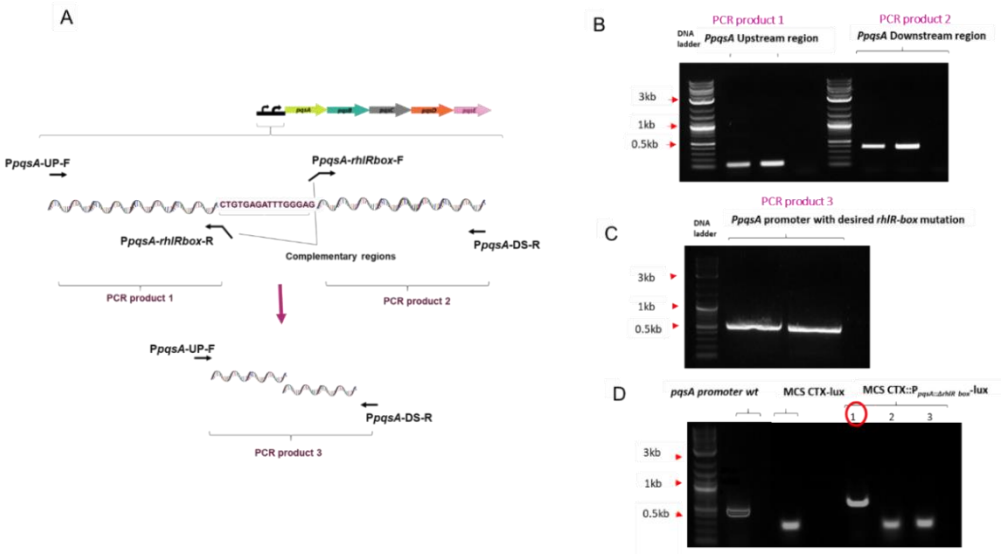


Figure S7.5. Site-direct mutagenesis of the *rhlR-box* within the *pqsA* promoter by overlapping PCR.

A) Schematic representation of the overlap extension PCR to create site specific mutation using primers P*pqsA*-UP-F/P*pqsA-ΔrhlRbox*-R and P*pqsA*-Δr*hlRbox*-F/P*pqsA*-DS-R, respectively (Table 2.2). PCR products 1 and 2 with complementary sequences re-amplify to generate PCR product 3, that holds the desired mutation. B) PCR products 1 and 2, corresponding to the upstream and downstream region of *pqsA,* respectively, are shown in agarose gel. C) PCR products 1 and 2 were combined and re-amplified by using flanking primers P*pqsA*-UP-F/P*pqsA*-DS-R to generate PCR product 3, corresponding to the P*pqsA* promoter with a mutated *rhlR-box*. D) Amplification of the MCS alone is including as a negative control (107bp). PCR product 3 inserted into the MCS of the pMiniCTX-*lux*. Deletion of the *rhlR-box* was confirmed by sequencing using primers MCS-CTX-SEQ-F/R (Table 2.2). Quick-Load 2-Log (NEB) was used as DNA Ladder.

S6. Impact of IPTG on transcription and translation of *pqsA* in *P. aeruginosa* PAO1-L and derivative mutant

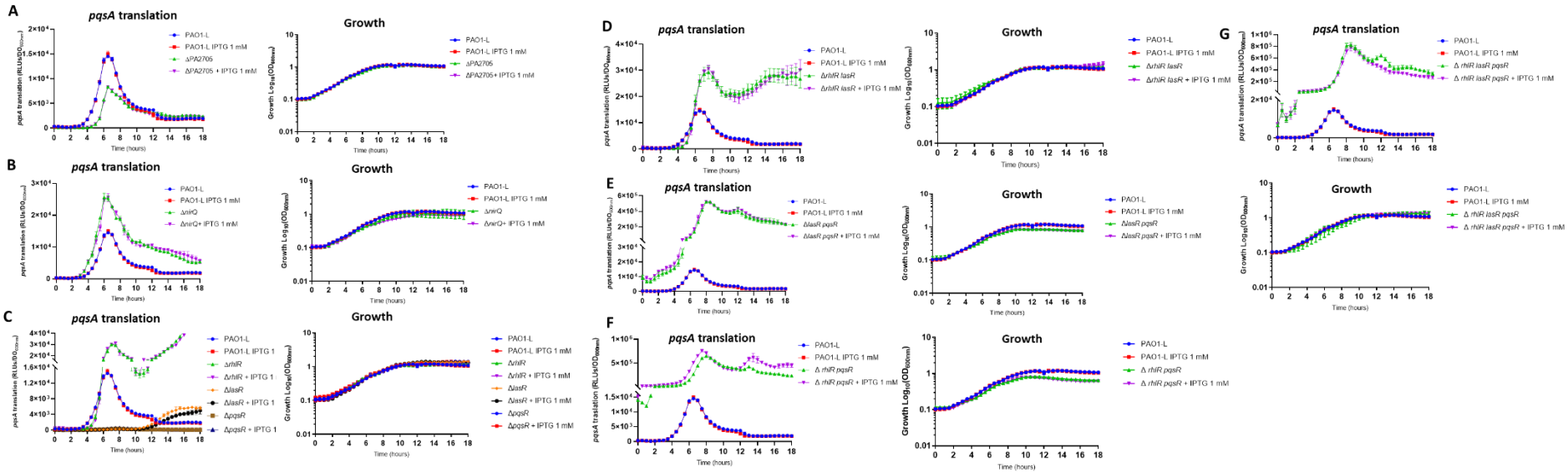


Figure S7.6.1. Impact of IPTG alone on the translation of *pqsA* in *P. aeruginosa* PAO1-L and mutant derivatives.

Normalised bioluminescence production for strains *P. aeruginosa* PAO1-L, A) *ΔPA2705,* B) *ΔnirQ,* C) *Δrhlr, ΔlasR, ΔpqsR,* D) *ΔrhlR lasR,* E) *ΔlasR pqsR,* F) *ΔrhlR pqsR and* G) *ΔrhlR lasR pqsR* carrying the construct $P_{pqsA\ long}$*-luxCDABE* (Gm^R). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. The Log10 growth curve of the OD_{600nm} is shown. Error bars represent 2x standard errors calculated across three biological replicates.

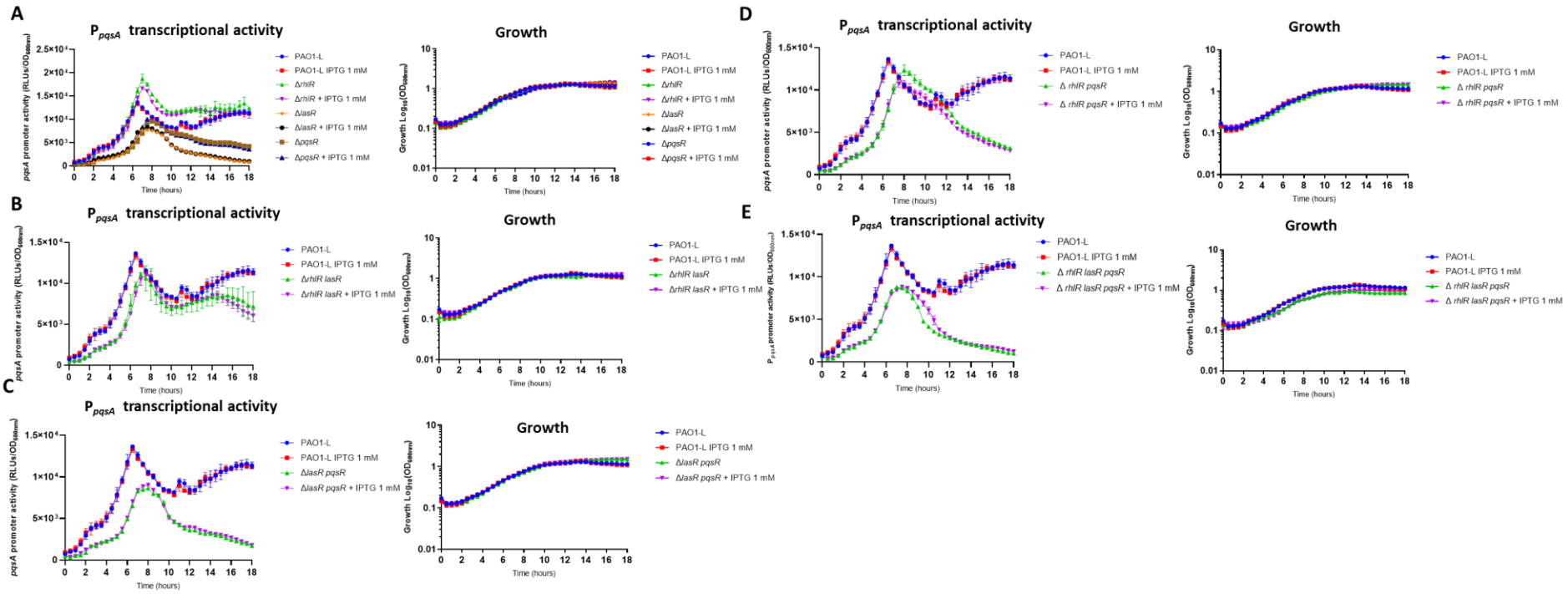
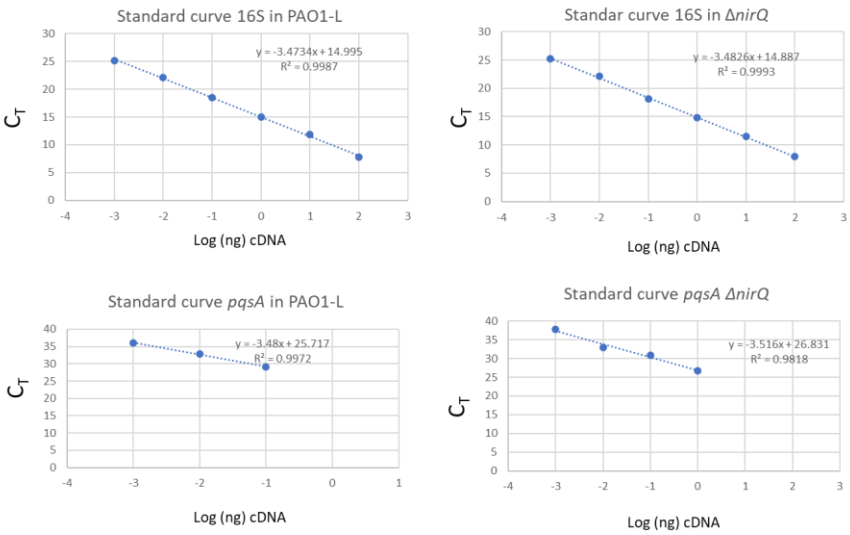


Figure S7.6.2. Impact of IPTG alone on the transcription of *pqsA* in *P. aeruginosa* PAO1-L and mutant derivatives.

Normalised bioluminescence production for strains *P. aeruginosa PAO1-L,* A) *Δrhlr, ΔlasR, ΔpqsR,* B) *ΔrhlR lasR,* C) *ΔlasR pqsR,* D) *ΔrhlR pqsR and* E) *ΔrhlR lasR pqsR* carrying the construct $P_{pqsA\ long}$ *-luxCDABE* - (Gm^R). Strains were grown in LB media for 18 hours in a TECAN plate reader and both OD_{600nm} and the relative light units (RLUs) were measured. The Log10 growth curve of the OD_{600nm} is shown. Error bars represent 2x standard errors calculated across three biological replicates.

