REGULATORY CASCADES INVOLVING CYCLIC DI-GMP SIGNALLING IN *PSEUDOMONAS AERUGINOSA*

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Regulatory cascades involving cyclic di-GMP signalling in *Pseudomonas aeruginosa*

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This thesis is presented for the degree of Doctor of Philosophy of Imperial College London and Diploma of Imperial College London 2013.

Candidate's Declaration

I, Joana Alves Moscoso, hereby confirm that this thesis represents my own work and that any external contributions to the research are duly acknowledged.

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Abstract

Cyclic di-GMP (c-di-GMP) has emerged as a bacterial second messenger that regulates a variety of cellular processes, in particular those associated with the switch between a motile and a sessile lifestyle. At low levels of c-di-GMP the motile lifestyle is favoured whereas at high levels of c-di-GMP the formation of biofilms is promoted.

In *Pseudomonas aeruginosa*, over 50 genes encoding proteins involved in the synthesis, hydrolysis or sensing of c-di-GMP are found and many remain uncharacterized. Herein, an analysis of a collection of mutants was performed and supported the idea that the sophisticated c-di-GMP network operates at high specificity.

In addition to c-di-GMP, *P. aeruginosa* has a regulatory pathway, the Gac pathway, that is known to control the bacterium lifestyle switch. By investigating a particular mutant affected in this pathway, a link between the Gac pathway and c-di-GMP was established.

Furthermore, the Gac pathway not only influences biofilm formation, but it is also crucial in determining the bacterium mode of infection. In other words, biofilm formation correlates to a chronic mode of infection where the bacterium has an active type VI secretion system (T6SS), and a motile phenotype correlates to an acute infection where the type III secretion system (T3SS) is active. Interestingly, by artificially modulating the levels of c-di-GMP it was demonstrated that c-di-GMP regulation goes beyond the control of the motile/sessile phenotypes and is able to inversely regulate the T3SS and T6SS.

Finally, the link between c-d-GMP and the Gac pathway was consolidated by showing that the Gac system impacts the expression of a few c-di-GMP related proteins and one protein, SadC, was identified as a central component of the network. Overall this work largely contributed to reconcile two independent concepts involved in the regulation of the *P. aeruginosa* lifestyle.

Acknowledgements

First of all, I would like to thank my supervisor Alain Filloux, for being always very supportive and for having accepted me in his group. The last 4 years working with you have been a real pleasure and the way you manage the team and our expectations are a real inspiration to me. I feel that I have come a long way and learned a lot, both professionally and at the personal level, throughout my PhD. Overall, this journey has been a good one and I am truly thankful for your mentoring, patience, encouragement and understanding.

Secondly, I would like to thank my parents who are always there for me and have been absolutely essential in my pursuit of happiness and professional fulfillment. You have set the standard high and it is that ambition that you passed on that keeps me going. I feel that I have made the right choices up to now and that is only possible because you have given me the tools to choose freely but conscienciously, in a right balance between being able to express myself and respecting who we are as a family. Obrigada Papa! Obrigada Mama! You have been amazing!

I would also like to thank my brother, Frederico, who I know will always be there for me and who gave me the two sweetest things in my life so far: my nephew Carlinhos and my niece Clarinha. And because you are a grown up now, thank you to you Carlinhos as well (you better improve your English, by the way, so that you can understand this properly!). You have changed my life when I was only 15 years old and I still cannot define if I am more of an aunt to you or an older sister! Anyway, I love you to pieces.

Now, I would like to thank my collaborators, those who replied to my requests and those who have accommodated me in their labs. On the three occasions that I went away to learn a new technique, first in Nottingham, then in Basel and finally in Marseille, I had an absolutely great time and always felt welcome and well supervised. Thank you again Stephan and Mary, Tina, Jutta and Ursi, and Christopher for your time. In addition, I would like to take this opportunity to also thank my previous “bosses” because without them I would not be here today. I will always be grateful to Victoria Shingler and Lisandro Bernardo for their mentoring in Sweden, and I will always remember Naresh Verma for hosting me in Australia.

To my colleagues in the Filloux’s group, thank you for the constant support, brainstorming moments and great atmosphere. This group is indeed something special and I always felt “home” at work which is very valuable to me and I treasure that feeling a lot. Many people came in and out during these 4 years, but a special thanks goes to Nicolas Barraud who was fantastic in my early days, Helga

Mikkelsen who has a brain like no other, Melissa Sivanesson who has the sweetest heart, Nadine Lossi who shared with me great moments, Abdou Hachani who brings the “wow” factor to everything around him, Cerith Jones who has been a constant presence, Daniela Muhl who you can always count on, Kailyn Hui who is the magician that gets things done and Eleni Manoli who brings some shine to the office and lab. A few words go also to the new people in the group, Luke Allsopp and Sara Planamente, and the other people in the floor, especially those from the group of Gad Frankel. I will also remember with kindness my students Evelina Musial and Qi Pan, and the visiting PhD student from Spain, Isabel Cortés, with whom I enjoyed working and shared good moments.

Finally, I would like to thank my dearest friends. You make my life fun and bearable! Without you I would have gone nuts a very long time ago! Thank you, thank you, thank you! With a big smile! In London, special thanks go to Antonio Santos, Lena Barrera, Veronique Peiffer, and the crowds from the LUSO team and then the PARSUK team. We will keep in touch for sure! In Portugal and around the world, thanks to my friends from Valença, specially Angela Pinheiro, my friends from Porto, specially Ana Oliveira and Joana Torres, my friend Karina Herrera, my friend Karsten Oelkers, my “friend” Ricardo Azevedo, my “sister” Susana Martins, and last but definitely not least, my friend Sofia Koch.

I would like to also thank Heather Combe and Niki Gounaris at Imperial College and finally, to FCT (Fundação para a Ciência e Tecnologia) for financing my PhD.

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Abbreviations

A	Alanine
A-site	Active site
ABC	ATP-binding cassette
ACs	Adenylyl cyclases
Ap	Ampicillin
ATP	Adenosine-5’-triphosphate
cAMP	Cyclic AMP (cyclic adenosine 3’,5’-monophosphate)
cGMP	Cyclic GMP (cyclic guanosine 3’,5’-monophosphate)
Cb	Carbenincillin
CBS	Domain found in cystathionine beta-synthase
cdAMP	Cyclic di-AMP (cyclic dimeric adenosine 3’,5’-monophosphate)
c-di-GMP	Cyclic di-GMP (cyclic dimeric guanosine 3’,5’-monophosphate)
c-di-GMP*	Radioactive labelled ^{32}P-c-di-GMP
cDNA	Complementary DNA
cGAMP	Cyclic GMP-AMP (cyclic guanosine monophosphate–adenosine monophosphate)
Cache	Domain found in animal calcium channels and prokaryotic chemotaxis receptors
CHASE	Cyclase/histidine kinase-associated sensing extracellular domain
Crp	Cyclic AMP receptor protein
Cup	Chaperone usher pathway
CV	Crystal violet
D	Aspartate

DDM	n-dodecyl-β-D-maltopyranoside
DNA	Deoxyribonucleic acid
DRaCALA	Differential radial capillary action of ligand assay
DSF	Difusible signalling factor
E	Glutamic acid
EGTA	Ethylene glycol tetra-acetic acid
F	Phenylalanine
G	Glycine
g	Grams
GAF	Domain found in cGMP-specific PDEs, ACs and FhlA
gDNA	Genomic DNA
GFP	Green fluorescent protein
Gm	Gentamycin
GMP	Guanosine monophosphate
GTP	Guanosine-5'-triphosphate
H	Histidine
h	Hours
HAMP	Domain found in H kinases, ACs, MCPs
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
I-site	Inhibition site
IPTG	Iso-propanyl-β-D-thiogalactopyranoside
K	Lysine
Km	Kanamycin

L	Leucine
LB	Luria Bertani
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MASE	Membrane associated sensory domain
MCP	Methyl accepting chemotaxis protein
MF	Microfermentor
MES	2-(*N*-morpholino)ethanesulfonic acid
mL	Mililiters
min	minutes
MTP	Microtiter plate
OD_{600}	Optical density measured at 600 nm
P	Proline
PAS	Domain found in Per, Arnt and Sim proteins
PBPb	Periplasmic substract-binding protein domain found in bacteria
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIA	Pseudomonas isolation agar
PGA	Poly-β-1,6-*N*-acetylglucosamine
pGpG	Phosphoguanylyl guanosine
PNPase	Polynucleotide phosphorylase
(p)ppGpp	Guanosine pentaphosphate or tetraphosphate
qRT-PCR	Real time reverse transcription PCR
REC	Response regulator receiver domain

RFU	Relative fluorescence units
RBS	Ribosome binding site
RT-PCR	Reverse transcription PCR
S	Serine
sec	seconds
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sm	Streptomycin
sRNA	Small non-coding RNA
T	Threonine
T3SS	Type III secretion system
T6SS	Type VI secretion system
Tc	Tetracyclin
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-D-galactoside
Y	Tyrosine
7TMR-DISM	Seven transmembrane receptors with diverse intracellular signalling modules

1) Introduction

Chapter 1 – Introduction

Bacteria are ubiquitous unicellular microorganisms. Not only they are abundant, they are also widespread in the environment, thriving in the most diverse ecosystems including extreme conditions or the organisms of other forms of life. The success of bacteria relies on a combination of factors and the ability to sense what surrounds them and quickly adapt is one of them. Fascinated by this, my work focussed on the molecular mechanisms used by bacteria to sense, signal and change their behaviour. This chapter starts with a general overview of *Pseudomonas aeruginosa*, a versatile opportunistic human pathogen that was used as model organism. It then explains how bacteria use regulatory cascades to adapt to the environment and further depicts an important pathway, the Gac pathway. Last but not least, this chapter gives a state of the art review of the c-di-GMP signalling network and presents the aims of the study.

1.1 *Pseudomonas aeruginosa*, an opportunistic pathogen

Pseudomonas aerugionsa is the type species of the *Pseudomonas* genus (Palleroni & Moore, 2004). These are rod-shaped, polarly flagellated, aerobic Gram-negative microorganisms that belong to the class of *Gammaproteobacteria* where many medically relevant bacteria are grouped. In *P. aeruginosa*, the species epithet “aeruginosa” is reminiscent of blue/green colour, an attribute observed in the pus of *P. aeruginosa* infected wounds and in laboratory cultures of the bacterium. This colour derives from its ability to produce and secrete two pigments, pyocyanin which is blue and pyoverdin which is fluorescent green (Gessard, 1984).

Since its discovery, *P. aeruginosa* has been extensively used as a model organism to study key aspects of bacterial biology, from basic metabolism to host/pathogen interaction. To date, the complete genome sequence of eleven *P. aeruginosa* strains is available in the Pseudomonas database (www.pseudomonas.com) (Winsor *et al.*, 2009) and three strains are most widely used in laboratories for research purposes: PAO1 (wound isolate), PA14 (burn isolate, more virulent than PAO1) and PAK (also a clinical isolate) (Liberati *et al.*, 2006; Stover *et al.*, 2000).

1.1.1 Acute versus chronic infections

Pseudomonas species are ubiquitous and can be found in soil, water, plants and animals. In humans, *P. aeruginosa* can be part of the normal microbiome (mainly in stools but occasionally in the skin, nasal mucosa, and throat) where it lives without being noticed (Morrison & Wenzel, 1984). However, in hospital patients and in people with a genetic disease called cystic fibrosis, infections of *P. aeruginosa* are prevalent and constitute a major public health concern. Even though the prevalence of nosocomial infections is lower in high-income countries compared to low-income countries (WHO, 2011), an average of 4 million people acquire an infection in hospitals every year in Europe (ECDC, 2012). Overall, *P.aeruginosa* is among the most common causative agents of hospital acquired pneumonia, urinary tract infections, catheter and surgery derived infections, and septicaemia (ECDC, 2012; Gaynes *et al.*, 2005). In addition to these acute-like infections, *P. aerugionsa* chronically colonizes the lungs of people with cystic fibrosis who have impaired ability of clearing their airways from inhaled microorganisms, and becomes the major causative agent of morbidity and mortality in these patients (Emerson *et al.*, 2002; Folkesson *et al.*, 2012; Williams & Davies, 2012).

The biology behind the ability of *P. aeruginosa* to cause both acute and chronic infections is complex and dependent on many factors but is known to rely in its capacity to adopt distinct lifestyles and control the production of different virulence factors (Figure 1.1). On the one hand, acute infections are associated with a motile lifestyle and involve the secretion of toxins via the type II and type III secretion systems (Bleves *et al.*, 2010; Mahenthiralingam *et al.*, 1994; Woods *et al.*, 1986). On the other hand, chronic infections are linked to a sessile lifestyle in which the bacterial cells produce exopolysaccharides and express the type VI secretion system (Costerton *et al.*, 1999; Hoiby *et al.*, 2001; Mougous *et al.*, 2006).

Sometimes during the long-term colonization of the lung in cystic fibrosis patients, *P. aeruginosa* undergoes genetic adaptation which results in the appearance of different colony morphotypes, specifically the mucoid colonies and the small colony variants (Damkiaer *et al.*, 2013; Smith *et al.*, 2006). The *mucoid phenotype* is mainly due to the production of an exopolysaccharide called alginate (Govan & Deretic, 1996) whereas the *small colony variants* are characterized by slow growth and have an autoaggregative phenotype that is dependent on the production of the Pel and Psl exopolysaccharides (Haussler *et al.*, 1999; Haussler *et al.*, 2003; Kirisits *et al.*, 2005). Curiously, the molecular origin of these phenotypes varies and can be under both negative and positive selective pressure over the course of an infection (Malone *et al.*, 2012; Starkey *et al.*, 2009).