S7. qRT-PCR

Among 4 candidate endogenous genes *(proC, rpoS, rpoD* and *16S*), 16S showed the less variation (<0.5) in Ct between the calibrator (PAO1-L) and treated (Δ*nirQ* mutant) sample, hence it was chosen as endogenous gene for further analysis. PCR efficiencies for *16S* and *pqsA* expression were calculated from different standard curves (**Figure S7.7**). PCR efficiency was over 90% in all samples.



PCR EFFICIENCY		PAO1-L		ΔnirQ	
y=mx+b	y=mx+b mx= slope b= intercep	*pqsA*	16S	*pqsA*	16S
		b=25.717 s=-3.48	b=14.995 s=-3.4734	b=26.831 s=-3.516	b=14.887 s=-3.4826
PCR efficiency	E= 10^(-1/S)	1.917809342	1.940449495	1.924926303	1.93705428
%PCR efficiency	%E=(E-1)*100	91.78093422	94.04494945	92.4926303	93.70542798

Figure S7.7. PCR efficiencies of 16S and *pqsA* in *P. aeruginosa* wild type and *nirQ* mutant.

100, 10, 1, 0,1, 0.01 and 0.001 ng of cDNA samples from PAO1-L and Δ*nirQ* were amplified in a 96 well-plate using 7500 applied biosystem RT-PCR machine. PCR efficiency was calculated by using the calculating the slope each standard curve in PAO1-L samples (calibrator) and Δ*nirQ* mutant samples using 16s-RTPCR and pqsA-RTPCR primers set.

S8. Growth curves of *rhlR, lasR* and *pqsR* mutants and their derivatives using $P_{pqsA\ long}$ -lux and $P_{pqsA\ long}$ '-' -*luxCDABE* reporters.

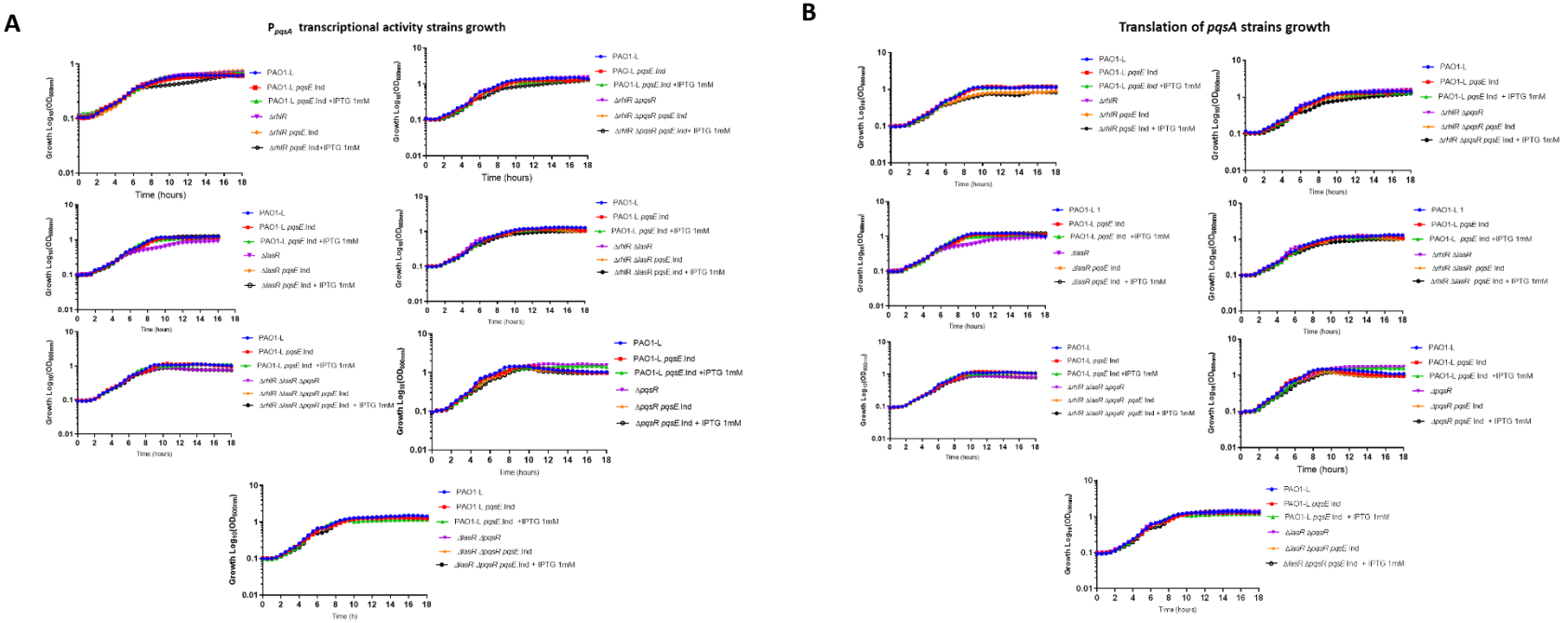


Figure S7.8. Growth curves of *P. aeruginosa* PAO1-L mutants and the *pqsE* Ind strains.

Growth curves of *P. aeruginosa PAO1-L, ΔrhlR, ΔlasR, ΔpqsR, ΔrhlR lasR, ΔlasR pqsR, ΔrhlR pqsR, ΔrhlR lasR pqsR* and the *pqsE* Ind mutants carrying the transcriptional fusion A) $P_{pqsA\ long}$ -*lux*- (Gm^R) and the translational fusion B) $P_{pqsA\ long}$ '-' -*luxCDABE* - (Gm^R). Strains were grown in LB media for 18 hours in a TECAN plate reader and the OD_{600nm} were measured. The Log10 growth curve of the OD_{600nm} is shown. Error bars represent 2x standard errors calculated across three biological replicates.

S9. Construction of the alternative transcripts *pqsx-T1* and *pqsx-T2*

The creation of the alternative transcripts *Term1* and *Term2* was performed as described in **Chapter 2, section 2.6.6** and it is illustrated in **Figure S7.9**

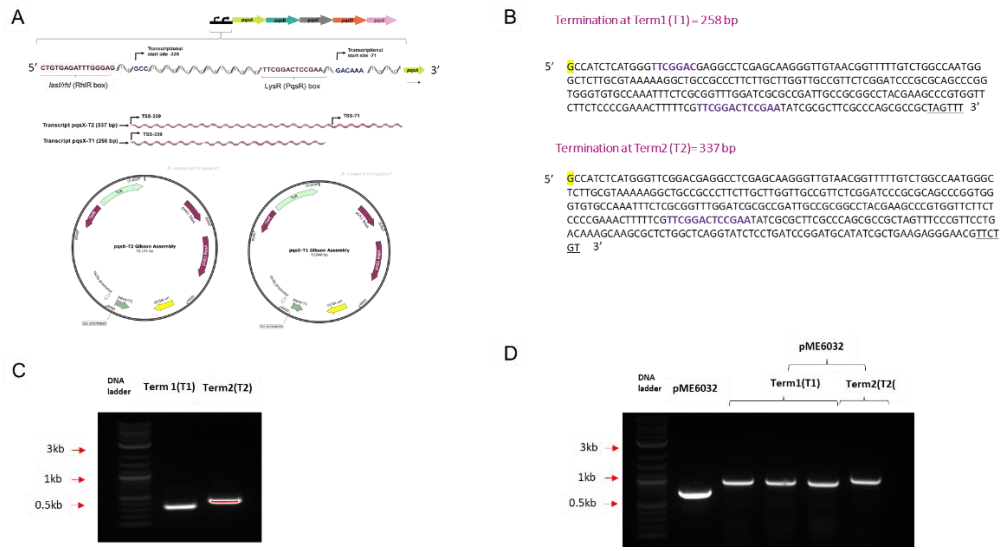


Figure S7.9. Construction of the alternative transcripts Term 1 (*pqsX-T1*) and Term2 (*pqsX-T2*) in pME6032.

A) Visual representation of the alternative transcripts Term1 (T1) and Term2 (T2) formed within the *pqsA* promoter and then inserted into pME6032 shuttle vector. B) DNA nucleotide sequences corresponding to regions of the transcripts T1 and T2, that included 258 and 337 nucleotides, respectively are shown. The transcriptional start site (TSS) is highlighted in yellow. C) PCR product of Term1(T1) and Term2(T2) at ~0.2kb and ~0.3 kb, respectively are shown in agarose gel. The resulting PCR products were incorporated into pME6032 using Gibson Assembly. D) Successful incorporation of Term1 (T1) and Term2 (T2) into pME6032 was confirmed by PCR and DNA sequencing. Quick-Load 2-Log (NEB) was used as DNA Ladder.

S10. Lrs1 transcript in PA14

Figure S7.10. Lrs1 sequence in PA14.

Genome-wide detection of sRNAs in intergenic regions (IGRs) (Wurtzel et al. 2012), identified *lrs1* (249 bp, underlined) at -268 bp upstream respective the *pqsA* TSS (+1). Mutation of *lrs1* performed in Wurtzel et al. (2012), Chuang et al., (2019) and Vrla et al*., (2020)* included +191 bp from the 5’ end of the Lrs1 sequence and it is highlighted in grey. The *LysR-box is* highlighted in green, and it is placed +217 bp from the *5’* end of Lrs1. Five-point insertions are present in PA14, highlighted in red.

8 Bibliography

Albus, A. M., Pesci, E. C., Runyen-Janecky, L. J., West, S. E. H., & Iglewski, B. H. (1997). Vfr controls quorum sensing in Pseudomonas aeruginosa. *Journal of Bacteriology*, *179*(12), 3928–3935. https://doi.org/10.1128/jb.179.12.3928-3935.1997

Allegretta, G., Maurer, C. K., Eberhard, J., Maura, D., Hartmann, R. W., Rahme, L., & Empting, M. (2017). In-depth profiling of MvfR-regulated small molecules in Pseudomonas aeruginosa after Quorum Sensing inhibitor treatment. *Frontiers in Microbiology*, *8*(MAY), 1–12. https://doi.org/10.3389/fmicb.2017.00924

Arai, H. (2011). Regulation and function of versatile aerobic and anaerobic respiratory metabolism in Pseudomonas aeruginosa. *Frontiers in Microbiology*, *2*(MAY), 1–13. https://doi.org/10.3389/fmicb.2011.00103

Arai, H., Igarashi, Y., & Kodama, T. (1994). Structure and ANR-Dependent Transcription of the nir Genes for Denitrification from Pseudomonas aeruginosa. *Bioscience, Biotechnology, and Biochemistry*, *58*(7), 1286–1291. https://doi.org/10.1271/bbb.58.1286

Arai, H., Kodama, T., & Igarashi, Y. (1999). Effect of nitrogen oxides on expression of the nir and nor genes for denitrification in Pseudomonas aeruginosa. *FEMS Microbiology Letters*, *170*(1), 19–24. https://doi.org/10.1016/S0378-1097(98)00517-5

Aslantas, Y., & Surmeli, N. B. (2019). *Effects of N-Terminal and C-Terminal Polyhistidine Tag on the Stability and Function of the Thermophilic P450 CYP119*. *2019*.

Azam, M. W., & Khan, A. U. (2019). Updates on the pathogenicity status of Pseudomonas aeruginosa. *Drug Discovery Today*, *24*(1), 350–359. https://doi.org/10.1016/j.drudis.2018.07.003

Baker, T. A., & Sauer, R. T. (2012). ClpXP, an ATP-powered unfolding and protein-degradation machine. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1823*(1), 15–28. https://doi.org/10.1016/j.bbamcr.2011.06.007

Baker, Y. R., Hodgkinson, J. T., Florea, B. I., Alza, E., Galloway, W. R. J. D., Grimm, L., Geddis, S. M., Overkleeft, H. S., Welch, M., & Spring, D. R. (2017). Identification of new quorum sensing autoinducer binding partners in: Pseudomonas aeruginosa using photoaffinity probes. *Chemical Science*, *8*(11), 7403–7411. https://doi.org/10.1039/c7sc01270e

Bala, A., Gupta, R. K., Chhibber, S., & Harjai, K. (2013). Detection and

quantification of quinolone signalling molecule: A third quorum sensing molecule of Pseudomonas aeruginosa by high performance-thin layer chromatography. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *930*, 30–35. https://doi.org/10.1016/j.jchromb.2013.04.027

Bassler, B. L., & Losick, R. (2006). Bacterially Speaking. *Cell*, *125*(2), 237–246. https://doi.org/10.1016/j.cell.2006.04.001

Blanc, D. S., Petignat, C., Janin, B., Bille, J., & Francioli, P. (1998). Frequency and molecular diversity of Pseudomonas aeruginosa upon admission and during hospitalization: A prospective epidemiologic study. *Clinical Microbiology and Infection*, *4*(5), 242–247. https://doi.org/10.1111/j.1469-0691.1998.tb00051.x

Bolanos-garcia, V. M., & Davies, O. R. (2006). *Structural analysis and classification of native proteins from E . coli commonly co-purified by immobilised metal affinity chromatography*. *1760*, 1304–1313. https://doi.org/10.1016/j.bbagen.2006.03.027

Boucher, R. C. (2007). Evidence for airway surface dehydration as the initiating event in CF airway disease. *Journal of Internal Medicine*, *261*(1), 5–16. https://doi.org/10.1111/j.1365-2796.2006.01744.x

Bouffartigues, E., Duchesne, R., Bazire, A., Simon, M., Maillot, O., Dufour, A., Feuilloley, M., Orange, N., & Chevalier, S. (2014). Sucrose favors Pseudomonas aeruginosa pellicle production through the extracytoplasmic function sigma factor SigX. *FEMS Microbiology Letters*, *356*(2), 193–200. https://doi.org/10.1111/1574-6968.12482

Boulette, M. L., Baynham, P. J., Jorth, P. A., Kukavica-Ibrulj, I., Longoria, A., Barrera, K., Levesque, R. C., & Whiteley, M. (2009). Characterization of alanine catabolism in Pseudomonas aeruginosa and its importance for proliferation in vivo. *Journal of Bacteriology*, *191*(20), 6329–6334. https://doi.org/10.1128/JB.00817-09

Boursier, M. E., Moore, J. D., Heitman, K. M., Shepardson-Fungairino, S. P., Combs, J. B., Koenig, L. C., Shin, D., Brown, E. C., Nagarajan, R., & Blackwell, H. E. (2018). Structure-Function Analyses of the N-Butanoyl L-Homoserine Lactone Quorum-Sensing Signal Define Features Critical to Activity in RhlR [Research-article]. *ACS Chemical Biology*, *13*(9), 2655–2662. https://doi.org/10.1021/acschembio.8b00577

Bredenbruch, F., Geffers, R., Nimtz, M., Buer, J., & Häussler, S. (2006). The Pseudomonas aeruginosa quinolone signal (PQS) has an iron-chelating activity. *Environmental Microbiology*, *8*(8), 1318–1329. https://doi.org/10.1111/j.1462-2920.2006.01025.x

Bredenbruch, F., Nimtz, M., Wray, V., Morr, M., & Mu, R. (2005). Biosynthetic

Pathway of Pseudomonas aeruginosa 4-Hydroxy-2-Alkylquinolines. *Journal of Bacteriology*, *187*(11), 3630–3635. https://doi.org/10.1128/JB.187.11.3630

Bretones, D. P. (2016). *Linking fatty acid derivatives with signalling and cross - talkin Pseudomonas aeruginosa*. *Thesis Submitted To The University of Nottingham For The Degree Of Doctor Of Philosophy.*

Brouwer, S., Pustelny, C., Ritter, C., Klinkert, B., Narberhaus, F., & Häussler, S. (2014). The PqsR and RhlR transcriptional regulators determine the level of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa by producing two different pqsABCDE mRNA Isoforms. *Journal of Bacteriology*, *196*(23), 4163–4171. https://doi.org/10.1128/JB.02000-14

Browning, D. F., & Busby, S. J. W. (2016). Local and global regulation of transcription initiation in bacteria. *Nature Reviews Microbiology*, *14*(10), 638–650. https://doi.org/10.1038/nrmicro.2016.103

Burgess, R. R. (1996). Purification of overproduced Escherichia coli RNA polymerase σ factors by solubilizing inclusion bodies and refolding from Sarkosyl. *Methods in Enzymology*, *273*(1993), 145–149. https://doi.org/10.1016/s0076-6879(96)73014-8

Busby, S., & Ebright, R. H. (1999). Transcription activation by catabolite activator protein (CAP). *Journal of Molecular Biology*, *293*(2), 199–213. https://doi.org/10.1006/jmbi.1999.3161

Butland, G., Peregrin-Alvarez, J. M., Li, J., Yang, W., Yang, X., Canadien, V., Starostine, A., Richards, D., Beattie, B., Krogan, N., Davey, M., Parkinson, J., Greenblatt, J., & Emili, A. (2005). Interaction network containing conserved and essential protein complexes in Escherichia coli. *Nature*, *433*(7025), 531–537. https://doi.org/10.1038/nature03239

Button, B., & Boucher, R. C. (2008). Role of mechanical stress in regulating airway surface hydration and mucus clearance rates. *Respiratory Physiology and Neurobiology*, *163*(1–3), 189–201. https://doi.org/10.1016/j.resp.2008.04.020

Calfee, M. W., Coleman, J. P., & Pesci, E. C. (2001). Interference with Pseudomonas quinoline signal synthesis inhibits virulence factor expression by Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(20), 11633–11637. https://doi.org/10.1073/pnas.201328498

Cao, H., Krishnan, G., Goumnerov, B., Tsongalis, J., Tompkins, R., & Rahme, L. G. (2001). *A quorum sensing-associated virulence gene of Pseudomonas aeruginosa encodes a LysR-like transcription regulator with a unique self-regulatory mechanism*. *98*(25), 10–15.

Carey, J. (1988). Gel retardation at low pH resolves trp repressor-DNA complexes for quantitative study. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(4), 975–979. https://doi.org/10.1073/pnas.85.4.975

Carloni, S., Macchi, R., Sattin, S., Ferrara, S., & Bertoni, G. (2017). The small RNA ReaL: a novel regulatory element embedded in the Pseudomonas aeruginosa quorum sensing networks. *Environmental Microbiology*, *19*(10), 4220–4237. https://doi.org/10.1111/1462-2920.13886

Castang, S., McManus, H. R., Turner, K. H., & Dove, S. L. (2008). H-NS family members function coordinately in an opportunistic pathogen. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(48), 18947–18952. https://doi.org/10.1073/pnas.0808215105

Cecchini, G., Schröder, I., Gunsalus, R. P., & Maklashina, E. (2002). Succinate dehydrogenase and fumarate reductase from Escherichia coli. *Biochimica et Biophysica Acta - Bioenergetics*, *1553*(1–2), 140–157. https://doi.org/10.1016/S0005-2728(01)00238-9

Chambedin, M., Mcgrath, J., Waskeu, L., Studier, F. W., Ring, J., Chamberlin, M., Davanloo, P., Rosenberg, A. H., Dunn, J. J., Studier, F. W., Richardson, C. C., Moffatt, B. A., Niles, E. G., Studier, F. W., Moss, B., Tabor, S., Struhl, K., Krippl, B., Bernstein, K. E., … Studier, F. W. (1990). *[6] Use of T7 RNA Polymerase to Direct Expression of Cloned Genes*. *185*(1986).

Chatterjee, J., Miyamoto, C. M., Zouzoulas, A., Franz Lang, B., Skouris, N., & Meighen, E. A. (2002). MetR and CRP bind to the Vibrio harveyi lux promoters and regulate luminescence. *Molecular Microbiology*, *46*(1), 101–111. https://doi.org/10.1046/j.1365-2958.2002.03128.x

Chisnall B., Johnson C., K. Y. and C. Y. W. (2014). Insoluble Protein Purification with Sarkosyl: Facts and Precautions. In: Chen Y. (eds) Structural Genomics. *Brenner's Encyclopedia of Genetics: Second Edition*, *1091*, 576–580. https://doi.org/10.1016/B978-0-12-374984-0.01487-X

Choi, Y., Park, H. Y., Park, S. J., Park, S. J., Kim, S. K., Ha, C., Im, S. J., & Lee, J. H. (2011). Growth phase-differential quorum sensing regulation of anthranilate metabolism in Pseudomonas aeruginosa. *Molecules and Cells*, *32*(1), 57–65. https://doi.org/10.1007/s10059-011-2322-6

Chrunyk, B. A., Evans, J., Lillquist, J., Young, P., & Wetzel, R. (1993). Inclusion body formation and protein stability in sequence variants of interleukin-1β. *Journal of Biological Chemistry*, *268*(24), 18053–18061. https://doi.org/10.1016/s0021-9258(17)46810-4

Chuang, S. K., Vrla, G. D., Fröhlich, K. S., & Gitai, Z. (2019). Surface association sensitizes Pseudomonas aeruginosa to quorum sensing. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-12153-1

Chugani, S. A., Whiteley, M., Lee, K. M., D'Argenio, D., Manoil, C., & Greenberg, E. P. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(5), 2752–2757. https://doi.org/10.1073/pnas.051624298

Croda-García, G., Grosso-Becerra, V., Gonzalez-Valdez, A., Servín-González, L., & Soberón-Chávez, G. (2011). Transcriptional regulation of Pseudomonas aeruginosa rhlR: Role of the CRP orthologue Vfr (virulence factor regulator) and quorum-sensing regulators LasR and RhlR. *Microbiology*, *157*(9), 2545–2555. https://doi.org/10.1099/mic.0.050161-0

De Boer, A. P. N., Van Der Oost, J., Reijnders, W. N. M., Westerhoff, H. V., Stouthamer, A. H., & Van Spanning, R. J. M. (1996). Mutational analysis of the nor gene cluster which encodes nitric-oxide reductase from Paracoccus denitrificans. *European Journal of Biochemistry*, *242*(3), 592–600. https://doi.org/10.1111/j.1432-1033.1996.0592r.x

de Kievit, T., Seed, P. C., Nezezon, J., Passador, L., & Iglewski, B. H. (1999). RsaL, a novel repressor of virulence gene expression in Pseudomonas aeruginosa. *J Bacteriol*, *181*(7), 2175–2184.