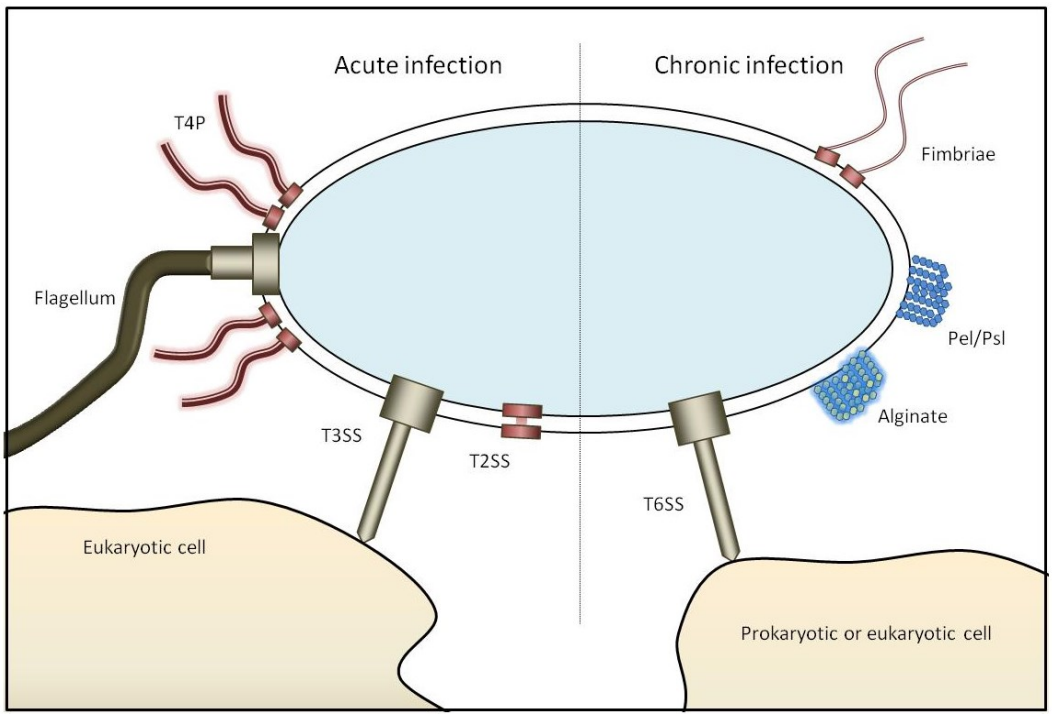

Figure 1.1 Simplified diagram of the virulence determinants of *P. aeruginosa*.

Acute infections are associated with a motile lifestyle and the secretion of toxins via the T2SS and T3SS. Swimming motility is driven by the polar flagellum and twitching motility is mediated by the production of type 4 pili (T4P). The T2SS secretes toxins to the extracellular medium like the elastases LasA and LasB or the exotoxin A. The T3SS secretes the exoenzymes S, T, Y and U into host cells. Chronic infections have been associated with the formation of biofilms which are sessile communities of bacteria. The formation of biofilms is facilitated by the production of fimbriae and adhesins that allow surface attachment and in *P. aeruginosa* it is strongly associated with the production of the Pel and Psl exopolysaccharides which are major components of the extracellular matrix of the biofilms. Sometimes, clinical isolates from chronically infected cystic fibrosis patients have a mucoid phenotype and in this case the exopolysaccharide alginate is produced. Also associated with chronic infections is the expression of the T6SS as high titers of antibodies against it are found in the serum of cystic fibrosis patients (Mougous *et al.*, 2006).

Below, the most important biological attributes that contribute for the definition of a *P. aeruginosa* infection, including the formation of biofilms and the expression of secretion systems, are described.

1.1.2 Biofilm development: from formation to dispersal

Scientists routinely cultivate bacteria in suspensions where a planktonic and free swimming mode of growth is favoured. However, in nature, it is more likely that bacterial cells live attached to a surface,

forming a cooperative and highly dynamic structure called *biofilm*. The ability of bacteria to attach to surfaces was first noticed by Claude Zobell in 1943 (Zobell, 1943) but the same observation only reappeared in the literature and was extensively studied much later by William Costerton (Costerton *et al.*, 1978; Costerton *et al.*, 1987; Hall-Stoodley *et al.*, 2004). Today, the biofilm developmental cycle is the focus of many studies and *Pseudomonas* spp. have been used as one main model organism. However, most of these studies have been carried out in *in vitro* conditions and one should bear in mind that during infection the dynamics of biofilm development may vary.

For *P. aeruginosa*, the study of biofilms is of clinical relevance for two main reasons. Firstly, the formation of biofilms in the lungs of cystic fibrosis patients is associated with the establishment of a chronic infection (Singh *et al.*, 2000). Secondly, the growth of biofilms on the surfaces of medical devices such as catheters or ventilators is a major cause of nosocomial infections worldwide (Donlan, 2001).

Based on microscopic, genetic and biochemical studies, the biofilm developmental cycle is consensually divided in five different stages: (i) reversible attachment, (ii) irreversible attachment, (iii) formation of microcolonies, (iv) macrocolony and biofilm maturation, and (v) biofilm dispersal (Sauer *et al.*, 2002; Tolker-Nielsen *et al.*, 2000; Webb *et al.*, 2003). A more detailed insight on the peculiarities of each stage follows and is illustrated in Figure 1.2.

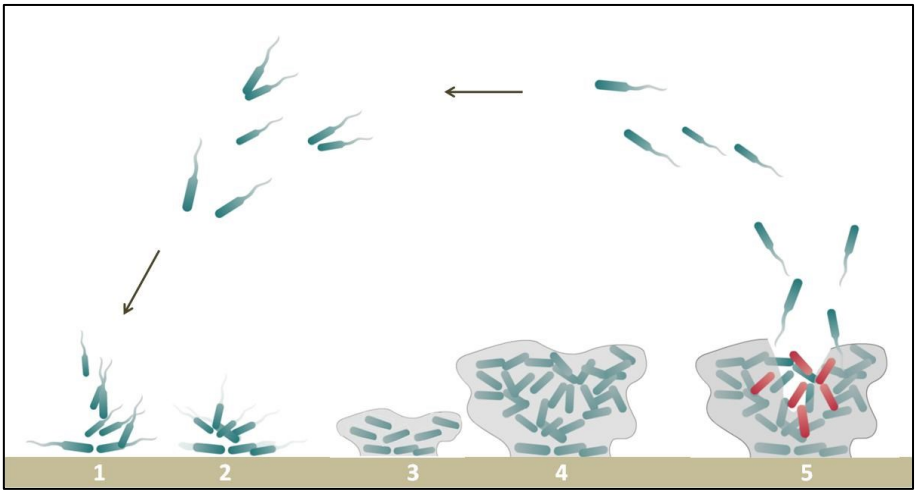

Figure 1.2 Stages of biofilm development.

The biofilm developmental cycle is divided into five stages: (1) reversible attachment, (2) irreversible attachment, (3) formation of microcolonies, (4) macrocolony and biofilm maturation and (5) biofilm dispersal. Initial reversible attachment occurs via the cell pole when planktonic cells enter in contact with a surface. Then, reorientation of the attached cells and loss of swimming motility contribute to

a more stable association with the surface. Following this irreversible attachment, clusters of cells start to appear and are referred to as microcolonies. Growth of these microcolonies forms macrocolonies. An extracellular matrix composed of exopolysaccharides, proteins and extracellular DNA helps maintaining the biofilm structure and protects the cells from external threats. The biofilm life cycle comes full circle when cells from the biofilm revert to a motile state and are released to the environment. Phage induced cell death is indicated by the bacteria coloured in red and has been associated with the progression to dispersal.

Stage1: Reversible attachment

The planktonic cells enter in contact with a surface (biotic or abiotic) and establish an initial polar and reversible attachment.

Stage2: Irreversible attachment

Following the first contact with a surface, the lateral reorientation of the attached cells, loss of swimming motility and expression of adhesins contribute to a more stable association of the bacterial cells with the surface.

Stage 3: Formation of microcolonies

Clusters of cells start to be visible and are referred to as *microcolonies*. The formation of microcolonies can occur either by clonal growth of the attached cells or by active translocation of cells across the surface (twitching motility).

Stage 4: Macrocolony and biofilm maturation

Growth of the microcolonies into mushroom-like structures forms *macrocolonies* which are separated by aqueous channels. An extracellular matrix composed of exopolysaccharides, extracellular DNA (Whitchurch *et al.*, 2002) and proteins maintains the biofilm structure and protects the cells against harmful compounds or adverse conditions.

Stage 5: Biofilm dispersal

The biofilm developmental cycle comes full circle when cells from the biofilm revert to a motile lifestyle and are released to the environment where they can colonize new areas (McDougald *et al.*, 2011). During biofilm maturation, phage-induced death of cells within the biofilm is part of the natural course of development and promotes biofilm dispersal (Webb *et al.*, 2003).

Both the biofilm formation and dispersal stages of the biofilm developmental cycle constitute critical moments that are potential targets to prevent or eradicate an infection. For instance, biofilm formation could be prevented through the development of compounds that hamper the attachment

of the bacteria (Valle *et al.*, 2006); or eradication of established biofilms could be induced by promoting dispersal (Christensen *et al.*, 2013). For this reason, understanding the regulatory cascades underlying biofilm development, as well as of other key traits of *P. aeruginosa* biology, is of pivotal relevance.

1.1.3 Extracellular appendages and exopolysaccharides

Regardless of the lifestyle that *P. aeruginosa* adopts (motile versus sessile), this bacterium is able to assemble a number of extracellular appendages that are involved in motility or adhesion. These include a unipolar flagellum, type IV pili (T4P), Cup fimbriae or the CdrA adhesin. In addition to these and during the sessile mode of growth, *P. aeruginosa* produces a range of extracellular polymeric substances such as the alginate, Pel and Psl exoplysaccharides.

The unipolar flagellum – swimming and swarming motility

Many bacteria can swim in liquid environments due to the presence of flagella. In general, this flagella-driven swimming motility is due to the rotation of long flagellin polymers (FliC, PA1092) that are hooked to the cell surface and are controlled by a flagellar motor. Rotation of the flagella is powered by a proton flux across the cytoplasmic membrane and depends on the interaction between proteins of the rotor (rotating part) and the stator (non-rotating, membrane associated part) that constitute the flagellar motor.

In *P. aeruginosa*, a single flagellum at one cell pole is found and approximately 50 genes are involved in its assembly and function (Dasgupta *et al.*, 2003). Curiously, two sets of stator proteins instead of just one, MotAB (PA4953-PA4954) and MotCD (PA1460-PA1461), are found in the PAO1 genome (Toutain *et al.*, 2005). Besides driving swimming motility in liquid environments, and upon production of the rhamnolipid surfactant, the *P. aeruginosa* flagellum also drives swarming motility in semisolid surfaces (Overhage *et al.*, 2008).

The Type IV Pili (T4P) – twitching motility

T4P are common extracellular appendages in bacteria that can serve different functions. In *P. aeruginosa* there are three types of T4P – T4Pa, T4Pb and Flp – which differ in function and assembly system. One of them, the T4Pa (here referred to as T4P for simplicity reasons), is associated with

twitching motility and assembles at the flagellated pole of the cells. This type of surface associated motility mediated by T4P is independent of the presence of a functional flagellum and results from the repeated extension, tethering and retraction of long and thin pili (Burrows, 2012).

Like for the flagellum, more than 50 genes in the genome of *P. aeruginosa* PAO1 strain are involved in the regulation, expression or function of the T4P. For its biogenesis, at least four different complexes of proteins are required: the pilus complex, the outer membrane complex, the inner membrane motor complex and the alignment complex (Burrows, 2012).

The pilus complex forms the actual surface appendage and is composed by the major pilin subunit PilA (PA4525) and the minor pilins FimU (PA4550), PilE (PA4556), PilV (PA4551), PilW (PA4552) and PilX (PA4553). The minor pilins have been proposed to form the initiation platform for pilus assembly and are thought to sit at the tip of each pilus. For pili to exit the cell, the outer membrane complex is required. This is composed by the secretin PilQ (PA5048) that forms outer membrane channels and the lipoprotein PilF (PA3805) that mediates PilQ oligomerization and ensures its correct localization. At the inner membrane, the motor complex is responsible for the continuous assembly and disassembly of the pilus complex and comprises the PilB (PA4526), PilC (PA4527), PilD (PA4528), PilT (PA0395) and PilU (PA0396) proteins. Finally, the alignment complex is composed of FimV (PA3115), PilM (PA5044), PilN (PA5045), PilO (PA5046) and PilP (PA5047) and serves to align the outer and inner membrane complexes for proper pilus biogenesis.

The twitching motility resulting from the function of T4P has been shown to be important for biofilm development at two different stages: the initial stages where it provides the bacteria with the ability to attach to a surface, and the later stages where it plays a role in biofilm differentiation (piliated cells are seen at the cap portion of the mushroom-like biofilms whereas nonpiliated cells localize to the stalk portion) (Klausen *et al.*, 2003a; Klausen *et al.*, 2003b).

The Cup fimbriae

Cup (chaperone usher pathway) fimbriae result from the polymerisation of fimbrial subunits at the cell surface via a chaperone usher pathway (Vallet *et al.*, 2001). In *P. aeruginosa* PAO1 strain, there are four gene clusters encoding for Cup fimbriae: *cupA* (PA2128-PA2132), *cupB* (PA4081-PA4086), *cupC* (PA0992-PA0994) and *cupE* (PA4648-PA4653). An additional *cupD* cluster is encoded in a pathogenicity island in the PA14 strain.

Although the *cup* gene clusters in *P. aeruginosa* show some variability, a typical *cup* cluster encodes (i) a pilin subunit; (ii) a chaperone that binds the pilin subunits in the periplasm; (iii) an adhesin that

is presented at the tip of the fimbriae; and (iv) an outer membrane usher responsible for the translocation of the pilin subunits across the membrane.

Some observations suggest that Cup fimbriae are important surface appendages during biofilm development. For instance, CupA fimbriae have been shown to be required at the early stages of attachment, CupB and CupC play a role in microcolony formation and CupE is involved in shaping the biofilm structure during macrocolony maturation (Giraud *et al.*, 2011; Ruer *et al.*, 2007; Vallet *et al.*, 2001).

The CdrA adhesin

Not much is known about the CdrA (PA4625) adhesin but it has been shown that it binds the Psl exopolysaccharide. It is encoded in an operon with its outer membrane transporter, CdrB (PA4624), and expression of CdrA promotes auto-aggregation in liquid cultures and biofilm formation on surfaces (Borlee *et al.*, 2010).

The alginate, Pel and Psl exopolysaccharides

Exopolysaccharides are major components of the extracellular biofilm matrix. In *P. aeruginosa* isolates from cystic fibrosis lungs, the mucoid phenotype is dependent on the production of alginate whereas in other isolates, including the small colony variants, the formation of biofilm is dependent on Pel or Psl exopolysaccharides (Figure 1.3).

Alginate is a polymer of D-mannuronic acid residues randomly modified by acetylation and epimerization. The genes for the production and export of alginate are encoded by the *alg* operon composed of twelve genes (PA3540-PA3551) and by *algC* (PA5322). In total, this involves the enzymes required for the synthesis of the polymer precursors in the cytoplasm, AlgA, AlgC and AlgD; the membrane proteins required for the polymerization and transport of the D-mannuronic acid polymer to the periplasm, Alg8 and Alg44; the enzymes required for its periplasmic modification, AlgF, AlgG, AlgI, AlgJ and possibly, AlgX; a periplasmic alginate lyase, AlgL; a periplasmic scaffold protein, AlgK; and an outer membrane porin, AlgE, responsible for the export of alginate to the extracellular medium (Franklin *et al.*, 2011; Mann & Wozniak, 2012).