Dekimpe, V., & Déziel, E. (2009). Revisiting the quorum-sensing hierarchy in Pseudomonas aeruginosa: The transcriptional regulator RhlR regulates LasR-specific factors. *Microbiology*, *155*(3), 712–723. https://doi.org/10.1099/mic.0.022764-0

Deziel, E., Lepine, F., Milot, S., He, J., Mindrinos, M. N., Tompkins, R. G., & Rahme, L. G. (2004). Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proceedings of the National Academy of Sciences*, *101*(5), 1339–1344. https://doi.org/10.1073/pnas.0307694100

Diggle, S. P., Matthijs, S., Wright, V. J., Fletcher, M. P., Chhabra, S. R., Lamont, I. L., Kong, X., Hider, R. C., Cornelis, P., Cámara, M., & Williams, P. (2007). The Pseudomonas aeruginosa 4-Quinolone Signal Molecules HHQ and PQS Play Multifunctional Roles in Quorum Sensing and Iron Entrapment. *Chemistry and Biology*, *14*(1), 87–96. https://doi.org/10.1016/j.chembiol.2006.11.014

Diggle, S. P., Winzer, K., Chhabra, S. R., Worrall, K. E., Cámara, M., & Williams, P. (2003). *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*. *50*, 29–43. https://doi.org/10.1046/j.1365-2958.2003.03672.x

Ding, F., Oinuma, K. I., Smalley, N. E., Schaefer, A. L., Hamwy, O., Greenberg, E. P., & Dandekar, A. A. (2018). The Pseudomonas aeruginosa orphan

quorum sensing signal receptor QscR regulates global quorum sensing gene expression by activating a single linked operon. *MBio*, *9*(4). https://doi.org/10.1128/mBio.01274-18

Djapgne, L., Panja, S., Brewer, L. K., Gans, J. H., Kane, M. A., & Woodson, S. A. (2018). *crossm Regulatory RNAs Promote 2-Alkyl-4-Quinolone Production through Redundant Regulation of the antR mRNA*. *200*(10), 1–17.

Drees, S. L., Ernst, S., Belviso, B. D., Jagmann, N., Hennecke, U., & Fetzner, S. (2018). PqsL uses reduced flavin to produce 2-hydroxylaminobenzoylacetate, a preferred PqsBC substrate in alkyl quinolone biosynthesis in Pseudomonas aeruginosa. *Journal of Biological Chemistry*, *293*(24), 9345–9357. https://doi.org/10.1074/jbc.RA117.000789

Drees, S. L., & Fetzner, S. (2015). PqsE of Pseudomonas aeruginosa acts as pathway-specific thioesterase in the biosynthesis of alkylquinolone signaling molecules. *Chemistry and Biology*, *22*(5), 611–618. https://doi.org/10.1016/j.chembiol.2015.04.012

Eckweiler, D., Schniederjans, M., Zimmermann, A., Jensen, V., Scharfe, M., Geffers, R., & Ha, S. (2012). *The Pseudomonas aeruginosa Transcriptome in Planktonic Cultures and Static Biofilms Using RNA Sequencing*. *7*(2). https://doi.org/10.1371/journal.pone.0031092

El Bakkouri, M., Gutsche, I., Kanjee, U., Zhao, B., Yu, M., Goret, G., Schoehn, G., Burmeister, W. P., & Houry, W. A. (2010). Structure of RavA MoxR AAA+ protein reveals the design principles of a molecular cage modulating the inducible lysine decarboxylase activity. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(52), 22499–22504. https://doi.org/10.1073/pnas.1009092107

El Zoeiby, A., Sanschagrin, F., Havugimana, P. C., Garnier, A., & Levesque, R. C. (2001). In vitro reconstruction of the biosynthetic pathway of peptidoglycan cytoplasmic precursor in Pseudomonas aeruginosa. *FEMS Microbiology Letters*, *201*(2), 229–235. https://doi.org/10.1016/S0378-1097(01)00268-3

Essar, D. W., Eberly, L., Hadero, A., & Crawford, I. P. (1990). Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: Interchangeability of the two anthranilate synthase and evolutionary implications. *Journal of Bacteriology*, *172*(2), 884–900. https://doi.org/10.1128/jb.172.2.884-900.1990

Farrow, J. M., Lynn Hudson, L., Wells, G., Coleman, J. P., & Pesci, E. C. (2015). CysB negatively affects the transcription of pqsR and Pseudomonas quinolone signal production in Pseudomonas aeruginosa. *Journal of Bacteriology*, *197*(12), 1988–2002. https://doi.org/10.1128/JB.00246-15

Farrow, J. M., & Pesci, E. C. (2007). Two distinct pathways supply anthranilate as a precursor of the Pseudomonas quinolone signal. *Journal of Bacteriology*, *189*(9), 3425–3433. https://doi.org/10.1128/JB.00209-07

Farrow, J. M., Sund, Z. M., Ellison, M. L., Wade, D. S., Coleman, J. P., & Pesci, E. C. (2008). PqsE functions independently of PqsR-Pseudomonas quinolone signal and enhances the rhl quorum-sensing system. *Journal of Bacteriology*, *190*(21), 7043–7051. https://doi.org/10.1128/JB.00753-08

Farrow, J., & Pesci, E. C. (2017). Distal and proximal promoters co-regulate pqsR expression in Pseudomonas aeruginosa. *Physiology & Behavior*, *104*(1), 78–91. https://doi.org/10.1111/mmi.13611.Distal

Filiatrault, M J, Wagner, V. E., Bushnell, D., Haidaris, C. G., & Iglewski, B. H. (2005). Effect of Anaerobiosis and Nitrate on Gene Expression in. *Society*, *73*(6), 3764–3772. https://doi.org/10.1128/IAI.73.6.3764

Filiatrault, Melanie J., Picardo, K. F., Ngai, H., Passador, L., & Iglewski, B. H. (2006). Identification of Pseudomonas aeruginosa genes involved in virulence and anaerobic growth. *Infection and Immunity*, *74*(7), 4237–4245. https://doi.org/10.1128/IAI.02014-05

Fletcher, M. P., Diggle, S. P., Cámara, M., & Williams, P. (2007). Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules. *Nat Prot*, *2*(5), 1254–1262. https://doi.org/10.1038/nprot.2007.158

Florez, C., Raab, J. E., Cooke, A. C., & Schertzer, J. W. (2017). Membrane Distribution of the Pseudomonas Quinolone Signal Modulates Outer Membrane Vesicle Production in Pseudomonas aeruginosa Catalina. *MBio*, 1–13.

Folch, B., Déziel, E., & Doucet, N. (2013). Systematic Mutational Analysis of the Putative Hydrolase PqsE: Toward a Deeper Molecular Understanding of Virulence Acquisition in Pseudomonas aeruginosa. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0073727

Francis, V. I., Stevenson, E. C., & Porter, S. L. (2017). Two-component systems required for virulence in Pseudomonas aeruginosa. *FEMS Microbiology Letters*, *364*(11), 1–22. https://doi.org/10.1093/femsle/fnx104

Fuchs, E. L., Brutinel, E. D., Jones, A. K., Fulcher, N. B., Urbanowski, M. L., Yahr, T. L., & Wolfgang, M. C. (2010). The Pseudomonas aeruginosa Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. *Journal of Bacteriology*, *192*(14), 3553–3564. https://doi.org/10.1128/JB.00363-10

Gaberc-porekar, V., Fonda, I., & Podobnik, B. (2005). *Production of Nonclassical Inclusion Bodies from Which Correctly*. 632–639.

Gad, E. A. M., El-Sukkary, M. M. A., & Ismail, D. A. (1997). Surface and thermodynamic parameters of sodium N-acyl sarcosinate surfactant solutions. *JAOCS, Journal of the American Oil Chemists' Society*, *74*(1), 43–47. https://doi.org/10.1007/s11746-997-0117-x

Galán, B., Dinjaski, N., Maestro, B., De Eugenio, L. I., Escapa, I. F., Sanz, J. M., García, J. L., & Prieto, M. A. (2011). Nucleoid-associated PhaF phasin drives intracellular location and segregation of polyhydroxyalkanoate granules in Pseudomonas putida KT2442. *Molecular Microbiology*, *79*(2), 402–418. https://doi.org/10.1111/j.1365-2958.2010.07450.x

Gallagher, L. A., McKnight, S. L., Kuznetsova, M. S., Pesci, E. C., & Manoil, C. (2002). Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. *Journal of Bacteriology*, *184*(23), 6472–6480. https://doi.org/10.1128/JB.184.23.6472-6480.2002

Galperin, M. Y., & Koonin, E. V. (2000). Who's your neighbor? New computational approaches for functional genomics. *Nature Biotechnology*, *18*(6), 609–613. https://doi.org/10.1038/76443

Gambello, M. J., Kaye, S., & Iglewski, B. H. (1993). LasR of Pseudomonas aeruginosa is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infection and Immunity*, *61*(4), 1180–1184. https://doi.org/10.1128/iai.61.4.1180-1184.1993

García-Reyes, S., Cocotl-Yañez, M., Soto-Aceves, M. P., González-Valdez, A., Servín-González, L., & Soberón-Chávez, G. (2021). PqsR-independent quorum-sensing response of Pseudomonas aeruginosa ATCC 9027 outlier-strain reveals new insights on the PqsE effect on RhlR activity. *Molecular Microbiology*, *116*(4), 1113–1123. https://doi.org/10.1111/mmi.14797

García-Reyes, S., Cocotl-Yañez, M., Soto-Aceves, M. P., González-Valdez, A., Servín-González, L., & Soberón-Chávez, G. (2021). PqsR-independent quorum-sensing response of Pseudomonas aeruginosa ATCC 9027 outlier-strain reveals new insights on the PqsE effect on RhlR activity . *Molecular Microbiology*, *February*, 1–11. https://doi.org/10.1111/mmi.14797

Gellatly, S. L., & Hancock, R. E. W. (2013). Pseudomonas aeruginosa: New insights into pathogenesis and host defenses. *Pathogens and Disease*, *67*(3), 159–173. https://doi.org/10.1111/2049-632X.12033

Gicquel, G., Bouffartigues, E., Bains, M., Oxaran, V., Rosay, T., Lesouhaitier, O., Connil, N., Bazire, A., Maillot, O., Bénard, M., Cornelis, P., Hancock, R. E. W., Dufour, A., Feuilloley, M. G. J., Orange, N., Déziel, E., & Chevalier, S. (2013). The extra-cytoplasmic function sigma factor sigX modulates biofilm and virulence-related properties in pseudomonas aeruginosa. *PLoS ONE*, *8*(11), 1–15. https://doi.org/10.1371/journal.pone.0080407

Glessner, A., Smith, R. S., Iglewski, B. H., & Robinson, J. B. (1999). Roles of

Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of twitching motility. *Journal of Bacteriology*, *181*(5), 1623–1629. https://doi.org/10.1128/jb.181.5.1623-1629.1999

Goenrich, M., Bartoschek, S., Hagemeier, C. H., Griesinger, C., & Vorholt, J. A. (2002). A glutathione-dependent formaldehyde-activating enzyme (Gfa) from Paracoccus denitrificans detected and purified via two-dimensional proton exchange NMR spectroscopy. *Journal of Biological Chemistry*, *277*(5), 3069–3072. https://doi.org/10.1074/jbc.C100579200

Gosset, G., Zhang, Z., Nayyar, S., Cuevas, W. A., & Saier, M. H. (2004). Transcriptome analysis of Crp-dependent catabolite control of gene expression in Escherichia coli. *Journal of Bacteriology*, *186*(11), 3516–3524. https://doi.org/10.1128/JB.186.11.3516-3524.2004

Gottesman S, Squires C, Pichersky E, Carrington M, Hobbs M, Mattick JS, Dalrymple B, Kuramitsu H, Shiroza T, Foster T, et al. (2014). Conservation of the Regulatory Subunit for the Clp ATP-Dependent Protease in Prokaryotes and Eukaryotes. *Proc Natl Acad Sci U S A.*, *5*(May), 1–7.

Govan, J. R. W., & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiological Reviews*, *60*(3), 539–574. https://doi.org/10.1111/j.1365-2672.2007.03706.x

Grant, S. G., Jessee, J., Bloom, F. R., & Hanahan, D. (1990). Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(12), 4645–4649.

Grodbergl, J., & Dunn, J. J. (1988). *ompT Encodes the Escherichia coli Outer Membrane Protease That Cleaves T7 RNA Polymerase during Purification*. *170*(3), 1245–1253.

Groleau, M., & Pereira, T. D. O. (2020). *crossm Regulation in Pseudomonas aeruginosa*.

Hand, N. J., & Silhavy, T. J. (2000). A practical guide to the construction and use of lac fusions in Escherichia coli. *Methods in Enzymology*, *326*(1985), 11–35. https://doi.org/10.1016/s0076-6879(00)26044-8

Hanson, P. I., & Whiteheart, S. W. (2005). AAA+ proteins: Have engine, will work. *Nature Reviews Molecular Cell Biology*, *6*(7), 519–529. https://doi.org/10.1038/nrm1684

Härtig, E., Schiek, U., Vollack, K. U., & Zumft, W. G. (1999). Nitrate and nitrite control of respiratory nitrate reduction in denitrifying Pseudomonas stutzeri by a two-component regulatory system homologous to NarXL of Escherichia coli. *Journal of Bacteriology*, *181*(12), 3658–3665.

https://doi.org/10.1128/jb.181.12.3658-3665.1999

Häussler, S., & Becker, T. (2008). The pseudomonas quinolone signal (PQS) balances life and death in Pseudomonas aeruginosa populations. *PLoS Pathogens*, *4*(9). https://doi.org/10.1371/journal.ppat.1000166

Hayashi, N. R., Arai, H., Kodama, T., & Igarashi, Y. (1998). The nirQ gene, which is required for denitrification of Pseudomonas aeruginosa, can activate the RubisCO from Pseudomonas hydrogenothermophila. *Biochimica et Biophysica Acta - General Subjects*, *1381*(3), 347–350. https://doi.org/10.1016/S0304-4165(98)00045-2

Hayashi, N. R., & Igarashi, Y. (2002). ATP binding and hydrolysis and autophosphorylation of CbbQ encoded by the gene located downstream of RubisCO genes. *Biochemical and Biophysical Research Communications*, *290*(5), 1434–1440. https://doi.org/10.1006/bbrc.2002.6366

Hazan, R., He, J., Xiao, G., Dekimpe, V., Apidianakis, Y., Lesic, B., Astrakas, C., Déziel, E., Lépine, F., & Rahme, L. G. (2010). Homeostatic interplay between bacterial cell-cell signaling and iron in virulence. *PLoS Pathogens*, *6*(3). https://doi.org/10.1371/journal.ppat.1000810

He, W., Li, C., & Lu, C. D. (2011). Regulation and characterization of the dadRAX Locus for D-Amino acid catabolism in Pseudomonas aeruginosa PAO1. *Journal of Bacteriology*, *193*(9), 2107–2115. https://doi.org/10.1128/JB.00036-11

Heeb, S., Blumer, C., & Haas, D. (2002). Regulatory RNA as Mediator in GacA / RsmA-Dependent Global Control of Exoproduct Formation in Pseudomonas Regulatory RNA as Mediator in GacA / RsmA-Dependent Global Control of Exoproduct Formation in Pseudomonas fluorescens CHA0. *Journal of Bacteriology*, *184*(4), 1046–1056. https://doi.org/10.1128/JB.184.4.1046

Heeb, S., Fletcher, M. P., Chhabra, S. R., Diggle, S. P., Williams, P., & Cámara, M. (2011). Quinolones: From antibiotics to autoinducers. *FEMS Microbiology Reviews*, *35*(2), 247–274. https://doi.org/10.1111/j.1574-6976.2010.00247.x

Hellman, L., & Fried, M. (2007). Electrophoretic Mobility Shift Assay (EMSA) for Detecting Protein- Nucleic Acid Interactions. *Nature Protocols*, *2*(8), 1849–1861. https://doi.org/10.1038/nprot.2007.249.Electrophoretic

Henrichfreise, B., Wiegand, I., Pfister, W., & Wiedemann, B. (2007). Resistance mechanisms of multiresistant Pseudomonas aeruginosa strains from Germany and correlation with hypermutation. *Antimicrobial Agents and Chemotherapy*, *51*(11), 4062–4070. https://doi.org/10.1128/AAC.00148-07

Heurlier, K., De, V., Pessi, G., Reimmann, C., & Haas, D. (2003). Negative Control of Quorum Sensing by RpoN (Sigma 54) in Pseudomonas aeruginosa PAO1. *Journal of Bacteriology*, *185*(7), 2227–2235. https://doi.org/10.1128/JB.185.7.2227

Higgins, S., Heeb, S., Rampioni, G., Fletcher, M. P., Williams, P., & Cámara, M. (2018). Differential regulation of the phenazine biosynthetic operons by quorum sensing in Pseudomonas aeruginosa PAO1-N. *Frontiers in Cellular and Infection Microbiology*, *8*(JUL), 1–13. https://doi.org/10.3389/fcimb.2018.00252

Hiroyuki Arai, Tohru Kodama, Y. I. (1998). *The Role of the nirQOP Genes in Energy Conservation during Anaerobic Growth of Pseudomonas aeruginosa*.

Hmelo, L. R., Borlee, B. R., Almblad, H., Love, M. E., Randall, T. E., Tseng, B. S., Lin, C., Irie, Y., Storek, K. M., Yang, J. J., Siehnel, R. J., Howell, P. L., Singh, P. K., Tolker-Nielsen, T., Parsek, M. R., Schweizer, H. P., & Harrison, J. J. (2015). Precision-engineering the Pseudomonas aeruginosa genome with two-step allelic exchange. *Nature Protocols*, *10*(11), 1820–1841. https://doi.org/10.1038/nprot.2015.115

Hoffman, L. R., Kulasekara, H. D., Emerson, J., Houston, L. S., Burns, J. L., Ramsey, B. W., & Miller, S. I. (2009). Pseudomonas aeruginosa lasR mutants are associated with cystic fibrosis lung disease progression. *Journal of Cystic Fibrosis*, *8*(1), 66–70. https://doi.org/10.1016/j.jcf.2008.09.006

Hohenester, E., & Engel, J. (2002). Domain structure and organisation in extracellular matrix proteins. *Matrix Biology*, *21*(2), 115–128. https://doi.org/10.1016/S0945-053X(01)00191-3

Holloway, B. W. (1955). Genetic recombination in Pseudomonas aeruginosa. *Journal of General Microbiology*, *13*(3), 572–581. https://doi.org/10.1099/00221287-13-3-572

Hong, P., Koza, S., & Bouvier, E. S. P. (2012). A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. *Journal of Liquid Chromatography and Related Technologies*, *35*(20), 2923–2950. https://doi.org/10.1080/10826076.2012.743724

Inamatsu, T. (1991). Pseudomonas aeruginosa bacteremia. *Nippon Rinsho. Japanese Journal of Clinical Medicine*, *49*(10), 2331–2335. https://doi.org/10.5694/j.1326-5377.1993.tb138046.x

Jerker Porath, Jan carlsson, I. O. & G. B. (1975). *methal affinity IMAC for protein purification.pdf*. https://doi.org/258, pages598–599 (1975)

Julia Emerson, Margaret Rosenfeld, S. M., & Bonnie Ramsey, and R. L. G. (2002).

Pseudomonas aeruginosa and other predictors of mortality and morbidity in young children with cystic fibrosis.pdf. https://doi.org/10.1002/ppul.10127

Junier, I., & Rivoire, O. (2016). Conserved units of co-expression in bacterial genomes: An evolutionary insight into transcriptional regulation. *PLoS ONE*, *11*(5), 1–25. https://doi.org/10.1371/journal.pone.0155740

Kang, C., Kim, S., Kim, H., Park, S., Choe, Y., Oh, M., Kim, E., & Choe, K. (2003). Pseudomonas aeruginosa Bacteremia: Risk Factors for Mortality and Influence of Delayed Receipt of Effective Antimicrobial Therapy on Clinical Outcome . *Clinical Infectious Diseases*, *37*(6), 745–751. https://doi.org/10.1086/377200

Kang, H., Gan, J., Zhao, J., Kong, W., Zhang, J., Zhu, M., Li, F., Song, Y., Qin, J., & Liang, H. (2017). Crystal structure of Pseudomonas aeruginosa RsaL bound to promoter DNA reaffirms its role as a global regulator involved in quorum-sensing. *Nucleic Acids Research*, *45*(2), 699–710. https://doi.org/10.1093/nar/gkw954

Kanjee, U., Gutsche, I., Alexopoulos, E., Zhao, B., El Bakkouri, M., Thibault, G., Liu, K., Ramachandran, S., Snider, J., Pai, E. F., & Houry, W. A. (2011). Linkage between the bacterial acid stress and stringent responses: The structure of the inducible lysine decarboxylase. *EMBO Journal*, *30*(5), 931–944. https://doi.org/10.1038/emboj.2011.5

Kay, E., Humair, B., Dénervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C., & Haas, D. (2006). Two GacA-dependent small RNAs modulate the quorum-sensing response in Pseudomonas aeruginosa. *Journal of Bacteriology*, *188*(16), 6026–6033. https://doi.org/10.1128/JB.00409-06

Kievit, T. R. D. E., Gillis, R., Marx, S., & Brown, C. (2001). Quorum-Sensing Genes in Pseudomonas aeruginosa Biofilms : Their Role and Expression Patterns. *Applied and Environmental Microbiology*, *67*(4), 1865–1873. https://doi.org/10.1128/AEM.67.4.1865

Kindrachuk, K. N., Fernández, L., Bains, M., & Hancock, R. E. W. (2011). Involvement of an ATP-dependent protease, PA0779/AsrA, in inducing heat shock in response to tobramycin in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, *55*(5), 1874–1882. https://doi.org/10.1128/AAC.00935-10

Knoten, C. A., Wells, G., Coleman, J. P., & Pesci, E. C. (2014). *A Conserved Suppressor Mutation in a Tryptophan Auxotroph Results in Dysregulation of Pseudomonas Quinolone Signal Synthesis*. *196*(13), 2413–2422. https://doi.org/10.1128/JB.01635-14

Kolb, A. (1993). Transcriptional Regulation by cAMP and its Receptor Protein. *Annual Review of Biochemistry*, *62*(1), 749–795.

https://doi.org/10.1146/annurev.biochem.62.1.749

Koskenkorva, T., Aro-Kärkkäinen, N., Bachmann, D., Arai, H., Frey, A. D., & Kallio, P. T. (2008). Transcriptional activity of Pseudomonas aeruginosa fhp promoter is dependent on two regulators in addition to FhpR. *Archives of Microbiology*, *189*(4), 385–396. https://doi.org/10.1007/s00203-007-0329-3

Kruger, E., Volker, U. W. E., & Hecker, M. (1994). *Stress Induction of clpC in Bacillus subtilis and Its Involvement in Stress Tolerance*. *176*(11), 3360–3367.