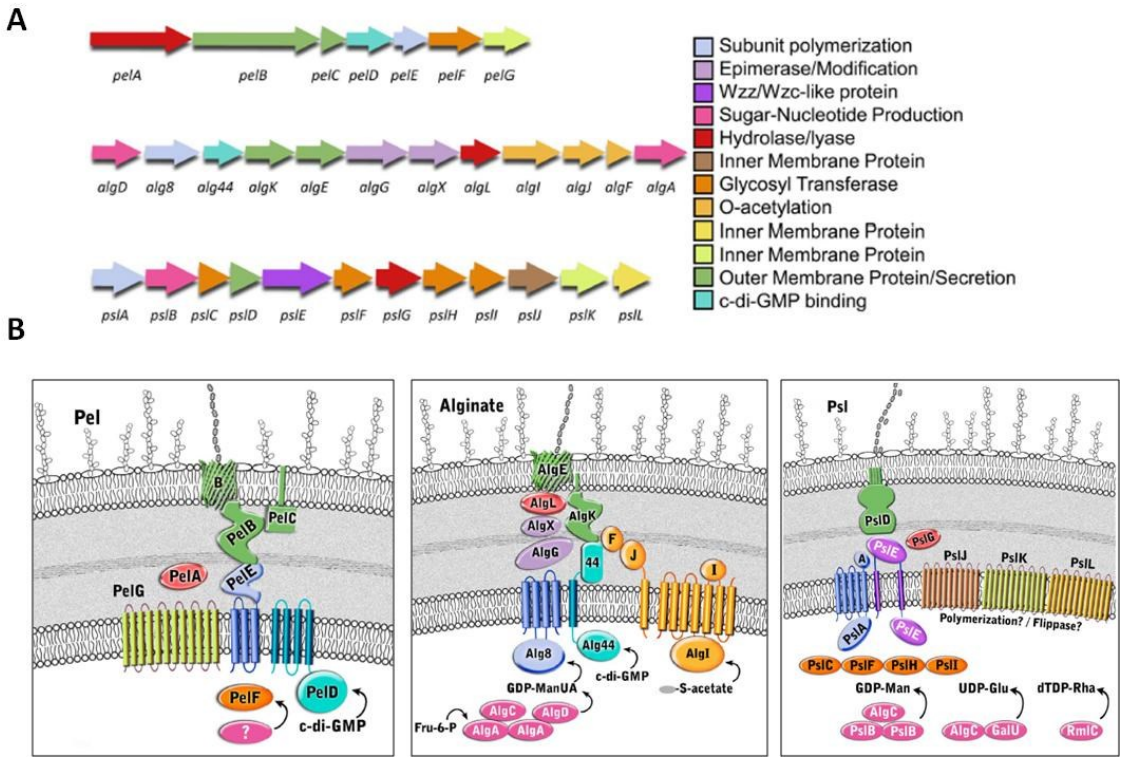

Figure 1.3 The biosynthetic complexes of Pel, alginate and Psl exopolysaccharides.

Figure retrieved from Franklin *et al.* (2011). **(A)** Genetic organization of the *pel*, *alg* and *psl* operons, coloured according to the description shown on the right. **(B)** Models of the organization of the biosynthetic complexes of the Pel, alginate and Psl exopolysaccharides, coloured as depicted in A.

The Pel exopolysaccharide is a glucose-rich polymer whose precise chemical composition or biosynthetic pathway is not known. The genes involved in its biogenesis however, are encoded in the *pel* operon (PA3058-PA3064) and based on structural homology analysis, a model for its production and export has been proposed (Coulon *et al.*, 2010; Franklin *et al.*, 2011; Friedman & Kolter, 2004a). In this case, the polymer precursors are thought to derive from the central carbon metabolism of the cells and the cytoplasmic PelF protein is involved in the polymerization process. After, the membrane proteins PelD, PelE and PelG are thought to be required for the transport of the polymer to the periplasm; PelA may be involved in the periplasmic modification and/or degradation of the polymer; and PelB and PelC are putative proteins associated with the outer membrane that serve as scaffold proteins and/or secretion platform for the exclusion of the Pel exopolysaccharide.

Finally, the Psl exopolysaccharide is a polymer composed of a repeating pentamer containing D-mannose, L-rhamnose, and D-glucose, and anchored to the cell surface in a helical pattern (Ma *et al.*, 2009). The genes required for the production and export of Psl are encoded in the *psl* operon which is composed of twelve genes (PA2231-PA2242). These include one cytoplasmic enzyme involved in

the synthesis of the mannose precursor, PslB; four cytoplasmic proteins that participate in the synthesis of the pentamer, PslC, PslF, PslH and PslI; five membrane proteins involved in the polymerazition and transport of the polymer to the periplasm, PslA, PslE, PslJ, PslK and PslL; a periplasmic protein for the modification and/or degradation of the polymer, PslG; and a protein associated with the outer membrane for the export of the Psl exopolysaccharide, PslD (Franklin *et al.*, 2011; Friedman & Kolter, 2004b). Curiously, part of the *psl* exopolysaccharide cluster is missing in the PA14 strain.

1.1.4 Secretion of proteins across the membrane

Like other Gram-negative bacteria, *P. aeruginosa* uses an array of different multiprotein complexes, called *secretion systems*, to translocate proteins to the extracellular medium or to target cells. Overall, these secretion systems play a crucial role in modulating the interactions between the bacteria and the environment or the bacteria and other organisms (like competitor microorganisms or host cells). A total of six different types of secretion systems have been recognized in Gram-negative bacteria and are named type I secretion system (T1SS) to type VI secretion system (T6SS). In the Gram-positive bacteria belonging to the *Mycobacterium* genus, a seventh type of secretion system has also been described for the translocation of proteins across the highly impermeable cell wall (Abdallah *et al.*, 2007) but it should be kept in mind that the nomenclature of the secretion systems is relative to Gram-negative bacteria and the translocation across the outer membrane.

In the Gram-negative *P. aeruginosa*, all but the T4SS are found (Bleves *et al.*, 2010). Generally, secretion systems are divided according to the mode of transport across the cell envelope: in one step, when the proteins are translocated directly from the cytoplasm to the extracellular medium or target cells (T1SS, T3SS, T4SS and T6SS); or in two steps, when the proteins are first transported to the periplasm and only after to the extracellular medium (T2SS and T5SS). A brief description of the secretion systems found in *P. aeruginosa* follows (Figure 1.4).

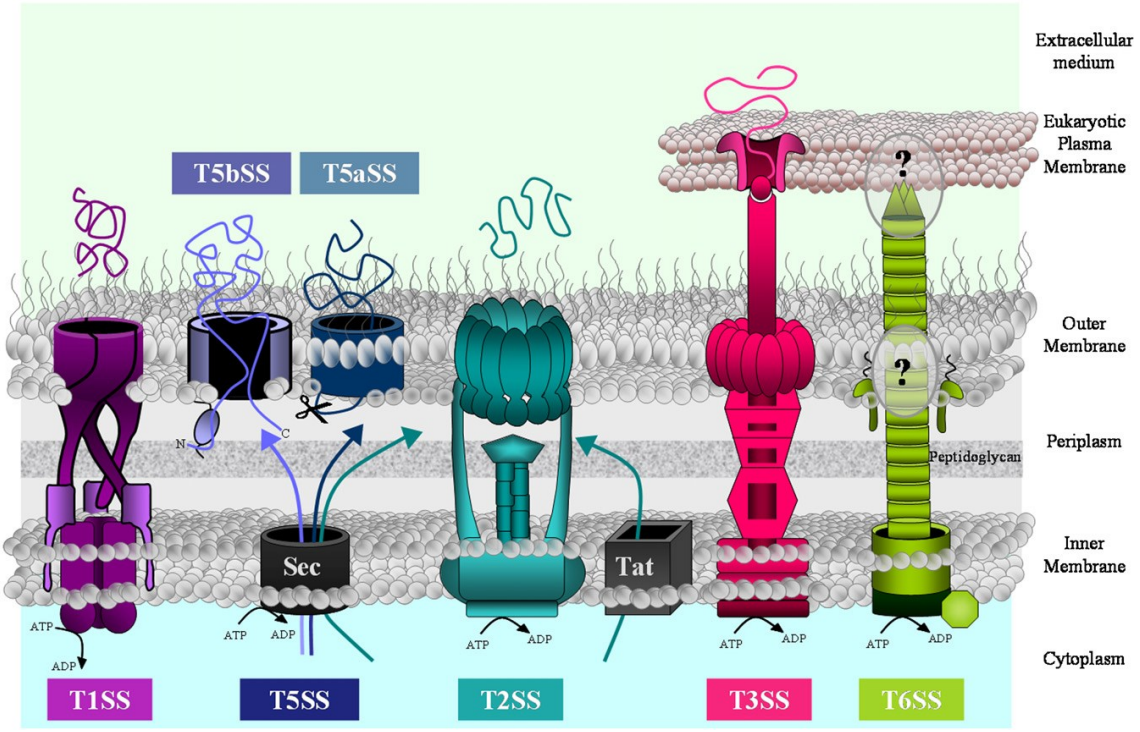

Figure 1.4 Secretion systems found in *P. aeruginosa*.

P. aeruginosa has five of the six secretion pathways found in Gram-negative bacteria. In the T1SS, T3SS and T6SS the effector proteins are translocated directly from the cytoplasm to the extracellular medium or target cells. In the T2SS and T5SS, the effector proteins are translocated from the periplasm to the outside and the translocation across the inner membrane is carried out by the Sec or Tat systems. The T3SS is known to transport toxins into the cytosol of eukaryotic cells. The T6SS is known to transport toxins into the periplasm and cytoplasm of bacterial cells and into the cytosol of eukaryotic cells. Figure retrieved from Bleves *et al.* (2010).

Type I secretion system (T1SS)

T1SSs are composed of an inner membrane ABC (ATP-binding cassette) transporter, an outer membrane protein and an adaptor protein that connects the first two. A total of four T1SSs have been found in *P. aeruginosa* but only the proteins secreted by two of them are known (Ma *et al.*, 2003). In the Apr(D-F) system (PA1246-1248), the alkaline protease and virulence factor AprA (PA1249) is secreted. In the Has(D-F) system (PA3404-PA3406), the haem acquisition protein HasA (PA3407) is secreted (Wandersman & Delepelaire, 2004).

Type II secretion system (T2SS)

T2SSs involve two steps. Initially, the proteins need to be translocated across the inner membrane by the means of the widespread Sec or Tat systems. Secondly, the proteins in the periplasm are exported through a large protein complex, the T2SS, to the extracellular medium.

In *P. aeruginosa,* the *Sec system* is composed of three inner membrane proteins that form a channel, SecE (PA4276), SecG (PA4747) and SecY (PA4243); one cytoplasmic ATPase that provides the energy for the translocation, SecA (PA4403); one chaperone that presents the unfolded protein to the ATPase, SecB (PA5128); and several other proteins that participate in the translocation process, cleavage of signal peptides or insertion of transmembrane domains in the inner membrane. On the other hand, the *Tat system* is composed of only three inner membrane proteins, TatA (PA5068), TatB (PA5069) and TatC (PA5070), and is responsible for the specific translocation of folded proteins that have an N-terminal signal peptide with a double arginine (RR) motif (Ma *et al.*, 2003).

Once in the periplasm, a complex of at least ten proteins is involved in the transport of the proteins across the outer membrane. Two complete sets of these proteins are found: the Xcp system (PA3095-PA3105), involved in the secretion of many proteolytic enzymes and virulence factors like the LasB elastase (PA3724) and the ToxA exotoxin A (PA1148); and the Hxc system (PA0677-PA0687), involved in the specific secretion of the LapA alkaline phosphatase (PA0688) under phosphate limiting conditions (Bleves *et al.*, 2010).

Interestingly, the T2SS machinery resembles the T4P assembly system in such a way that a similar protein arrangement is visible: a pseudopilus complex composed of a major pseudopilin (XcpT) is capped with minor pseudopilins (XcpU, XcpV, XcpW and XcpX) and spans the periplasm; an inner membrane and alignment complex comprises the ATPase motor XcpR and the proteins XcpP, XcpS, XcpY and XcpZ; and an outer membrane complex composed of the XcpQ secretin forms a channel (Filloux, 2004). Mechanistically, the XcpP protein is seen as a platform that recruits the periplasmic proteins to be exported and the pseudopilus is likely to work as a piston that pushes the recruited proteins through the channel formed by the XcpQ secretin (Douzi *et al.*, 2011).

Type III secretion system (T3SS)

T3SSs are responsible for the assembly of a needle structure that allows the injection of bacterial effectors directly into the cytosol of host cells. A total of 42 genes involved in the assembly and regulation of the T3SS are encoded in the *P. aeruginosa* PAO1 genome. Most of them are arranged in five operons that cluster together, except the three known secreted effectors (ExoY, PA2191; ExoS, PA3841; and ExoT, PA0044) and the cytoplasmic effector chaperone (SpcS, PA3842) which are

encoded elsewhere. In the PA14 strain, the additional effector ExoU and its chaperone SpcU are found (Hauser, 2009).

All the structural components of the T3SS are thought to be encoded within the five-operon cluster, including the inner membrane associated baseplate complex, the outer membrane complex and the needle complex. Briefly, at the baseplate, the cytoplasmic ATPase PscN (PA1697) is likely to energize the system; at the outer membrane complex, the secretin PscC (PA1716) allows the passage of the needle; and at the needle complex, PscF (PA1719) is the building block of the needle. Once assembled, three proteins at the tip of the needle, PopB (PA1708), PopD (PA1709) and PcrV (PA1706), are involved in the formation of a pore in the membrane of the host cells. The subsequent injection of the effector proteins helps the bacteria to evade the host immune system and is highly cytotoxic, promoting the establishment of an acute infection (Hauser, 2009).

Type V secretion system (T5SS)

Like T2SSs, T5SSs operate in two steps. First, the Sec system translocates the proteins across the inner membrane. Second, an autotransporter or a two-partner secretion pathway exports the proteins to the extracellular medium. There, they can remain associated to the cell surface or be released to the environment.

In an *autotransporter pathway*, the protein that exits the cell assists its own passage through the outer membrane. Three autotransporters are found in *P. aeruginosa* (PA0328, PA3535, PA5112) and they are characterized by the following modular organization: an N-terminal signal peptide that allows its translocation via the Sec pathway to the periplasm; a central domain that is the actual effector domain to be secreted; and a C-terminal domain that alone or upon oligomerization forms a β-barrel at the outer membrane and allows the secretion of the effector domain (Bleves *et al.*, 2010).

In a *two-partner secretion pathway*, the domain forming the outer membrane pore is in a different protein. These two proteins are encoded in an operon and the secreted protein has an additional N-terminal motif that is recognized by the outer membrane component (Ma *et al.*, 2003). In *P. aeruginosa*, five two-partner secretion pathways have been identified and one of them corresponds to the CdrA adhesin and the CdrB outer membrane protein.