Kuehn, M. J., & Kesty, N. C. (2005). Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes and Development*, *19*(22), 2645–2655. https://doi.org/10.1101/gad.1299905

Kurnasov, O., Jablonski, L., Polanuyer, B., Dorrestein, P., Begley, T., & Osterman, A. (2003). Aerobic tryptophan degradation pathway in bacteria: Novel kynurenine formamidase. *FEMS Microbiology Letters*, *227*(2), 219–227. https://doi.org/10.1016/S0378-1097(03)00684-0

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., & Haas, D. (1992). Global control in Pseudomonas fluorescens mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(5), 1562–1566. https://doi.org/10.1073/pnas.89.5.1562

Lawson, C. L., Swigon, D., Murakami, K. S., Darst, S. A., Berman, H. M., & Ebright, R. H. (2004). Catabolite activator protein: DNA binding and transcription activation. *Current Opinion in Structural Biology*, *14*(1), 10–20. https://doi.org/10.1016/j.sbi.2004.01.012

Ledgham, F., Ventre, I., Soscia, C., Foglino, M., Sturgis, J. N., & Lazdunski, A. (2003). Interactions of the quorum sensing regulator QscR: Interaction with itself and the other regulators of Pseudomonas aeruginosa LasR and RhlR. *Molecular Microbiology*, *48*(1), 199–210. https://doi.org/10.1046/j.1365-2958.2003.03423.x

Lenz, A. P., Williamson, K. S., Pitts, B., Stewart, P. S., & Franklin, M. J. (2008). Localized gene expression in Pseudomonas aeruginosa biofilms. *Applied and Environmental Microbiology*, *74*(14), 4463–4471. https://doi.org/10.1128/AEM.00710-08

Leoni, L., Orsi, N., & Visca, P. (2000). Functional Analysis of PvdS, an Iron Starvation Sigma Factor of. *Society*, *182*(6), 1481–1491.

Lequette, Y., Lee, J. H., Ledgham, F., Lazdunski, A., & Greenberg, E. P. (2006). A distinct QscR regulon in the Pseudomonas aeruginosa quorum-sensing circuit. *Journal of Bacteriology*, *188*(9), 3365–3370.

https://doi.org/10.1128/JB.188.9.3365-3370.2006

Li, Z., Kosorok, M. R., Farrell, P. M., Laxova, A., West, S. E. H., Green, C. G., Collins, J., Rock, M. J., & Splaingard, M. L. (2005). Longitudinal development of mucoid Pseudomonas aeruginosa infection and lung disease progression in children with cystic fibrosis. *Journal of the American Medical Association*, *293*(5), 581–588. https://doi.org/10.1001/jama.293.5.581

Liang, H., Li, N., Dong, Z., Surette, M. G., & Duan, K. (2008). The YebC family protein PA0964 negatively regulates the Pseudomonas aeruginosa quinolone signal system and pyocyanin production. *Journal of Bacteriology*, *190*(18), 6217–6227. https://doi.org/10.1128/JB.00428-08

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Longo, F., Rampioni, G., Bondì, R., Imperi, F., Fimia, G. M., Visca, P., Zennaro, E., & Leoni, L. (2013). A New Transcriptional Repressor of the Pseudomonas aeruginosa Quorum Sensing Receptor Gene lasR. *PLoS ONE*, *8*(7), 1–9. https://doi.org/10.1371/journal.pone.0069554

Malgaonkar, A., & Nair, M. (2019). Quorum sensing in Pseudomonas aeruginosa mediated by RhlR is regulated by a small RNA PhrD. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-018-36488-9

Mandsberg, L. F., Ciofu, O., Kirkby, N., Christiansen, L. E., Poulsen, H. E., & Høiby, N. (2009). Antibiotic resistance in Pseudomonas aeruginosa strains with increased mutation frequency due to inactivation of the DNA oxidative repair system. *Antimicrobial Agents and Chemotherapy*, *53*(6), 2483–2491. https://doi.org/10.1128/AAC.00428-08

Mashburn-Warren, L., Howe, J., Brandenburg, K., & Whiteley, M. (2009). Structural requirements of the Pseudomonas quinolone signal for membrane vesicle stimulation. *Journal of Bacteriology*, *191*(10), 3411–3414. https://doi.org/10.1128/JB.00052-09

Mashburn-Warren, L., Howe, J., Garidel, P., Richter, W., Steiniger, F., Roessle, M., Brandenburg, K., & Whiteley, M. (2008). Interaction of quorum signals with outer membrane lipids: Insights into prokaryotic membrane vesicle formation. *Molecular Microbiology*, *69*(2), 491–502. https://doi.org/10.1111/j.1365-2958.2008.06302.x

Mashburn, L. M., & Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature*, *437*(7057), 422–425. https://doi.org/10.1038/nature03925

Mathlouthi, A., Pennacchietti, E., & De Biase, D. (2018). Effect of temperature,

pH and plasmids on in vitro biofilm formation in Escherichia coli. *Acta Naturae*, *10*(4), 129–132. https://doi.org/10.32607/20758251-2018-10-4-129-132

Mavrodi, D. V., Bonsall, R. F., Delaney, S. M., Soule, M. J., Phillips, G., & Thomashow, L. S. (2001). Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from Pseudomonas aeruginosa PAO1. *Journal of Bacteriology*, *183*(21), 6454–6465. https://doi.org/10.1128/JB.183.21.6454-6465.2001

Mavrodi, D. V., Peever, T. L., Mavrodi, O. V., Parejko, J. A., Raaijmakers, J. M., Lemanceau, P., Mazurier, S., Heide, L., Blankenfeldt, W., Weller, D. M., & Thomashow, L. S. (2010). Diversity and evolution of the Phenazine Biosynthesis Pathways. *Applied and Environmental Microbiology*, *76*(3), 866–879. https://doi.org/10.1128/AEM.02009-09

Mawla, G. D., Hall, B. M., Cárcamo-Oyarce, G., Grant, R. A., Zhang, J. J., Kardon, J. R., Ribbeck, K., Sauer, R. T., & Baker, T. A. (2021). ClpP1P2 peptidase activity promotes biofilm formation in Pseudomonas aeruginosa. *Molecular Microbiology*, *115*(6), 1094–1109. https://doi.org/10.1111/mmi.14649

McGrath, S., Wade, D. S., & Pesci, E. C. (2004). Dueling quorum sensing systems in Pseudomonas aeruginosa control the production of the Pseudomonas quinolone signal (PQS). *FEMS Microbiology Letters*, *230*(1), 27–34. https://doi.org/10.1016/S0378-1097(03)00849-8

McKnight, S. L., Iglewski, B. H., & Pesci, E. C. (2000). The Pseudomonas quinolone signal regulates rhl quorum sensing in Pseudomonas aeruginosa. *Journal of Bacteriology*, *182*(10), 2702–2708. https://doi.org/10.1128/JB.182.10.2702-2708.2000

Medina, G., Juárez, K., Díaz, R., & Soberón-Chávez, G. (2003). Transcriptional regulation of Pseudomonas aeruginosa rhlR, encoding a quorum-sensing regulatory protein. *Microbiology*, *149*(11), 3073–3081. https://doi.org/10.1099/mic.0.26282-0

Meighen, E. A. (1993). Bacterial bioluminescence: organization, regulation, and application of the lux genes . *The FASEB Journal*, *7*(11), 1016–1022. https://doi.org/10.1096/fasebj.7.11.8370470

Mercenier, A., Simon, J. P., Vander Wauven, C., Haas, D., & Stalon, V. (1980). Regulation of enzyme synthesis in the arginine deiminase pathway of Pseudomonas aeruginosa. *Journal of Bacteriology*, *144*(1), 159–163. https://doi.org/10.1128/jb.144.1.159-163.1980

Milton, D. L., Toole, R. O., & Horstedt, P. (1996). Flagellin A is essential for the virulence of Vibrio anguillarum . These include : Flagellin A Is Essential for the Virulence of Vibrio anguillarum. *Microbiology*, *178*(5), 1310–1319.

https://doi.org/8631707

Mukherjee, S., Moustafa, D. A., Stergioula, V., Smith, C. D., Goldberg, J. B., & Bassler, B. L. (2018). The PqsE and RhlR proteins are an autoinducer synthase–receptor pair that control virulence and biofilm development in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(40), E9411–E9418. https://doi.org/10.1073/pnas.1814023115

Mukherjee, S., Moustafa, D., Smith, C. D., Goldberg, J. B., & Bassler, B. L. (2017). The RhlR quorum-sensing receptor controls Pseudomonas aeruginosa pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. *PLoS Pathogens*, *13*(7), 1–25. https://doi.org/10.1371/journal.ppat.1006504

Nadal Jimenez, P., Koch, G., Thompson, J. A., Xavier, K. B., Cool, R. H., & Quax, W. J. (2012). The Multiple Signaling Systems Regulating Virulence in Pseudomonas aeruginosa. *Microbiology and Molecular Biology Reviews*, *76*(1), 46–65. https://doi.org/10.1128/MMBR.05007-11

Neuwald, A. F., Aravind, L., Spouge, J. L., & Koonin, E. V. (1999). AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Research*, *9*(1), 27–43. https://doi.org/10.1101/gr.9.1.27

Nilsson, J., Sta, S., Lundeberg, J., & Uhle, M. (1997). *Affinity Fusion Strategies for Detection , Purification , and Immobilization of Recombinant Proteins*. *16*, 1–16.

Oglesby, A. G., Farrow, J. M., Lee, J. H., Tomaras, A. P., Greenberg, E. P., Pesci, E. C., & Vasil, M. L. (2008). The influence of iron on Pseudomonas aeruginosa physiology: A regulatory link between iron and quorum sensing. *Journal of Biological Chemistry*, *283*(23), 15558–15567. https://doi.org/10.1074/jbc.M707840200

Oton, E. (2018). *Pseudomonas aeruginosa PQS mediated virulence regulation and interference*. *Thesis Submitted To The University of Nottingham For The Degree Of Doctor Of Philosophy.*

Pachori, P., Gothalwal, R., & Gandhi, P. (2019). Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review. *Genes and Diseases*, *6*(2), 109–119. https://doi.org/10.1016/j.gendis.2019.04.001

Pelzmann, A., Ferner, M., Gnida, M., Meyer-Klaucke, W., & Meyer, O. (2009). The CoxD protein of Oligotropha carboxidovorans is a predicted AAA+ ATPase chaperone involved in the biogenesis of the CO dehydrogenase [CuSMoO2] cluster. *Journal of Biological Chemistry*, *284*(14), 9578–9586. https://doi.org/10.1074/jbc.M805354200

Pesci, E C, Pearson, J. P., Seed, P. C., Pesci, E. C., Pearson, J. P., Seed, P. C., & Iglewski, B. H. (1997). Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa Regulation of las and rhl Quorum Sensing in Pseudomonas aeruginosa. *Strain*, *179*(10), 3127–3132. https://doi.org/10 3127-3132

Pesci, Everett C., Milbank, J. B. J., Pearson, J. P., Mcknight, S., Kende, A. S., Greenberg, E. P., & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(20), 11229–11234. https://doi.org/10.1073/pnas.96.20.11229

Petrova, O. E., Cherny, K. E., & Sauer, K. (2014). The Pseudomonas aeruginosa diguanylate cyclase GcbA, a homolog of P. Fluorescens GcbA, promotes initial attachment to surfaces, but not biofilm formation, via regulation of motility. *Journal of Bacteriology*, *196*(15), 2827–2841. https://doi.org/10.1128/JB.01628-14

Ponting, C. P., Aravind, L., Schultz, J., Bork, P., & Koonin, E. V. (1999). *Eukaryotic Signalling Domain Homologues in Archaea and Bacteria . Ancient Ancestry and Horizontal Gene Transfer*.