Type VI secretion system (T6SS)

Like T3SSs, T6SSs are responsible for the translocation of proteins directly into target cells. However, the activity of the T6SS in *P. aeruginosa* is commonly associated with the chronic mode of infection rather than acute, and with the ability of the bacterium to defend itself against other bacteria, at least in the case of the best characterized cluster so far (Hood *et al.*, 2010; Potvin *et al.*, 2003).

In *P. aeruginosa* PAO1, three clusters encoding for three T6SS machineries have been identified (H1, H2 and H3). Most of the available literature focuses on the understanding of the machinery encoded by the H1 cluster (PA0070-PA0095) (Coulthurst, 2013; Filloux, 2013). Structurally, the H1-T6SS machinery resembles an inverted bacteriophage with a contractile tail and a puncturing device at the tip of the tail tube. Encoded in the H1 cluster are twenty five genes involved in the assembly and function of the T6SS. This includes the components IcmF1 (PA0077) that anchors the system to the membrane; Hcp1 (PA0085) which forms the tail tube that extends to the target cell; VgrG1 (PA0091) which sits at the tip of the tube to form the puncturing device (Hachani *et al.*, 2011; Pukatzki *et al.*, 2006); TssB1 (PA0083) and TssC1 (PA0084) which form the contractile tail sheath at the the baseplate of the system (Lossi *et al.*, 2013); and ClpV1 (PA0090), a cytoplasmic ATPase involved in the disassembly of the sheath (Mougous *et al.*, 2006).

Mechanistically, the contraction of the TssB1/TssC1 sheath allows the pushing of the Hcp1 tube which displays the VgrG1 puncturing device on top and perforates the membrane of target cells. Subsequently, the delivery of effector proteins to the targeted cells is thought to contribute to their killing. So far, three toxins that are delivered to the periplasm or the cytoplasm of the recipient cells, Tse1 (PA1844), Tse2 (PA2702), and Tse3 (PA3484), have been identified as H1-T6SS effectors (Hood *et al.*, 2010). Interestingly, each one of these toxins has a corresponding antitoxin that prevents the killing of the donor cell and constitutes a *toxin-antitoxin system*.

1.2 Regulatory cascades in *P. aeruginosa*

Bacteria respond to variations in the environment by sensing extracellular signals that are translated across the membrane into an intracellular readout and lead to a biological output. Signal transduction pathways are the regulatory cascades of reactions that drive this signalling and allow the integration of extracellular signals (or *first messengers*) into intracellular messages. Generally, signal transduction pathways involve either (i) direct binding to an effector protein, (ii) a cascade of phosphorylation reactions or (iii) a combination of phosphorylation and methylation reactions (Figure 1.5). Ultimately, regulatory cascades result in the modulation of gene expression at the

transcriptional, posttranscriptional or posttranslational/allosteric level, helping bacteria to shape their biological behaviour.

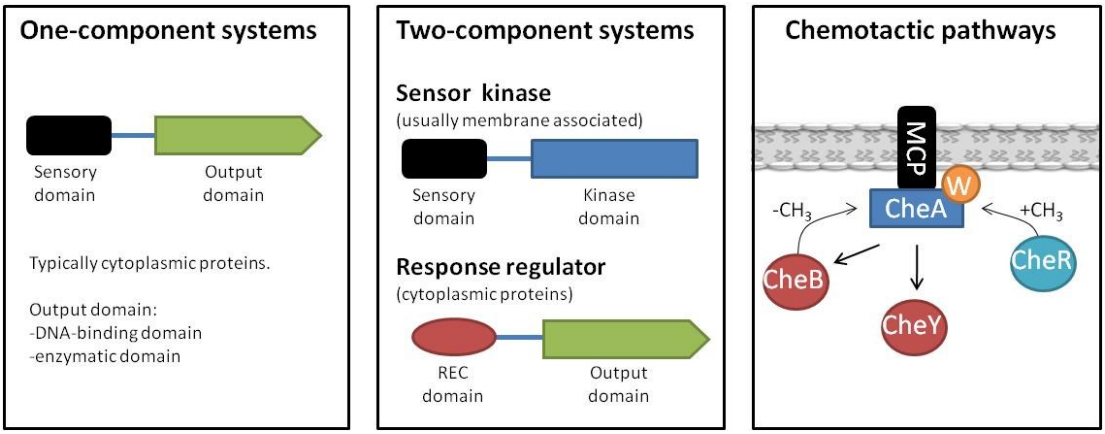

Figure 1.5 Signalling transduction pathways in bacteria.

Three categories of regulatory cascades are considered based on their complexity: direct binding of a signal to an effector protein (one-component systems), a cascade of phosphorylation reactions (two-component systems), and a combination of phosphorylation and methylation reactions (chemotactic pathways). One-component systems are typically cytoplasmic proteins with a sensory domain and an output domain that can either be a DNA-binding domain or an enzymatic domain. Two-component systems involved two proteins, one sensor and one response regulator. Upon sensing of a stimulus, the sensor undergoes autophosphorylation in an H (histidine) residue of the kinase domain and transfers the phosphoryl group into a D (aspartate) residue in the REC domain of the response regulator. Sometimes, the kinase domain in the sensor is followed by a REC domain. In these cases, the two-component systems are referred to as hybrid if a third component which is a Hpt domain protein exists, or unorthodox if the Hpt domain follows the REC domain in the sensor protein. The signal transduction pathway involved in chemotaxis comprises phosphorylation and methylation reactions. In the canonical chemotactic pathways there is a receptor protein (MCP) that sits in the inner membrane, an adaptor protein (CheW, represented by W), a kinase (CheA), a methyltransferase (CheR), and two response regulators (CheY and CheB). CheB functions as a methylesterase and together with CheR resets the system. CheY consists of only the REC domain and goes to the flagellum proteins to modulate motility.

1.2.1 Regulation by direct binding to effector proteins

Proteins that exert their regulatory action upon direct sensing of a signal are abundant in bacterial genomes and can be referred to as *one-component systems* (Ulrich *et al.*, 2005). Usually, these proteins have two domains, an N-terminal *input domain* involved in binding to small molecules, and

a C-terminal *output domain*. This is either a helix-turn-helix DNA binding domain or, less frequently, an enzymatic domain (Figure 1.5).

The majority of one-component systems lack transmembrane domains and are therefore more likely to be involved in sensing signals directly from the cytoplasm (Ulrich *et al.*, 2005). These signals can be extracellular signals that diffuse or are transported across the membrane, or intracellular signals originated after sensing of an extracellular stimulus, i.e. *second messengers*.

A typical example of a one-component system is LasR (PA1430) or RhlR (PA3477), proteins involved in quorum sensing signalling in *P. aeruginosa*. *Quorum sensing* is the bacterial adaptive response to cell density and an important signalling mechanism in bacteria that will not be discussed in detail in this thesis. Both LasR and RhlR have a modular organization where the input domain is an autoinducer binding domain and the output domain is a DNA binding domain. *Autoinducers* are the signals used in quorum sensing mediated regulation and binding of those signals promotes conformational changes (usually homodimerization) of the receptor proteins, resulting in binding of the output domain to its DNA targets. In the case of LasR and RhlR, the autoinducers are 3-oxo-C12-homoserine lactone and C4-homoserine lactone, respectively, and they act as a signal that activates transcription of a large number of DNA targets (Suarez-Moreno *et al.*, 2012).

Despite the fact that one-component systems are usually cytoplasmic proteins, there are some examples of one-component systems that are membrane associated (but not necessarily involved in direct sensing of signals in the extracellular medium). Two such examples in *P. aeruginosa* are RbdA (PA0861, Table 1.5) and MucR (PA1727, Table 1.5).

In RbdA, the N-terminus harbours an input PAS domain just after a predicted transmembrane domain, and the C-terminus contains two output domains with putative enzymatic activities (see section 1.4.2 for more details). *PAS domains* (named after the proteins where they are commonly found) are very common in bacteria and have a three-dimensional structure where small molecules like haem, flavin or adenine, bind to a ligand-binding pocket and respond to the presence of factors like oxygen or light (Table 1.1) (Galperin, 2004). In the characterization of RbdA, the protein was shown to be involved in biofilm dispersal via the activity of only one of its enzymatic domains and an intact PAS domain was essential for the catalytic activity (An *et al.*, 2010; Roy *et al.*, 2012). It was suggested that RbdA senses oxygen and that binding of oxygen to the PAS domain suppresses the activity of the output domain, preventing biofilm dispersal.

In the case of MucR, the N-terminus contains a MHYT domain and the C-terminus has two output domains with putative enzymatic activities (see section 1.4.3 for more details). A *MHYT domain* consists of six transmembrane regions with cytoplasmic loops rich in arginine residues and periplasmic loops rich in charged amino acids. MHYT domains have been bioinformatically predicted to bind metals and sense oxygen, carbon monoxide or nitric oxide, but this is still largely unexplored (Table 1.1) (Galperin *et al.*, 2001). Even for MucR, the signal or impact of the MHYT domain in the enzymatic activity of the output domains is unknown but the protein has been shown to be involved in alginate production and biofilm dispersal (Hay *et al.*, 2009; Li *et al.*, 2013).

Another input domain often found in membrane associated one-component systems is the *CHASE domain* that is bioinformatically predicted to have an extracellular input domain and is found associated with enzymatic output domains (Table 1.1) (Zhulin *et al.*, 2003). A total of four proteins with this domain are found in *P. aeruginosa*, namely PA0847 (Table 1.5), PA2072 (Table 1.5), PA4036 and PA4112, but up to now they remain uncharacterized.

Table 1.1 Input domains relevant for this study.

Domain	Description	Reference
PAS	Domain found in Per, Arnt and Sim proteins. Internal sensor of oxygen, redox potential and light.	Taylor & Zhulin, 1999 Galperin, 2004
REC	Domain found in response regulators. Contains a phosphoacceptor site. Usually, the phosphoryl group is received from a cognate sensor.	Pao & Saier, 1995
HAMP	Linker domain found in H kinases, ACs, MCPs.	Aravind & Ponting, 1999; Parkinson, 2010
GAF	Domain found in cGMP-specific PDEs, ACs and FhlA. Can serve as an interface for protein interactions or binding to ligands including cyclic nucleotides in eukaryotic cells, chromophores in plants or sodium in cyanobacteria.	Aravind & Ponting, 1997; Cann, 2007; Heikaus *et al.*, 2009
MHYT	Named based on its conserved amino acid pattern. Predicted to bind metals and sense oxygen, carbon monoxide or nitric oxide. Consists of six transmembrane regions connected by short arginine-rich cytoplasmic loops and periplasmic loops rich in charged amino acids. Three of the transmembrane regions have highly conserved methionine and histidine residues located near the outer face of the cytoplasmic membrane.	Galperin *et al.*, 2001

Domain	Description	Reference
CHASE	Cyclase/histidine kinase-associated sensing extracellular domain.	Zhulin *et al.*, 2003
MASE	Membrane associated sensory domain.	Nikolskaya *et al.*, 2003
PBPb or SBP_bac_3	Periplasmic substract-binding protein domain found in bacteria	Bermejo *et al.*, 2010; Tam & Saier, 1993
CBS	Intracellular modules that pair together and bind ligands.	Bateman, 1997; Scott *et al.*, 2004
7TMR-DISM	Consists of seven transmembrane segments with a periplasmic ligand-binding domain.	Anantharaman & Aravind, 2003
Cache	Frequently found in animal calcium channels and prokaryotic chemotaxis receptors. It is an extracellular domain that is predicted to have a role in small-molecule recognition.	Anantharaman & Aravind, 2000

1.2.2 Regulation by phosphorylation

Regulatory cascades that use phosphorylation as a means of information transfer are usually composed of two proteins and are therefore called *two-component systems*. In bacteria, they are less abundant than one-component systems but are more complex, allowing a more versatile signalling, and are evolutionary younger (Ulrich *et al.*, 2005). In highly adaptable microorganisms like *P. aeruginosa*, the prevalence of these systems is generally high and indeed, in the complete genome sequence of *P. aeruginosa* PAO1, a total of 135 two-component system genes are found (more than double the average of other bacterial species) (Barakat *et al.*, 2011; Capra & Laub, 2012; Rodrigue *et al.*, 2000; Stover *et al.*, 2000). Among the 135 genes, 84 form a recognizable pair and the remaining are either atypical or orphan sensors and response regulators.

In two-component systems, the input and output domains are located in two different proteins, the sensor kinase and the response regulator (Figure 1.5). The sensor kinase is usually a membrane protein with an extracellular N-terminal input domain and a C-terminal transmitter domain able to bind ATP (adenosine triphosphate) and undergo autophosphorylation. The response regulator is a cytoplasmic protein with an N-terminal *REC domain* (receiver domain) and a C-terminal output domain that can bind DNA or, less frequently, have enzymatic activity (Galperin, 2010a).

In a classical two-component system, binding of a signal to the input domain of the sensor triggers *autophosphorylation* (i.e. the attachment of a phosphoryl group from ATP to an amino acid) of a histidine (H) residue in the transmitter domain. Subsequently, the phosphoryl group is transferred to an aspartate (D) residue in the REC domain of a cognate response regulator, causing conformational changes that activate the output domain. Sometimes, the transfer of the phosphoryl group is not done in one direct step to the REC domain of the response regulator but in three steps, creating a *phosphorelay* (Ventre et al., 2004). In this case, the phosphoryl group is firstly transferred to an extra REC domain located in the sensor kinase, then to an *Hpt domain* (H phosphotransfer domain, found in tandem with the sensor or as a cytoplasmic protein) and finally, to the REC domain of the response regulator. Additionally, many classical sensor kinases are bifunctional showing phosphorylation activity in the active form and phosphatase activity in the absence of an input signal. This phosphatase activity is not so well understood as the phosphorylation reactions but it is thought to be important to fine-tune the activity of the response regulators output domain and to prevent cross-talk between different two-component systems in the same cell (Huynh & Stewart, 2011).

Given their abundance in *P. aeruginosa*, the biological processes regulated by two-component systems are diverse, ranging from nutrient metabolism and motility control to virulence and antibiotic resistance (Table 1.2) (Gooderham & Hancock, 2009; Mikkelsen *et al.*, 2011; Ventre *et al.*, 2004). Among all the two-component systems characterized so far, one of them stands out for its global role on the inverse regulation of phenotypes associated with acute and chronic infections. This system is named the Gac system and is depicted below. Another remarkable example of the regulation exerted by two-component systems is related to the control of the biofilm development which has been shown to be mediated by the synchronized and sequential action of at least three two-component systems: the Bfi, Bfm and Mfi systems.

Table 1.2 Two-component systems of known function in *P. aeruginosa*.