Prieto, M. A., Bühler, B., Jung, K., Witholt, B., & Kessler, B. (1999). PhaF, a polyhydroxyalkanoate-granule-associated protein of Pseudomonas oleovorans GPo1 involved in the regulatory expression system for pha genes. *Journal of Bacteriology*, *181*(3), 858–868. https://doi.org/10.1128/jb.181.3.858-868.1999

Bates, Robyn Hannah. (2013). Pseudomonas aeruginosa – Host Interactions And The Identification Of Novel Modulators Of Quorum Sensing-Mediated Virulence. Thesis Submitted To The University of Nottingham For The Degree Of Doctor Of Philosophy.

Qing, G., Ma, L. C., Khorchid, A., Swapna, G. V. T., Mal, T. K., Takayama, M. M., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, G. T., Ikura, M., & Inouye, M. (2004). Cold-shock induced high-yield protein production in Escherichia coli. *Nature Biotechnology*, *22*(7), 877–882. https://doi.org/10.1038/nbt984

Rampioni, G. C. P., Fletcher, M. P., Wright, V. J., Bruce, M., Rumbaugh, K. P., Heeb, S., Cámara, M., & Williams, and P. (2010). 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. *Environmental Microbiology*, *12*(6), 1659–1673. https://doi.org/10.1111/j.1462-2920.2010.02214.x

Rampioni, G., Falcone, M., Heeb, S., Frangipani, E., Fletcher, M. P., Dubern, J. F., Visca, P., Leoni, L., Cámara, M., & Williams, P. (2016). Unravelling the Genome-Wide Contributions of Specific 2-Alkyl-4-Quinolones and PqsE to

Quorum Sensing in Pseudomonas aeruginosa. *PLoS Pathogens*, *12*(11), 1–25. https://doi.org/10.1371/journal.ppat.1006029

Raynaud, C., Sheppard, D., Berry, J. L., Gurung, I., & Pelicic, V. (2021). PilB from Streptococcus sanguinis is a bimodular type IV pilin with a direct role in adhesion. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(22), 1–10. https://doi.org/10.1073/pnas.2102092118

Recinos, D. A., Sekedat, M. D., Hernandez, A., Cohen, T. S., Sakhtah, H., Prince, A. S., Price-Whelan, A., & Dietrich, L. E. P. (2012). Redundant phenazine operons in Pseudomonas aeruginosa exhibit environment-dependent expression and differential roles in pathogenicity. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(47), 19420–19425. https://doi.org/10.1073/pnas.1213901109

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., & Haas, D. (1997). The global activator GacA of Pseudomonas aeruginosa PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Molecular Microbiology*, *24*(2), 309–319. https://doi.org/10.1046/j.1365-2958.1997.3291701.x

Rickenberg, H. V. (1974). Cyclic AMP in prokaryotes. *Annual Review of Microbiology*, *28*(1), 353–369. https://doi.org/10.1146/annurev.mi.28.100174.002033

Rodríguez-Carmona, E., Cano-Garrido, O., Seras-Franzoso, J., Villaverde, A., & García-Fruitós, E. (2010). Isolation of cell-free bacterial inclusion bodies. *Microbial Cell Factories*, *9*, 71. https://doi.org/10.1186/1475-2859-9-71

Rommens, J. M., Iannuzzi, M. C., Kerem, B. S., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J. R., Tsui, L. C., & Collins, F. S. (1989). Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science*, *245*(4922), 1059–1065. https://doi.org/10.1126/science.2772657

Sadikot, R. T., Blackwell, T. S., Christman, J. W., & Prince, A. S. (2005). Pathogen-host interactions in pseudomonas aeruginosa pneumonia. *American Journal of Respiratory and Critical Care Medicine*, *171*(11), 1209–1223. https://doi.org/10.1164/rccm.200408-1044SO

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, *239*(4839), 487–491. https://doi.org/10.1126/science.2448875

Salvi, G., Rios, P. D. L., & Vendruscolo, M. (2005). *Effective Interactions Between Chaotropic Agents and Proteins*. *499*(March), 492–499.

Samanta Saha, R. T. and S. J. (2008). *Phenazine Pigments from Pseudomonas aeruginosa and Their Application as Antibacterial Agent and Food Colourants* (pp. 122–128). https://doi.org/10.3923/jm.2008.122.128

Sambrook, J., and Russell, R. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed. Co).

Savli, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U., & Vahaboglu, H. (2003). Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of Pseudomonas aeruginosa by real-time quantitative RT-PCR. *Journal of Medical Microbiology*, *52*(5), 403–408. https://doi.org/10.1099/jmm.0.05132-0

Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M., & Jahn, D. (2007). The anaerobic regulatory network required for Pseudomonas aeruginosa nitrate respiration. *Journal of Bacteriology*, *189*(11), 4310–4314. https://doi.org/10.1128/JB.00240-07

Schuster, M., Urbanowski, M. L., & Greenberg, E. P. (2004). Promoter specificity in Pseudomonas aeruginosa quorum sensing revealed by DNA binding of purified LasR. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(45), 15833–15839. https://doi.org/10.1073/pnas.0407229101

Schuster, Martin, & Greenberg, E. P. (2006). A network of networks: Quorum-sensing gene regulation in Pseudomonas aeruginosa. *International Journal of Medical Microbiology*, *296*(2–3), 73–81. https://doi.org/10.1016/j.ijmm.2006.01.036

Schuster, Martin, Lostroh, C. P., Ogi, T., & Greenberg, E. P. (2003a). Identi cation, Timing, and Signal Speci city of. *Society*, *185*(7), 2066–2079. https://doi.org/10.1128/JB.185.7.2066

Schuster, Martin, Lostroh, C. P., Ogi, T., & Greenberg, E. P. (2003b). Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: A transcriptome analysis. *Journal of Bacteriology*, *185*(7), 2066–2079. https://doi.org/10.1128/JB.185.7.2066-2079.2003

Schütz, C., & Empting, M. (2018). Targeting the Pseudomonas quinolone signal quorum sensing system for the discovery of novel anti-infective pathoblockers. *Beilstein Journal of Organic Chemistry*, *14*, 2627–2645. https://doi.org/10.3762/bjoc.14.241

Serate, J., Roberts, G. P., Berg, O., & Youn, H. (2011). Ligand responses of Vfr, the virulence factor regulator from Pseudomonas aeruginosa. *Journal of Bacteriology*, *193*(18), 4859–4868. https://doi.org/10.1128/JB.00352-11

Shah, N., Gislason, A. S., Becker, M., Belmonte, M. F., Fernando, W. G. D., & de

Kievit, T. R. (2020). Investigation of the quorum-sensing regulon of the biocontrol bacterium Pseudomonas chlororaphis strain PA23. *PloS One*, *15*(2), e0226232. https://doi.org/10.1371/journal.pone.0226232

Shine, J., & Dalgarno, L. (1975). *Determinant of cistron specificity in bacterial ribosomes*. *254*, 34–38.

Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B., & Jackson, R. W. (2011). Pseudomonas genomes: Diverse and adaptable. *FEMS Microbiology Reviews*, *35*(4), 652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x

Simon, R., Priefer, U., & Pühler, A. (1983). A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. *Bio/Technology*, *1*(9), 784–791. https://doi.org/10.1038/nbt1183-784

Smith, R. S., Wolfgang, M. C., & Lory, S. (2004). An Adenylate Cyclase-Controlled Signaling Network Regulates Pseudomonas aeruginosa Virulence in A Mouse Model of Acute Pneumonia. *Infection and Immunity*, *72*(3), 1677–1684. https://doi.org/10.1128/IAI.72.3.1677-1684.2004

Snider, J., Gutsche, I., Lin, M., Baby, S., Cox, B., Butland, G., Greenblatt, J., Emili, A., & Houry, W. A. (2006). Formation of a distinctive complex between the inducible bacterial lysine decarboxylase and a novel AAA+ ATPase. *Journal of Biological Chemistry*, *281*(3), 1532–1546. https://doi.org/10.1074/jbc.M511172200

Snider, J., & Houry, W. A. (2006). MoxR AAA+ ATPases: A novel family of molecular chaperones? *Journal of Structural Biology*, *156*(1), 200–209. https://doi.org/10.1016/j.jsb.2006.02.009

Sobota, J. M., & Imlay, J. A. (2011). Iron enzyme ribulose-5-phosphate 3-epimerase in Escherichia coli is rapidly damaged by hydrogen peroxide but can be protected by manganese. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(13), 5402–5407. https://doi.org/10.1073/pnas.1100410108

Soc, P. R., Ser, L., Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G., & Ausubelt, F. M. (1995). *Common Virulence Factors for Bacterial Pathogenicity in Plants and Animals*. *268*(June).

Solovyev, V. V. (2016). *V. Solovyev, A Salamov (2011) Automatic Annotation of Microbial Genomes and Metagenomic Sequences. In Metagenomics and its Applications in Agriculture, Biomedicine and Environmenta... January 2011*.

Sonnleitner, E., Gonzalez, N., Sorger-Domenigg, T., Heeb, S., Richter, A. S., Backofen, R., Williams, P., Hüttenhofer, A., Haas, D., & Bläsi, U. (2011). The

small RNA PhrS stimulates synthesis of the Pseudomonas aeruginosa quinolone signal. *Molecular Microbiology*, *80*(4), 868–885. https://doi.org/10.1111/j.1365-2958.2011.07620.x

Sonnleitner, E., & Haas, D. (2011). Small RNAs as regulators of primary and secondary metabolism in Pseudomonas species. *Applied Microbiology and Biotechnology*, *91*(1), 63–79. https://doi.org/10.1007/s00253-011-3332-1

Sonnleitner, E., Schuster, M., Sorger-Domenigg, T., Greenberg, E. P., & Bläsi, U. (2006). Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in Pseudomonas aeruginosa. *Molecular Microbiology*, *59*(5), 1542–1558. https://doi.org/10.1111/j.1365-2958.2006.05032.x

Spagnolo, A. M., Sartini, M., & Cristina, M. L. (2021). Pseudomonas aeruginosa in the healthcare facility setting. *Reviews in Medical Microbiology*, *32*(3), 169–175. https://doi.org/10.1097/mrm.0000000000000271

Squires, C. L., Pedersen, S., Ross, B. M., & Squires, C. (1991). *C1pB Is the Escherichia coli Heat Shock Protein F84 . 1*. *173*(14), 4254–4262.

Stickle, D. F., Liu, G., & Fried, M. G. (1994). Analysis of the Thermodynamic Linkage of DNA Binding and Ion Binding for Dimeric and Tetrameric forms of the Lac Repressor. *European Journal of Biochemistry*, *226*(3), 869–876. https://doi.org/10.1111/j.1432-1033.1994.00869.x

Storz, G., Vogel, J., & Wassarman, K. M. (2011). Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Molecular Cell*, *43*(6), 880–891. https://doi.org/10.1016/j.molcel.2011.08.022

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowallk, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., … Olson, M. V. (2000). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature*, *406*(6799), 959–964. https://doi.org/10.1038/35023079

Suh, S. J., Runyen-Janecky, L. J., Maleniak, T. C., Hager, P., MacGregor, C. H., Zielinski-Mozny, N. A., Phibbs, P. V., & West, S. E. H. (2002). Effect of vfr mutation on global gene expression and catabolite repression control of Pseudomonas aeruginosa. *Microbiology*, *148*(5), 1561–1569. https://doi.org/10.1099/00221287-148-5-1561

Tao, H., Liu, W., Simmons, B. N., Harris, H. K., Cox, T. C., & Massiah, M. A. (2010). Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS. *BioTechniques*, *48*(1), 61–64. https://doi.org/10.2144/000113304

Taylor, I. R., Paczkowski, J. E., Je, P. D., Henke, B. R., Smith, C. D., & Bassler, B.

L. (2021). *Inhibitor Mimetic Mutations in the Pseudomonas aeruginosa PqsE Enzyme Reveal a Protein − Protein Interaction with the Quorum-Sensing Receptor RhlR That Is Vital for Virulence Factor Production*. https://doi.org/10.1021/acschembio.1c00049

Taylor, I. R., Paczkowski, J. E., Jeffrey, P. D., Henke, B. R., Smith, C. D., & Bassler, B. L. (2021). Inhibitor Mimetic Mutations in the Pseudomonas aeruginosa PqsE Enzyme Reveal a Protein-Protein Interaction with the Quorum-Sensing Receptor RhlR That Is Vital for Virulence Factor Production. *ACS Chemical Biology*, *16*(4), 740–752. https://doi.org/10.1021/acschembio.1c00049

Thomas, C. M. (2001). mRNA stabilizing or destabilizing sites that influence the expression of transcriptional reporters. *Microbiology*, *August*, 1993–1995.

Timm, A., & Steinbüchel, A. (1992). Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of Pseudomonas aeruginosa PAO1. *European Journal of Biochemistry*, *209*(1), 15–30. https://doi.org/10.1111/j.1432-1033.1992.tb17256.x

Toyofuku, M., Nomura, N., Kuno, E., Tashiro, Y., Nakajima, T., & Uchiyama, H. (2008). Influence of the Pseudomonas quinolone signal on denitrification in Pseudomonas aeruginosa. *Journal of Bacteriology*, *190*(24), 7947–7956. https://doi.org/10.1128/JB.00968-08

Toyofuku, M., Uchiyama, H., & Nomura, N. (2012). Social behaviours under anaerobic conditions in Pseudomonas aeruginosa. *International Journal of Microbiology*, *2012*. https://doi.org/10.1155/2012/405191

Tsai, Y. C. C., Lapina, M. C., Bhushan, S., & Mueller-Cajar, O. (2015). Identification and characterization of multiple rubisco activases in chemoautotrophic bacteria. *Nature Communications*, *6*(May). https://doi.org/10.1038/ncomms9883

Urban, A., Neukirchen, S., Jaeger, K., Mikroorganismen, L. B. Der, & Bochum, D.-. (1997). *<refs 1 and 2 for OVERLAP extension PCR for mutagenesis.pdf>*. *25*(11), 2227–2228.

Voisard, C., Bull, C. T., Keel, C., Laville, J., Maurhofer, M., Schnider, U., Défago, G., & Haas, D. (2007). Biocontrol of Root Diseases by Pseudomonas fluorescens CHA0: Current Concepts and Experimental Approaches. *Molecular Ecology of Rhizosphere Microorganisms: Biotechnology and the Release of GMOs*, 67–89. https://doi.org/10.1002/9783527615810.ch6

Vrla, G. D., Esposito, M., Zhang, C., Kang, Y., Seyedsayamdost, M. R., & Gitai, Z. (2020). Cytotoxic alkyl-quinolones mediate surface-induced virulence in pseudomonas aeruginosa. *PLoS Pathogens*, *16*(9), 1–26. https://doi.org/10.1371/journal.ppat.1008867

Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, a, Engstrom, E., Coleman, J. P., Everett, C., Ling, E. a, & Pesci, E. C. (2005). Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, *187*(13), 4372–4380. https://doi.org/10.1128/JB.187.13.4372

Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P., & Pesci, E. C. (2005). Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa. *Journal of Bacteriology*, *187*(13), 4372–4380. https://doi.org/10.1128/JB.187.13.4372-4380.2005

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., & Iglewski, B. H. (2003). Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: Effects of growth phase and environment. *Journal of Bacteriology*, *185*(7), 2080–2095. https://doi.org/10.1128/JB.185.7.2080-2095.2003

Walker, T. S., Bais, H. P., De, E., Schweizer, H. P., Rahme, L. G., Fall, R., & Vivanco, J. M. (2004). *Pseudomonas aeruginosa -Plant Root Interactions . Pathogenicity , Biofilm Formation , and Root Exudation 1*. *134*(January), 320–331. https://doi.org/10.1104/pp.103.027888.such

Wauven, C. V., Pierard, A., Kley-Raymann, M., & Haas, D. (1984). Pseudomonas aeruginosa mutants affected in anaerobic growth on arginine: Evidence for a four-gene cluster encoding the arginine deiminase pathway. *Journal of Bacteriology*, *160*(3), 918–934. https://doi.org/10.1128/jb.160.3.928-934.1984

West, S. E. H., Sample, A. K., & Runyen-Janecky, L. J. (1994). The vfr gene product, required for Pseudomonas aeruginosa exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *Journal of Bacteriology*, *176*(24), 7532–7542. https://doi.org/10.1128/jb.176.24.7532-7542.1994

Westfall, L. W., Luna, A. M., San Francisco, M., Diggle, S. P., Worrall, K. E., Williams, P., Cámara, M., & Hamood, A. N. (2004). The Pseudomonas aeruginosa global regulator MvaT specifically binds to the ptxS upstream region and enhances ptxS expression. *Microbiology*, *150*(11), 3797–3806. https://doi.org/10.1099/mic.0.27270-0

Whiteley, M., Lee, K. M., & Greenberg, E. P. (1999). Identification of genes controlled by quorum sensing in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(24), 13904–13909. https://doi.org/10.1073/pnas.96.24.13904

Whittaker, C. A., & Hynes, R. O. (2002). *Distribution and Evolution of von Willebrand / Integrin A Domains : Widely Dispersed Domains with Roles in Cell Adhesion and Elsewhere* □. *13*(October), 3369–3387. https://doi.org/10.1091/mbc.E02

Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P. C., Gottesman, S., Ochsner, U. A., & Vasil, M. L. (2004). Identification of tandem duplicate regulatory small RNAs in Pseudomonas aeruginosa involved in iron homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(26), 9792–9797. https://doi.org/10.1073/pnas.0403423101

Winson, M. K., Camarat, M., Latifi, A., Foglino, M., Chhabrat, S. R. A. M., Daykint, M., Ballyt, M., Chapont, V., Salmond, G. P. C., Bycroftt, B. W., Lazdunskit, A., Stewart, G. S. A. B., & Williamst, P. (1995). *metabolites Pseudomonas*. *92*(September), 9427–9431.

Winsor, G. L., Griffiths, E. J., Lo, R., Dhillon, B. K., Shay, J. A., & Brinkman, F. S. L. (2016). Enhanced annotations and features for comparing thousands of Pseudomonasgenomes in the Pseudomonas genome database. *Nucleic Acids Research*, *44*(D1), D646–D653. https://doi.org/10.1093/nar/gkv1227

Wong, K. S., Bhandari, V., Janga, S. C., & Houry, W. A. (2017). The RavA-ViaA Chaperone-Like System Interacts with and Modulates the Activity of the Fumarate Reductase Respiratory Complex. *Journal of Molecular Biology*, *429*(2), 324–344. https://doi.org/10.1016/j.jmb.2016.12.008

Woolwine, S. C., Sprinkle, A. B., & Wozniak, D. J. (2001). Loss of Pseudomonas aeruginosa PhpA aminopeptidase activity results in increased algD transcription. *Journal of Bacteriology*, *183*(15), 4674–4679. https://doi.org/10.1128/JB.183.15.4674-4679.2001

Wu, M., Guina, T., Brittnacher, M., Nguyen, H., Eng, J., & Miller, S. I. (2005). The Pseudomonas aeruginosa proteome during anaerobic growth. *Journal of Bacteriology*, *187*(23), 8185–8190. https://doi.org/10.1128/JB.187.23.8185-8190.2005

Wurtzel, O., Yoder-himes, D. R., Han, K., Dandekar, A. A., & Edelheit, S. (2012). *The Single-Nucleotide Resolution Transcriptome of Pseudomonas aeruginosa Grown in Body Temperature*. *8*(9). https://doi.org/10.1371/journal.ppat.1002945

Xiao, G., He, J., & Rahme, L. G. (2006). Mutation analysis of the Pseudomonas aeruginosa mvfR and pqsABCDE gene promoters demonstrates complex quorum-sensing circuitry. *Microbiology*, *152*(6), 1679–1686. https://doi.org/10.1099/mic.0.28605-0

Yokoyama, K., Hayashi, N. R., Arai, H., Chung, S. Y., Igarashi, Y., & Kodama, T. (1995). Genes encoding RubisCO in Pseudomonas hydrogenothermophila are followed by a novel cbbQ gene similar to nirQ of the denitrification gene cluster from Pseudomonas species. *Gene*, *153*(1), 75–79. https://doi.org/10.1016/0378-1119(94)00808-6

Yu, S. (2013). *Assessment of … Assessment of …*. *1*(1), 86–88.

Yu, S., Jensen, V., Seeliger, J., Feldmann, I., Weber, S., Schleicher, E., Häussler, S., & Blankenfeldt, W. (2009). Structure elucidation and preliminary assessment of hydrolase activity of PqsE, the Pseudomonas quinolone signal (PQS) response protein. *Biochemistry*, *48*(43), 10298–10307. https://doi.org/10.1021/bi900123j

Yuan, Y., Leeds, J. A., & Meredith, T. C. (2012). Pseudomonas aeruginosa directly shunts β-oxidation degradation intermediates into de novo fatty acid biosynthesis. *Journal of Bacteriology*, *194*(19), 5185–5196. https://doi.org/10.1128/JB.00860-12

Zhang, Y. M., Frank, M. W., Zhu, K., Mayasundari, A., & Rock, C. O. (2008). PqsD is responsible for the synthesis of 2,4-dihydroxyquinoline, an extracellular metabolite produced by Pseudomonas aeruginosa. *Journal of Biological Chemistry*, *283*(43), 28788–28794. https://doi.org/10.1074/jbc.M804555200