Name	Sensor	Response regulator	Associated functions	References
Sensing of nutrients				
PfeRS	PfeS (PA2687)	PfeR (PA2686)	Iron assimilation	Dean *et al.*, 1996
NarLX	NarX (PA3878)	NarL (PA3879)	Nitrate assimilation	Van Alst *et al.*, 2007; Van Alst *et al.*, 2009
CbrAB	CbrA (PA4725)	CbrB (PA4726)	Carbon and nitrogen assimilation	Li & Lu, 2007; Nishijyo *et al.*, 2001

Name	Sensor	Response regulator	Associated functions	References
PhoBR	PhoR (PA5361)	PhoB (PA5360)	Phosphate assimilation, quorum sensing, T2SS	Anba *et al.*, 1990; Ball *et al.*, 2002; Faure *et al.*, 2013; Filloux *et al.*, 1988; Jensen *et al.*, 2006
Motility				
FleRS	FleS (PA1098)	FleR (PA1099)	Flagella-driven motility	Dasgupta *et al.*, 2003; Ritchings *et al.*, 1995
PilRS	PilS (PA4546)	PilR (PA4547)	T4P-mediated motility	Boyd *et al.*, 1994; Boyd & Lory, 1996; Boyd, 2000; Ethier & Boyd, 2000; Hobbs *et al.*, 1993; Jin *et al.*, 1994
Biofilm formation and Virulence				
GacAS	GacS (PA0928)	GacA (PA2586)	Exopolysaccharides, T4P, T3SS, T6SS, quorum sensing, iron homeostasis, cyanide production	Brencic & Lory, 2009; Brencic *et al.*, 2009; Kay *et al.*, 2006; Parkins *et al.*, 2001; Pessi & Haas, 2001; Reimmann *et al.*, 1997; Soscia *et al.*, 2007
Roc/Sad	RocS1 (PA3946) RocS2 (PA3044)	RocR (PA3947) RocA1 (PA3948) RocA2 (PA3045)	CupB and CupC frimbriae regulation, T3SS, antibiotic resistance, biofilm maturation	Kuchma *et al.*, 2005; Kulasekara *et al.*, 2005; Sivaneson *et al.*, 2011
BfmRS	BfmS (PA4102)	BfmR (PA4101)	Biofilm maturation	Petrova & Sauer, 2009
BfiRS	BfiS (PA4197)	BfiR (PA4196)	Biofilm initiation	Petrova & Sauer, 2009; Petrova & Sauer, 2010
PprAB	PprA (PA4293)	PprB (PA4296)	T4Pb/Flp pilin regulation, CupE fimbriae, quorum sensing, antibiotic resistance	Bernard *et al.*, 2009; de Bentzmann *et al.*, 2012; Dong *et al.*, 2005; Giraud *et al.*, 2011; Wang *et al.*, 2003
Fim/Alg	FimS (PA5262)	AlgR (PA5261)	Alginate production, cyanide production, T4P-mediated motility, quorum sensing	Carterson *et al.*, 2004; Lizewski *et al.*, 2002; Lizewski *et al.*, 2004; Morici *et al.*, 2007; Whitchurch *et al.*, 1996; Yu *et al.*, 1997
Kin/Alg	KinB (PA5484)	AlgB (PA5483)	Alginate production	Chand *et al.*, 2011; Goldberg & Dahnke, 1992; Ma *et al.*, 1997; Wozniak & Ohman, 1991
MfiRS	MfiS (PA5512)	MfiR (PA5511)	Microcolony formation	Petrova & Sauer, 2009
PvrRS	PvrS [PA14]	PvrR [PA14]	CupD regulation	Mikkelsen *et al.*, 2009a
RcsBC	RcsC [PA14]	RcsB [PA14]	CupD regulation	Mikkelsen *et al.*, 2009a; Nicastro *et al.*, 2009
Metal and Antibiotic resistance				
PhoPQ	PhoQ (PA1180)	PhoP (PA1179)	Antibiotic resistance	Barrow & Kwon, 2009; Macfarlane *et al.*, 1999;

Name	Sensor	Response regulator	Associated functions	References
ParRS	ParS (PA1798)	ParR (PA1799)	Antibiotic resistance	McPhee *et al.*, 2006 Fernandez *et al.*, 2010; Muller *et al.*, 2011
CzcRS	CzcS/CzrS (PA2524)	CzcR/CzrR (PA2523)	Metal and antibiotic resistance	Hassan *et al.*, 1999; Perron *et al.*, 2004
CopRS	CopS (PA2810)	CopR (PA2809)	Metal and antibiotic resistance	Caille *et al.*, 2007
PmrAB	PmrB (PA4777)	PmrA (PA4776)	Antibiotic resistance	Barrow & Kwon, 2009; McPhee *et al.*, 2003; McPhee *et al.*, 2006; Moskowitz *et al.*, 2004

Regulation of virulence: the Gac system

The Gac system is particularly well understood and has been related to the switch between the planktonic and biofilm lifestyles in *P. aeruginosa* (Figure 1.6). It is found in pretty much all the *Pseudomonas* spp. genomes sequenced to date and has orthologs in other *Gammaproteobacteria* species such as *Escherichia* spp., *Salmonella* spp., *Vibrio* spp. or *Legionella* spp. (Lapouge *et al.*, 2008).

The core GacAS two-component system

The GacAS two-component system is composed of the sensor GacS and the response regulator GacA. GacS (PA0928) is an *unorthodox sensor* that has a transmembrane domain and an HAMP domain at the N-terminus – *HAMP domains* function as linkers and were named after the proteins where they are commonly found (Table 1.1) (Parkinson, 2010) – followed by an H kinase domain, a REC domain and an Hpt domain at the C-terminus. GacA (PA2586) is a typical response regulator, with an N-terminal REC domain and a C-terminal helix-turn-helix DNA binding domain. What exactly drives autophosphorylation of GacS is not known, but when active, the phosphoryl group travels via the REC and Hpt domains of GacS to the REC domain of GacA (Goodman *et al.*, 2009; Zuber *et al.*, 2003). Once phosphorylated, GacA drives the expression of two small non coding RNAs (sRNAs), RsmY (PA0527.1) and RsmZ (PA3621.1), by directly binding to the upstream region of their promoters (Brencic *et al.*, 2009).

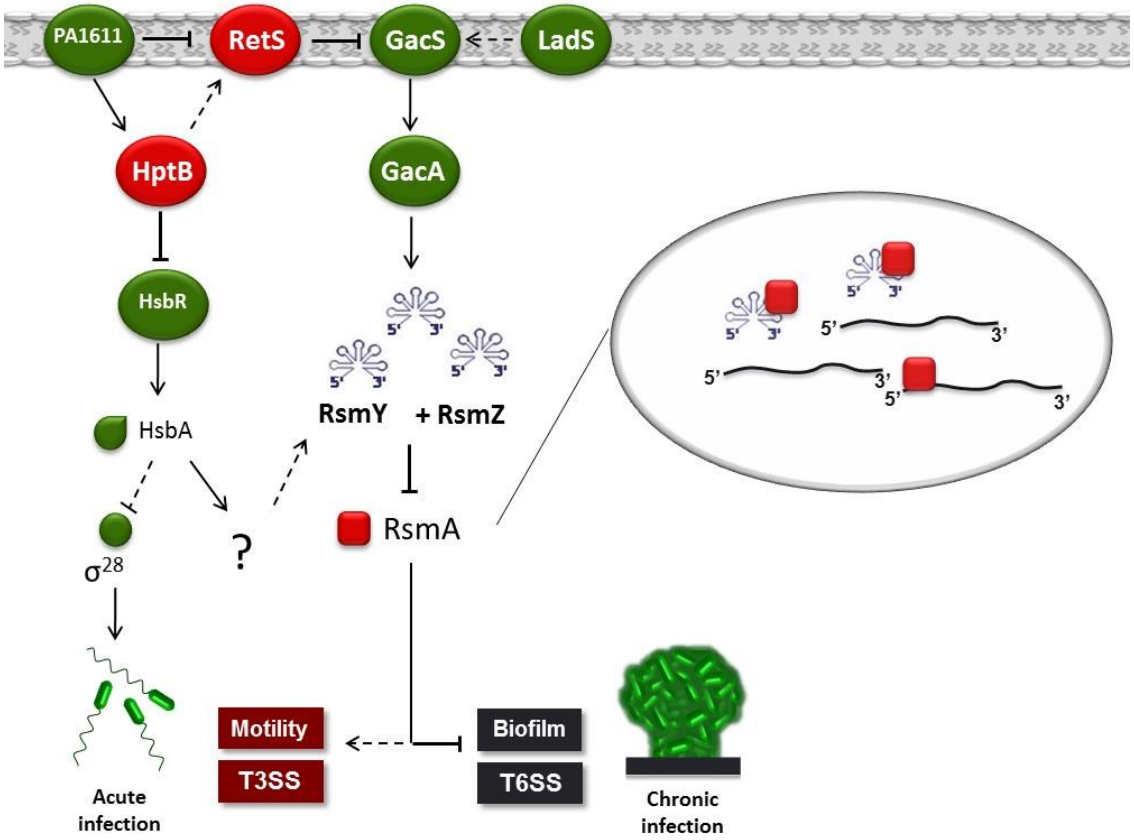

Figure 1.6 The Gac regulatory cascade in *P. aeruginosa* inversely regulates the bacterial lifestyle and mode of infection.

This complex cascade is composed of a central two-component system, GacS and GacA. Upon phosphorylation of the response regulator GacA by the sensor GacS, transcription of two sRNAs is initiated. These sRNAs, RsmY and RsmZ, bind to RsmA and antagonize its activity. RsmA is a translational repressor that functions by binding to GGA motifs in the ribosome binding site of target mRNAs. Known direct targets of RsmA are the H1-T6SS genes and the *psl* genes. Two additional sensors impact the function of GacAS - RetS has a negative impact whereas LadS has a positive impact. The RetS inhibition occurs by direct binding of the sensor to GacS. Another sensor, PA1611 is also able to bind RetS, counteracting its inhibitory effect on GacAS (Kong *et al.*, 2013). In addition, PA1611 phosphorylates HptB which then translates the phosphate to HsbR. Phosphorylation of HsbR inhibits its phosphatase activity and therefore HsbA, a putative anti-anti-sigma factor, remains phosphorylated and somehow prevents the expression of RsmY (Bordi *et al.*, 2010). The sigma factor required for the expression for RsmY is not known but HsbA was shown to bind to FlgM, a repressor of σ^{28} (FliA, PA1455) which is required in the flagellum biosynthesis (Bhuwan *et al.*, 2012). This Gac regulatory cascade is known to inversely regulate attributes related to the switch between acute and chronic infections. The proteins coloured in red behave as antagonists of the central GacAS and deletion mutants of the genes encoding those proteins have a hyperbiofilm phenotype. All the other proteins are coloured in green. Dashed arrows indicate unknown regulatory mechanism.

The antagonist sensor RetS

Although the chemical signal that activates GacS is not known, one other sensor, RetS (PA4856), can prevent and control its activity. RetS is an orphan sensor with an N-terminus transmembrane input domain and a C-terminus with the H kinase domain and two REC domains. The input domain is a *7TMR-DISM domain* (seven transmembrane receptors with diverse intracellular signalling modules) which consists of seven transmembrane segments with a periplasmic ligand-binding domain (Table 1.1) (Anantharaman & Aravind, 2003). Structural analysis of this domain in RetS revealed that it can form oligomers and that the periplasmic ligand-binding domain folds like a carbohydrate binding module (Vincent *et al.*, 2010). On the other hand, molecular and biochemical studies of the C-terminus of RetS revealed that RetS does not respond by autophosphorylation and instead, it seems to interact via its H kinase domain either with GacS or with another protein, PA1611 (Goodman *et al.*, 2009; Kong *et al.*, 2013; Laskowski & Kazmierczak, 2006). The formation of heterodimers between GacS and RetS inhibits GacA phosphorylation which is also prevented by the ability of RetS to promote the dephosphorylation of GacS (S. Porter, personal communication). Remarkably, deletion of *retS* or overexpression of PA1611 had the same impact on phenotypes like the activity of T3SS and biofilm formation, indicating that PA1611 can act as an antagonist of RetS. In addition, the impact observed on T3SS and biofilms was dependent on the central two-component system GacAS, demonstrating that indeed these proteins belong to the same pathway (Goodman *et al.*, 2004; Kong *et al.*, 2013).

The sensor PA1611 and HptB

PA1611 is a *hybrid sensor* kinase with transmembrane regions and a HAMP domain at the N-terminus and an H kinase and REC domain at the C-terminus. Despite the fact that the kinase activity of PA1611 is not required for the interaction with RetS, PA1611 alone is able to undergo autophosphorylation and create a phosphorelay that goes via an Hpt domain protein, HptB (PA3345), to the response regulator HsbR (PA3346) (Hsu *et al.*, 2008; Lin *et al.*, 2006). Interestingly, the regulon of an *hptB* deletion mutant was shown to correspond to a subset of the regulon of a *retS* deletion mutant (Figure 1.7) and the T3SS and biofilm phenotypes associated with the *hptB* mutant were dependent on GacAS (Bordi *et al.*, 2010).

The additional sensor LadS

Besides RetS, there are only three other genes in *P. aeruginosa* PAO1 that encode for proteins with a predicted 7TMR-DISM input domain: LadS, PA3462 and PA4929. LadS (PA3974) is another orphan and hybrid sensor that has a H kinase and REC domain at the C-terminus. Strikingly, the regulon of a

ladS deletion mutant partially overlaps with the regulon of a *retS* mutant and in more than 90% of the cases the genes are oppositely regulated (Figure 1.7) (Ventre *et al.*, 2006). Also, deletion of *ladS* causes a similar reduction on *rsmZ* expression as a deletion in *gacS*, supporting a model in which LadS acts synergistically with GacAS to activate the expression of the downstream *rsmY* and *rsmZ* targets (Ventre *et al.*, 2006). Like LadS, PA3462 is an orphan and hybrid sensor whose function was very recently shown to reduce the phosphorylation of GacS (Francis *et al.*, 2013) and PA4929 (Table 1.5) instead of having an H kinase transmitter domain at the C-terminus, it has an enzymatic output domain with putative catalytic activity.

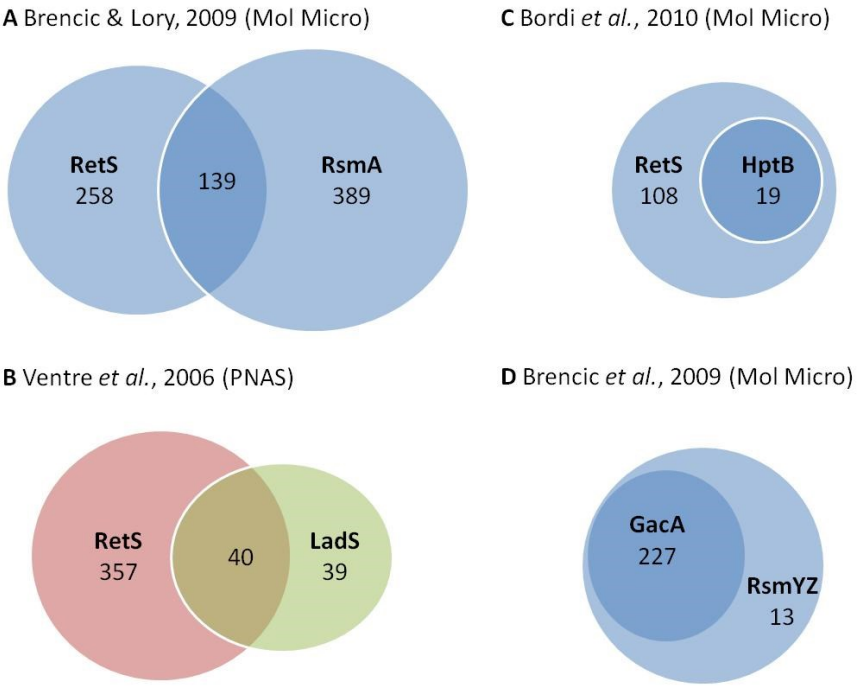

Figure 1.7 Venn diagrams showing the number of genes in the regulons of RetS, LadS, GacA, HptB, RsmYZ and RsmA.

(A) A total of 139 genes of the RetS and RsmA regulons overlap and are regulated in the same direction. **(B)** A total of 40 genes of the RetS and LadS regulons overlap and are antagonistically regulated with the exception of PA1001 which was regulated in the same orientation. **(C)** The regulon of HptB falls into the regulon of RetS. **(D)** The regulons of GacA and RsmYZ (RsmY and RsmZ) overlap almost in their entirety (227 genes) and are regulated in the same direction with the exception of PA2560.

The sRNAs: RsmY and RsmZ

At least three different sensors (RetS, PA1611 and LadS) feed into the GacAS two-component system to regulate the expression of two sRNAs, RsmY and RsmZ. Importantly, once expressed, these sRNAs

are both subjected to and exerting a regulatory action. For instance, it was demonstrated that two transcriptional regulators, MvaT (PA4315) and MvaU (PA2667), can bind AT-rich stretches upstream of *rsmZ* and inhibit its expression (Brencic *et al.*, 2009). Also, RsmZ seems to be regulated posttranscriptionally by the RNase CafA (PA4477) to allow the natural progression of the biofilm developmental cycle (Petrova & Sauer, 2010). On the other hand, RsmY was shown to be posttranscriptionally stabilized by an RNA-binding protein called Hfq (PA4944) (Sonnleitner *et al.*, 2006). It has also been demonstrated that HptB controls the levels of RsmY expression (Bordi *et al.*, 2010). In this case, the precise regulatory mechanism is not known, but a model has been proposed where the downstream targets of HptB, i. e. HsbR and HsbA, control the release of a sigma factor that impacts *rsmY* expression. Briefly, HptB leads to phosphorylation of the response regulator HsbR, therefore repressing the phosphatase activity of the HsbR output domain. Subsequently, the HsbR downstream target, a putative anti-anti-sigma factor named HsbA (PA3347), is not activated by dephosphorylation and prevents the release of the sigma factor required for *rsmY* expression (Bordi *et al.*, 2010). More recently, a model for HsbR was proposed in which HsbR can act as both a phosphatase and a S (serine) kinase of HsbA and HsbA was shown to bind to the anti-sigma factor FlgM which negatively regulates the σ^{28} sigma factor involved in the expression of flagellar genes (Bhuwan *et al.*, 2012). However, the authors of this study did not explore if σ^{28} is the sigma factor involved in the expression of RsmY.

The translational repressor RsmA

In relation to the regulatory role that RsmY and RsmZ exert, it has been shown that the two sRNAs can bind a small protein called RsmA (Heurlier *et al.*, 2004; Kay *et al.*, 2006). RsmA (PA0905) is a RNA-binding protein that acts as a posttranscriptional repressor by binding to GGA motifs in the ribosome binding site (RBS) of target RNAs and preventing translation (Brencic & Lory, 2009). In a very exciting work, Brencic and Lory at the Harvard Medical School, identified the regulon of RsmA (more than 500 genes) and showed that indeed the negative regulation exerted by RsmA is dependent on the direct binding of RsmA to the mRNA targets. Among many others, these targets were mRNA of genes belonging to operons responsible for T6SS or exopolysaccharide production. Remarkably, approximately one third of the RsmA regulon was positively regulated rather than negatively. This included genes of the T3SS and T4P. For this positive regulation, direct binding of RsmA to mRNA targets was not observed supporting the notion that RsmA can only act as a posttranscriptional repressor and suggesting that the positive regulation is indirect, probably exerted at the level of transcription via the negative regulation of regulatory factors (Brencic & Lory, 2009). Indeed, this seems to be the case for the control of motility in *Pseudomonas fluorescens*

where RsmA was shown to bind a sigma factor, σ^{22} (PA0762) and block its translation. By doing so, RsmA is indirectly preventing the expression of σ^{22} targets which include a transcriptional repressor of the flagellar genes (Martinez-Granero *et al.*, 2012). It remains to be investigated if the same mechanism operates in *P. aeruginosa*.

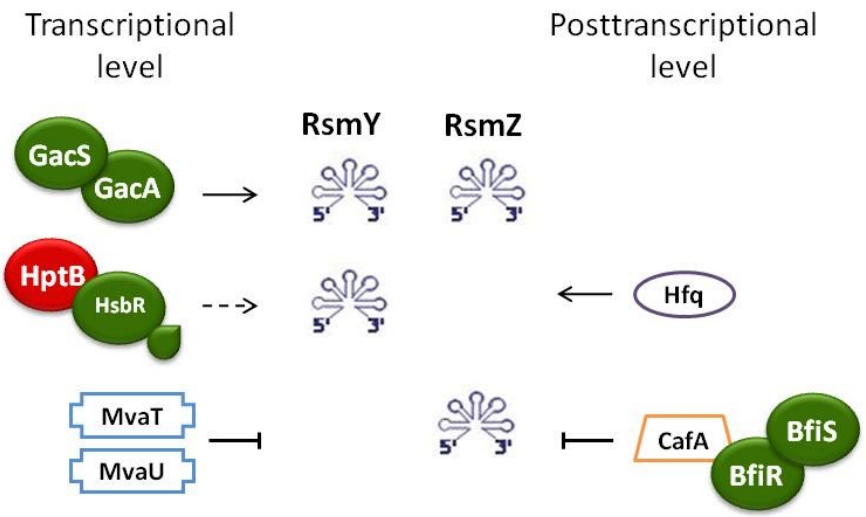

Figure 1.8 Regulation of the sRNAs, RsmY and RsmZ.

At the transcriptional level, the two-component system GacAS is required for the transcription of the two sRNAs whereas HptB indirectly affects positively *rsmY* expression and the transcriptional regulators MvaT and MvaU negatively affect the expression of *rsmZ*. The control exerted by GacA is likely dependent on GacA dimerization upon phosphorylation and occurs by binding of GacA to a conserved upstream activating sequence in the promoter regions (Humair *et al.*, 2010). HptB is an Hpt domain protein that phophorylates HsbR and HsbR has an output domain with phosphatase activity. This phosphatase activity was hypothesized to activate an anti-anti-sigma factor which binds an anti-sigma factor and realeases a sigma factor involved in the transcription of *rsmY* (Bordi *et al.*, 2010). MvaT and MvaU bind AT-rich stretches upstream of *rsmZ* and inhibit its expression (Brencic *et al.*, 2009). At the posttranscriptional level, the RNA-binding protein Hfq is known to stabilize RsmY (Sonnleitner *et al.*, 2006) and the RNase CafA is involved in degrading RsmZ. The production of CafA is transcriptionally regulated by binding of BfiR, the response regulator of the sensor BfiS, to the region upstream of *cafA* (Petrova & Sauer, 2010). Dashed arrows indicate unknown regulatory mechanism.

In summary, the Gac system is composed of a central two-component system, GacAS whose response regulator GacA drives the expression of two sRNAs upon phosphorylation. The sRNAs then compete for binding to RsmA, sequestering it and allowing translation of its mRNA targets. This leads to biofilm formation and expression of T6SS. In addition, other sensors have been shown to intersect with this central cascade. More specifically, LadS and PA1611 act in concert with GacAS to counteract the RsmA repression and promote biofilm formation; and RetS acts as an antagonist of

GacAS, preventing phosphorylation of GacA and promoting a motile lifestyle with the expression of the T3SS (Figure 1.6).

Regulation of biofilm development: the Bfi, Bfm and Mif systems

Two-component systems can regulate a broad range of phenotypes, like the Gac system, but they can also regulate specific traits in a very timely and ordered manner. This is the case, for instance, of the Bfi, Bfm and Mif systems which are activated one after the other to allow the progression of the biofilm developmental cycle from initial attachment to maturation.

These systems were identified when studying the phosphoteome of *P. aeruginosa* biofilms at different stages of development. Petrova and Sauer found three two-component systems that were phosphorylated at different stages: BfiRS at the initiation of biofilm formation, BfmRS during biofilm maturation and MifRS at the stage of microcolony formation (Petrova & Sauer, 2009). Although the three sensors, BfiS (PA4197), BfmS (PA4102) and MifS (PA5512), all have C-terminal H kinase domains, their predicted N-terminal structure is different: BfiS has a PAS domain, BfmS has transmembrane domains followed by a HAMP domain, and MifS harbours transmembrane domains. Regarding the response regulators, BfiR (PA4196), BfmR (PA4101) and MifR (PA5511), the same modular organization of an N-terminal REC domain and a C-terminal DNA binding domain is recognizable. However, the structure of their DNA binding domain is different and belongs to different families of transcriptional regulators – BfiR belongs to the NarL family, BfmR to the OmpR family and MfiR to the NtrC family (Ventre *et al.*, 2004).

In addition to these three two-component systems, the sensor SagS (PA2824) was shown to be activated prior to BfiS and to be able to interact with BfiS to modulate its autophosphorylation (Petrova & Sauer, 2011). Interestingly, SagS is a membrane associated hybrid sensor that was identified in the same screen that led to the characterization of RetS (Goodman *et al.*, 2004) and that is able to phosphorylate HptB (Hsu *et al.*, 2008). Furthermore, during the biofilm mode of growth it was shown that a *sagS* deletion mutant has elevated levels of both RsmY and RsmZ (Petrova & Sauer, 2011). This is in agreement with the concepts presented earlier. Firstly, deletion of *sagS* prevents phosphorylation of HptB and the sigma factor required for RsmY expression is released (Bordi *et al.*, 2010). Secondly, deletion of *sagS* prevents BfiS phosphorylation and this does not contribute for the degradation of RsmZ by CafA (Petrova & Sauer, 2010).

In summary, in the initial stages of biofilm development a synchronized action of at least four sensors is required. While it is not known how the communication occurs between BfiS and BfmS or BfmS and MifS, it has been shown that BfiS phosphorylation is SagS dependent (Petrova & Sauer, 2011).

Regulation by eukaryotic-like kinases

In eukaryotic cells, two-component systems as seen in prokaryotic cells are not widely distributed and instead pathways that use STY (serine, threonine or tyrosine) phosphorylation in posttranslational modifications are common. Curiously, in an analysis of the STY phosphoproteome of the *P. aeruginosa* PAO1 strain, a total of 57 proteins were identified. One of them, PpkA (PA0074) is a known kinase involved in the posttranslational control of the H1-T6SS (Ravichandran *et al.*, 2009). Briefly, the membrane associated PpkA undergoes autophosphorylation in a specific S residue upon sensing of a signal. The phosphoryl group is then transferred to a T residue in the Fha1 (PA0081) protein, allowing assembly of the T6SS machinery and secretion of the effector proteins. The kinase activity of PpkA is counteracted by its cognate phosphatase PppA (PA0075) which keeps Fha1 dephosphorylated and prevents secretion. The signal that drives autophosphorylation of PpkA is unknown but is thought to be perceived in the periplasm and be surface-contact dependent (Casabona *et al.*, 2012; Mougous *et al.*, 2007; Silverman *et al.*, 2011; Silverman *et al.*, 2012).

1.2.3 Regulation by phosphorylation and methylation

In addition to direct binding of a signal to an effector protein (one-component systems) and cascades of phosphorylation reactions (two-component systems), bacteria also have sophisticated regulatory mechanisms that use a combination of both phosphorylation and methylation processes and allow the sensing of the rate of change of different environmental cues. These regulatory cascades are referred to as *chemotactic pathways* and are usually involved in chemotaxis, i.e. the signal transduction pathway that allows flagellated bacteria to sense extracellular signals and swim towards favourable niches.

At least six proteins are involved in a typical *Escherichia coli* chemotactic pathway: a receptor (MCP, methyl accepting chemotaxis protein), an adaptor (CheW), a kinase (CheA), two response regulators (CheY and CheB) and a methyltransferase (CheR). Occasionally, an additional phosphatase (CheZ) is present (Figure 1.5) (Scott *et al.*, 2012). The functional organization of a chemotactic signalling

pathway starts with a signal being perceived by a MCP (usually membrane associated with an extracellular sensing domain) and then transduced through its C-terminus and by means of an adaptor protein, to a cytoplasmic kinase. When triggered, the kinase suffers autophosphorylation of an H residue and the phosphoryl group is then transferred to the two response regulators CheY and CheB. CheY, is an atypical response regulator as it only consists of the REC domain but CheB has a methylesterase output domain. Upon phosphorylation, CheY goes to the flagellar motor where it interacts with flagellum proteins to modulate motility and CheB demethylates the MCP affecting the rate at which CheA autophosphorylates. The methylesterase activity of CheB is counteracted by the methyltransferase activity of CheR which is constitutively active. Together, CheB and CheR work as a module that resets the system, guaranteeing that the bacterial behaviour is being constantly adapted to the surrounding environment. An additional level of control can be added by CheZ which acts as a phosphatase of CheY to quickly restart the system.

Based on homology to the CheA kinase, there are four chemotaxis clusters in the genome of *P. aeruginosa* PAO1 and a total of 26 MCP genes are found (Table 1.3) (Porter *et al.*, 2011; Parales *et al.*, 2004). Again, this high number of chemotaxis clusters and MCPs contrasts with other bacteria – for instance, *E. coli* has only one cluster and 5 MCPs. Interestingly, not all the four clusters found in *P. aeruginosa* are involved in chemotaxis. The *pil-chp* cluster (PA0408-PA0417) is involved in T4P-mediated twitching motility and the *wsp* cluster (PA3702-PA3708) is involved in c-di-GMP dependent biofilm formation.

A variety of MCPs

P. aeruginosa is known to have a chemotactic behaviour towards a variety of amino acids, sugars, organic acids, aromatic compounds and oligopeptides (Kato *et al.*, 2008). So far, only half of the MCPs have been characterized to date and in most of the cases, the data does not provide biochemical insights about their function. It has been shown, however, that MCPs in other model organisms tend to form homodimers and then oligomerise, appearing as cluster of different MCPs at the cell poles. By aggregating, these clusters of MCPs allow for both sensing of multiple signals and amplification of an input signal.

Table 1.3 MCPs found in *P. aeruginosa*.

MCP	Function	Transmembrane domains	Other domains	References
Chemotaxis				
PA0176 (McpB/ Aer2/TlpG)	Aerotaxis	--	PAS	Hong *et al.*, 2004; Watts *et al.*, 2011
PA0180 (McpA/CttP)	Low chloroethylenes	✓	--	Kim *et al.*, 2006
PA1561 (Aer/TplC)	Aerotaxis	✓	PAS	Hong *et al.*, 2004
PA2561 (CtpH)	Low inorganic phosphate	✓	HAMP	Wu *et al.*, 2000
PA2652	Malic acid	✓	Cache, HAMP	Alvarez-Ortega & Harwood, 2007
PA4307 (PctC)	L-aminoacids and high chloroethylenes	✓	Cache, HAMP	Shitashiro *et al.*, 2005; Taguchi *et al.*, 1997
PA4309 (PctA)				Kuroda *et al.*, 1995; Shitashiro *et al.*, 2005
PA4310 (PctB)				Shitashiro *et al.*, 2005; Taguchi *et al.*, 1997
PA4844 (CtpL)	High inorganic phosphate	✓	HAMP	Wu *et al.*, 2000
Other functions				
PA0411 (PilJ)	Type IV pili biogenesis	✓	HAMP	DeLange *et al.*, 2007
PA1423 (BdlA)	Biofilm dispersal	--	PAS	Morgan *et al.*, 2006
PA2573	Virulence	✓	HAMP	McLaughlin *et al.*, 2012
PA3708 (WspA)	Biofilm formation	✓	HAMP	O'Connor *et al.*, 2012
Unknown function				
PA1251	Polar localisation	✓	HAMP	--
PA1608		✓	HAMP	--
PA1646		✓	HAMP	--
PA1930 (McpS)		--	PAS	Bardy & Maddock, 2005
PA2654		✓	HAMP	--
PA2788		✓	HAMP	--
PA2867		✓	HAMP	--
PA2920		✓	HAMP	--
PA4290		✓	--	--
PA4520		✓	HAMP	--
PA4633		✓	HAMP	--
PA4915		✓	HAMP	--
PA5072		✓	HAMP	--

In terms of modular organization, MCPs usually have N-terminal transmembrane segments that place the input domain extracellularly; a cytoplasmic HAMP domain that allows transduction of the signal through the membrane; and a C-terminal kinase control module that harbours a methylation

glycine hinge and interacts with the downstream components of the cascade to modulate CheA autophosphorylation.

In *P. aeruginosa*, a few MCPs involved in chemotactic responses to amino acids (PctA, PctB, PctC), organic acids (PA2652), inorganic phosphate (CtpH, CtpL), oxygen (Aer, Aer2) and ethylene derivatives (CttP) have been identified (Table 1.3). Sometimes, like for Aer and Aer2, MCPs have PAS domains and lack transmembrane domains, serving as sensors of the redox status of the cell (Schweinitzer & Josenhans, 2010). Also, in the case of PctA, PctB, PctC and PA2652, an N-terminal input domain – called *Cache domain* for being frequently found in animal calcium channels and prokaryotic chemotaxis receptors –, is bioinformatically recognizable but its function remains unknown (Table 1.1) (Anantharaman & Aravind, 2000).

Interestingly, a few other MCPs have been implicated in functions other than chemotaxis. These include PilJ, WspA, BdlA and PA2573. Briefly, PilJ (PA0411) belongs to the Pil-Chp cluster and has been shown to be required for proper biogenesis of the T4P (DeLange *et al.*, 2007). WspA (PA3708) belongs to the Wsp cluster and does not seem to form clusters at the cell poles or respond to binding of small extracellular signal molecules. Instead, it localises to the periphery of the cells and responds to signals associated with growth on a surface (Guvener & Harwood, 2007; O'Connor *et al.*, 2012). BdlA (PA1423) is an orphan MCP with two N-terminal PAS domains and is required for biofilm dispersal. To become activated, BdlA is subjected to a quite complex posttranslational modification. Upon phosphorylation of a Y residue located after the second PAS domain, the protein suffers protease cleavage between the two PAS domains, leading to an interaction of the two cleaved fragments that causes structural modifications which activate downstream targets (Morgan *et al.*, 2006; Petrova & Sauer, 2012a; Petrova & Sauer, 2012b). Finally, another orphan MCP, PA2573, has been implicated in virulence and antibiotic resistance. It has been shown that PA2573 interacts with the REC domain of the response regulator PA2572 (Table 1.5) and that the regulon of PA2573 and PA2572 deletion mutants overlaps in the negative regulation of the T3SS and the modulation of various genes involved in antibiotic resistance (McLaughlin *et al.*, 2012).

The che *and* che2 *clusters*

These two clusters are related to flagella-driven swimming motility and include genes that share homology with the components of the chemotactic pathway found in *E. coli*.

The Che system is essential for chemotaxis and has homologues to CheA (PA1458), CheB (PA1459), CheW (PA1464), CheY (PA1456) and CheZ (PA1457). It misses a recognizable MCP, it encodes four

additional proteins – two of which are the MotCD homologues of the MotAB flagellum stator proteins –, and it lacks the methyltransferase CheR (PA3348) which is encoded elsewhere in the chromosome (Kato *et al.*, 1999; Masduki *et al.*, 1995). Next to CheR, a protein with homology to CheW at the N-terminus and CheY at the C-terminus, CheV (PA3349), is found (Alexander *et al.*, 2010). Interestingly, CheR was shown to be able to methylate the MCP PctA in response to addition of amino acids (Schmidt *et al.*, 2011).

The Che2 system is thought to play a role in chemotaxis when the cells enter stationary growth (Schuster *et al.*, 2004). This cluster encodes two MCPs (McpA and McpB) and all the other components of a chemotactic pathway: CheA2 (PA0178), CheB2 (PA0173), CheW2 (PA0177), CheY2 (PA0179) and CheR2 (PA0175). In addition, it has a CheD (PA0174) homologue which, in other microorganisms, functions as a MCP deamidase and is involved in the adaptation of the chemotactic response (Scott *et al.*, 2012). Intriguingly, both Che and Che2 protein complexes locate to the cell poles but do not colocalize and whereas McpB (PA0176) is required for Che2 protein complex formation, McpA (PA0180) seems to be recruited to the Che protein complex when cells enter stationary phase (Guvener *et al.*, 2006).

The pil-chp *cluster*

The *pil-chp* cluster is related to the function and biogenesis of the T4P (Whitchurch, 2004). It encodes one MCP (PilJ), one methylesterase (ChpB), two adaptor proteins (PilI and ChpC), two response regulators (PilG and PilH), and one methyltransferase (PilK). A kinase, ChpA (PA0413), is also encoded but this is an unusually complex protein harbouring an extra eight potential phosphorylation sites (six Hpt, one T and one S) at the N-terminus, and a REC domain at the C-terminus. In addition, two other genes are found, *chpD* and *chpE*, but their exact function is unknown. ChpD (PA0416) is a predicted transcriptional regulator and ChpE (PA0417) a small membrane protein (Whitchurch *et al.*, 2004).

It has been proposed that the functional organisation of the Pil-Chp system starts when PilJ, the MCP, senses a yet unknown signal that induces the autophosphorylation of ChpA with the help of one of the adaptor proteins PilI (PA0410) and/or ChpC (PA0415). The precise role of the extra phosphorylation sites of CheA is not well understood but the REC domain at the C-terminus has been shown to be essential for twitching motility (Leech & Mattick, 2006). Nonetheless, the phosphoryl group is thought to be transferred from CheA to the methylesterase ChpB (PA0414) and to the two response regulators PilG (PA0408) and PilH (PA0409) which then interact with downstream targets to control pilus extension and retraction, respectively (Bertrand *et al.*, 2010).

Given the complexity of the kinase ChpA, it is possible that the Pil-Chp system has additional roles that go beyond the direct control of twitching motility. For instance, at the tip of the N-terminus, a domain that resembles FimL (PA1822) is recognizable. FimL is a known regulator of the levels of an intracellular signalling cyclic nucleotide called cyclic AMP (cAMP). This cyclic nucleotide is known to regulate a variety of processes including T2SS, T3SS, T4P and flagella biogenesis (Inclan *et al.*, 2011; Nolan *et al.*, 2012; Whitchurch *et al.*, 2005). Remarkably, it has recently been described that mutations in *chp* genes have an effect on the levels of cAMP. Although the precise molecular mechanism of this effect is yet to be elucidated, if it turns out that there is a direct link between the two, a new and broader impact of the Pil-Chp pathway will be unravelled (Fulcher *et al.*, 2010).

The wsp *cluster*

The *wsp* cluster has been associated with biofilm formation and harbours homologues to a MCP (WspA), a methylesterase (WspF), two adaptor proteins (WspB and WspD) and a methyltransferase (WspC) (Hickman *et al.*, 2005). Similar to the Pil-Chp cluster, the kinase WspE (PA3704) has also a REC domain at the C-terminus (but lacks the remaining ChpA complexity). The WspR (PA3702) response regulator has the typical REC domain and in addition, a C-terminal output domain with enzymatic activity (Figure 1.9, Table 1.5).

The Wsp system is associated with the formation of biofilms. Upon sensing of a signal associated with growth on a surface and with the help of the adaptor proteins WspB (PA3707) and/or WspD (PA3705), the MCP WspA induces autophosphorylation of WspE (O'Connor *et al.*, 2012). The function of the REC domain of WspE is not known but the phosphoryl group is then transferred to the methylesterase WspF (PA3703) and the response regulator WspR. Once phosphorylated, WspR forms oligomers which are visible as patches in the bacterial cells (Guvener & Harwood, 2007; Huangyutitham *et al.*, 2013). Together, the phosphorylation and the clustering of WspR activate its output domain whose catalytic activity leads to the production of the intracellular signalling molecule cyclic di-GMP, stimulating biofilm formation. The *P. aeruginosa* c-di-GMP signalling network is described in the following pages and you can find more information about WspR in section 1.4.1.

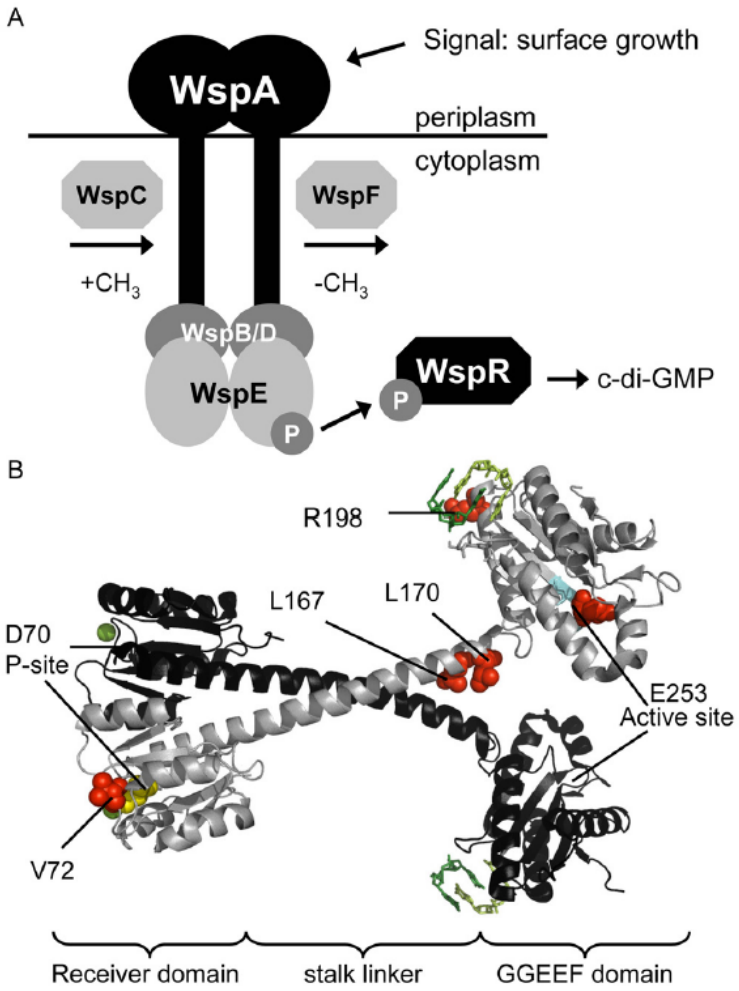

Figure 1.9 The Wsp system and WspR.

(A) Model of the Wsp signal transduction complex. WspA is a MCP protein that detects an unknown signal associated with growth on a surface. Upon reception of a stimulus, the H kinase WspE phosphorylates the response regulator WspR. Phosphorylated WspR produces c-di-GMP. The methyltransferase WspC and the methylesterase WspF likely play a role in adaptation to the surface signal. In a *wspF* deletion mutant WspR is constitutively active. The adaptor proteins WspB and WspD are important for function and proper localization of the Wsp complex. **(B)** Structure of a WspR dimer with bound c-di-GMP to the inhibition sites (I-sites). One molecule is shown in grey and the other in black. Cyan indicates the GGEEF motif which forms the A-site of the protein. The phosphorylation site is indicated in yellow (D70) and the green spheres are the magnesium ions needed for phosphorylation. The residues indicated in red are important for function. The V72 residue is located in the REC domain and mutation to D activates the GGDEF domain. The L167 and L170 residues are located in the linker region and affect WspR tetramerization. R198 mutation to A renders the I-site unable to bind c-di-GMP. E253 mutation to A changes the GGEEF motif and inactivates the DGC activity. Figure retrieved from Huangyutitham et al. (2013).

1.3 Cyclic nucleotide signalling in *P. aeruginosa*

Nucleotides are not only the building blocks of nucleic acids. They are also fundamental molecules in cellular energy transfer, via ATP (adenosine triphosphate), and serve as important signalling molecules in all domains of life. In eukaryotic cells, the cyclic nucleotides cAMP (cyclic adenosine 3',5'-monophosphate) and cGMP (cyclic guanosine 3',5'-monophosphate) have long been recognized as important signalling molecules that act as second messengers to regulate a great variety of cellular processes. More recently, a third cyclic nucleotide, cGAMP (cyclic guanosine monophosphate–adenosine monophosphate) has been identified and shown to have a role in triggering the innate immune system upon sensing of DNA in the cytosol (Davies *et al.*, 2012; Wu *et al.*, 2013; Yoon *et al.*, 2007).

In prokaryotic cells, cAMP, cGMP and cGAMP can be found but with the exception of cAMP, their role is limited to a few regulatory pathways or not well understood. Instead, cyclic dinucleotides like cdAMP (cyclic dimeric adenosine monophosphate) and c-di-GMP (cyclic dimeric guanosine monophosphate) have emerged as bacterial second messengers that are used to regulate a wide variety of processes in a very specific manner. Interestingly, c-di-GMP has been described as a ubiquitous but not essential molecule in the bacterial world (Galperin, 2010b; Romling, 2012; Solano *et al.*, 2009). In Gram-negative bacteria, it commonly forms an intricate network that regulates a variety of biological functions, including those associated with the transition between a motile and a biofilm lifestyle (Mills *et al.*, 2011; Parsek & Aldridge, 2010). In contrast, cdAMP seems to be an essential but not so widespread signalling molecule. In Gram-positive bacteria, cdAMP has been implicated in sensing of DNA integrity, control of ion transport across the membrane and cell wall homeostasis (Corrigan *et al.*, 2011; Corrigan *et al.*, 2013; Corrigan & Grundling, 2013; Oppenheimer-Shaanan *et al.*, 2011). Besides cyclic nucleotides, bacteria can also use the linear nucleotides (p)ppGpp (guanosine pentaphosphate or tetraphosphate) to mediate their response to stress conditions (a process known as *stringent response*) (Kalia *et al.*, 2013).

In *P. aeruginosa*, two cyclic nucleotides have known regulatory functions: cAMP and c-di-GMP (Coggan & Wolfgang, 2012). State of the art knowledge on the signalling mechanisms of each molecule in *P. aeruginosa* is described hereafter.

1.3.1 Cyclic AMP signalling

Cyclic AMP was first characterized in eukaryotic cells as a signalling molecule in the adrenaline pathway by Earl Sutherland, who later became a Nobel Laureate for his findings on the mechanisms of action of hormones (Kresge *et al.*, 2005). This cyclic nucleotide was then found in bacteria where it is mainly associated with the *carbon catabolite control*. Briefly, this is the response that bacterial cells undergo to avoid the unnecessary use of energy when more than one carbon source is available in the surrounding medium (Rojo, 2010).

In *E. coli*, the carbon catabolite control is well characterized in relation to glucose, the preferred carbon source, and lactose, a secondary carbon source. In very simple terms, the presence of glucose in the medium is concomitant with a repression of the *lac* operon which encodes proteins required for lactose degradation. This repression is owed to the presence of a cAMP receptor protein (Crp) that has a cAMP-binding domain at the N-terminus and a DNA-binding domain at the C-terminus. When glucose is depleted from the medium, the production of cAMP is stimulated by the glucose transporter system in the membrane, leading to the formation of cAMP-Crp complexes that act as transcription activators of the *lac* operon (Botsford, 1981). In *P. aeruginosa*, however, organic acids such as succinate and amino acids are the preferred carbon sources and carbon catabolite control does not operate in a similar fashion. Instead, the CbrAB two-component system (Table 1.2) is a key player in carbon catabolite control, acting through the regulation of the availability of a RNA-binding protein, Crc, that inhibits translation of genes involved in the catabolism of nonpreferred nutrients (Rojo, 2010).

Nonetheless, *P. aeruginosa* is able to produce cAMP and this is known to play a role in virulence. Typically, cAMP is produced from ATP by adenylyl cyclases (ACs), degraded by phosphodiesterases (PDEs) and perceived by cAMP-binding proteins. *P. aeruginosa* harbours three ACs (CyaA, CyaB and ExoY), one PDE (CpdA) and two cAMP-binding proteins (Vfr and CbpA).

CyaA (PA5272) is a cytoplasmic AC with catalytic activity that has been implicated in glucose starvation induced biofilm dispersal through a yet unknown mechanism that requires cAMP synthesis (Huynh *et al.*, 2012). In the case of CyaB (PA3217), the protein has a recognizable N-terminus MASE domain and a C-terminus AC domain. *MASE domains* are membrane associated sensory domains that were bioinformatically identified and whose function is largely unexplored (Nikolskaya *et al.*, 2003). Very recently however, a MASE domain has been characterized for the first time. In a protein from *Salmonella enterica*, mutation of a KKE (lysine – lysine – glutamic acid) motif located in one of the cytoplasmic loops of the MASE domain rendered the protein unable to mediate

aspartate chemotaxis, a function that was also dependent on the redox status of the cell (Table 1.1) (Lacey *et al.*, 2013).

Like CyaA, the AC CyaB has also catalytic activity. Both under laboratory growth conditions and during infection of an acute murine model, CyaB seems to be the dominant AC (Smith *et al.*, 2004; Wolfgang *et al.*, 2003).

The two CyaA and CyaB have been shown to be involved in the regulation of a total of 181 genes and most of them (162 genes) were shown to be also regulated by the cAMP-binding protein Vfr (Wolfgang *et al.*, 2003). Vfr (PA0652) has 67% sequence identity to Crc in *E. coli* and upon binding of cAMP acts as a transcriptional activator of different virulence factors including T4P, T2SS and T3SS genes. In contrast, it can act as a transcriptional repressor of a subset of other targets (45 out of 206 genes), including the transcription regulator of the genes involved in flagellar biogenesis (Dasgupta *et al.*, 2002; Wolfgang *et al.*, 2003). Curiously, Vfr also affects positively the transcription of the quorum sensing related LasR protein but this occurs in a cAMP independent manner (Fuchs *et al.*, 2010a).

The cAMP catalytic action of CyaA and CyaB is counteracted by the action of the PDE CpdA. CpdA (PA4969) was shown to be able to degrade cAMP upon binding of an iron cofactor and transcription of *cpdA* is under positive control of Vfr (Fuchs *et al.*, 2010b). Hence, CpdA is thought to act in concert with CyaA and CyaB to adjust the bacterial intracellular cAMP levels and fine tune the expression of different virulence traits. Overall, the modulation of cAMP concentration is known to be dependent or correlated to host cell contact, calcium concentration in the medium, activity of the Pil-Chp system, production of alginate and expression of *fimL* (Fulcher *et al.*, 2010; Inclan *et al.*, 2011; Jones *et al.*, 2010; Wolfgang *et al.*, 2003).

Interestingly, the third AC of *P. aeruginosa* is ExoY, an effector protein that is secreted via the T3SS into eukaryotic host cells. Unlike CyaA or CyaB, ExoY (PA2191) does not synthesize cAMP in the bacterial cells. Instead, the protein acts as a toxin in the T3SS targeted cells where it becomes capable of producing cAMP (Yahr *et al.*, 1998). The increased levels of cAMP generated in the host cells are an oedema factor but the precise mechanisms of action underlying this phenomenum are not well understood yet (Hritonenko *et al.*, 2011; Ochoa *et al.*, 2012; Prasain *et al.*, 2009).

In addition to Vfr, a second cAMP-binding protein, CbpA (PA4704), is found in *P. aeruginosa* genome. This protein has two cAMP-binding domains: one degenerated at the N-terminus and one conserved and functional at the C-terminus. Unfortunately, not much is known about CbpA, except that it is under positive regulation of Vfr and that it localizes to the cell poles in a cAMP dependent manner

(Endoh & Engel, 2009). It is noteworthy that the proteins CyaB, FimL and PilJ (the latter belonging to the Pil-Chp system), also appear localised at the cell poles although in this case the localisation seems to be bipolar whereas for CbpA it is unipolar (DeLange *et al.*, 2007; Inclan *et al.*, 2011).

1.3.2 Cyclic di-GMP signalling

Cyclic di-GMP was first identified by Moshe Benziman when studying cellulose biogenesis in an innocuous Gram-negative bacterium called *Gluconacetobacter xylinus* (Amikam *et al.*, 2010; Ross *et al.*, 1987). Coincidently, both c-di-GMP and two-component systems were described for the first time in the decade of 1980 but unlike two-component systems, research on c-di-GMP was hampered for more than 15 years. Only in the last decade the understanding of c-di-GMP signalling took a leap and today, c-di-GMP is known as a ubiquitous bacterial second messenger that modulates a variety of biological processes that shape the bacterial lifestyle. In particular, the role of c-di-GMP in the regulation of the transition between a planktonic/motile and a biofilm/sessile lifestyle has become evident. At high levels of c-di-GMP, bacteria tend to adopt a biofilm lifestyle while at low levels of c-di-GMP bacteria are directed towards a motile lifestyle (Gjermansen *et al.*, 2006; Hengge, 2009; Jenal & Malone, 2006; Simm *et al.*, 2004).

The intracellular levels of c-di-GMP are known to be controlled by the biosynthetic activity of diguanylate cyclases (DGCs), which are enzymes that carry a GGDEF domain; and by the hydrolytic activity of PDEs that have an EAL or HD-GYP domain (Figure 1.10) (Schirmer & Jenal, 2009). Sometimes proteins have a GGDEF domain followed by an EAL domain and this domain is referred to as GGDEF/EAL domain. Cyclic di-GMP is synthesized from two molecules of GTP (guanosine triphosphate) and is degraded into pGpG (phosphoguanylyl guanosine) and/or GMP (guanosine monophosphate). Once in the cell, c-di-GMP can bind an array of different *c-di-GMP receptors* that act as effector proteins, translating the signal message into a molecular readout.

The GGDEF domain

The GGDEF domain is approximately 170 amino acids long and exhibits a series of conserved motifs including a GGDEF (glycine – glycine – aspartic acid – glutamic acid – phenylalanine) motif that folds into a β-hairpin (Figure 1.11) (Galperin, 2010b). This motif, or the variant GGEEF motif, harbours the DGC catalytic activity and constitutes half of the active site (*A-site*) as production of c-di-GMP requires the formation of dimers. Often, another motif, the RxxD (arginine – x – x – aspartic acid;

where *x* is any amino acid) motif, acts as an inhibition site (*I-site*) where c-di-GMP binds and prevents the encounter of the two halves of the A-site, therefore exerting a negative feedback on the DGC catalytic activity. Interestingly, the RxxD motif is found just five residues upstream of the GGDEF motif but in the three-dimensional structure it adopts an antipodal localization in relation to the GGDEF motif (Table 1.4) (Chan *et al.*, 2004; De *et al.*, 2008).

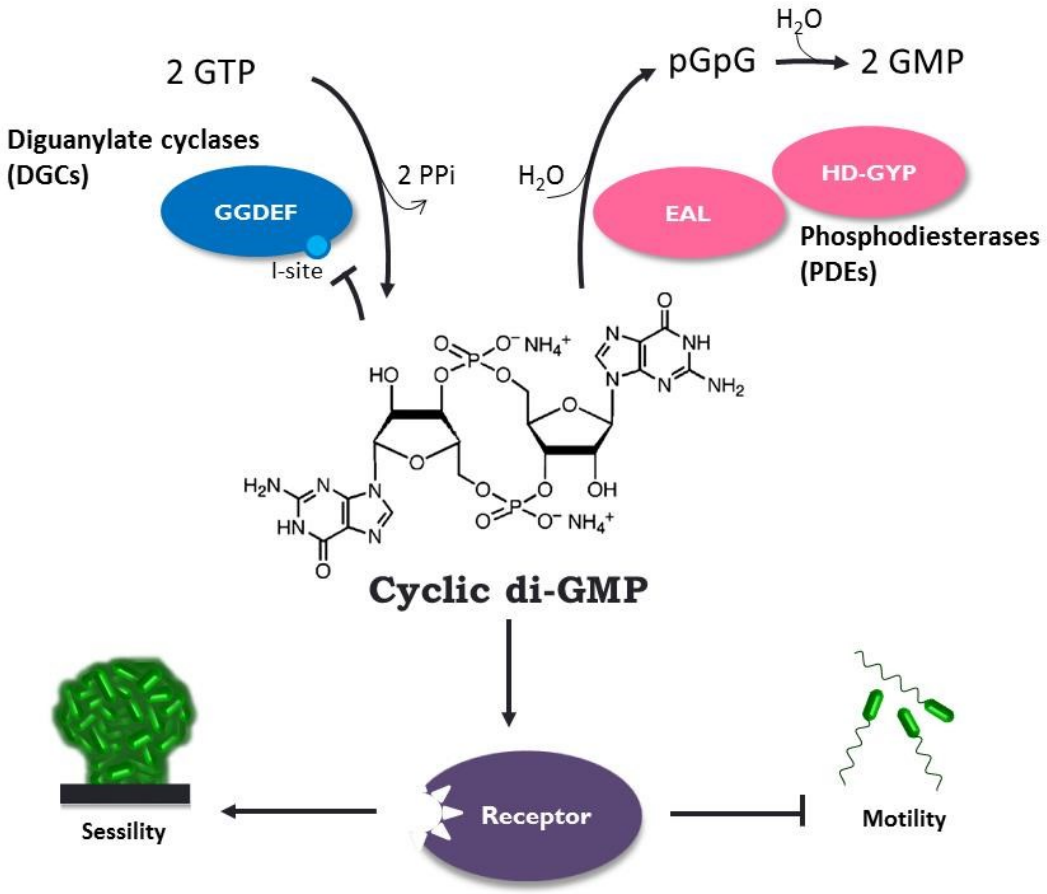

Figure 1.10 Cyclic di-GMP related proteins.

The second messenger is synthesized from two molecules of GTP by DGCs which have a GGDEF domain (in a reaction that releases two diphosphate) and degraded into pGpG or GMP by PDEs which have an EAL or HD-GYP domain. Often, the DGCs activity is inhibited by binding of c-di-GMP to the I-site. The I-site is an RxxD motif of the GGDEF domain that is located upstream of the GGDEF motif. Proteins that bind to c-di-GMP function downstream to transmit the signal and are referred to as c-di-GMP receptors. High levels of c-di-GMP are associated with a sessile lifestyle whereas low levels of c-di-GMP correlate to a motile lifestyle.

The EAL domain

The EAL domain is approximately 240 amino acids long and folds in a central β-barrel with flanking α-helices (Figure 1.11) (Galperin, 2010b). This domain is highly specific for c-di-GMP and its PDE activity requires the binding of a metal ion that is coordinated by five to ten conserved residues. In addition, EAL domains are able to form dimers and this seems to be structurally and biochemically relevant. Overall, the E residue of the EAL (glutamic acid – alanine – leucine) motif, or the variant EVL (glutamic acid – valine – leucine) motif, together with a few downstream residues and a conserved loop 6 (corresponding to β5-α5 loop), are known to be important for coordinating ion binding, dimerization and catalysis (Minasov *et al.*, 2009; Rao *et al.*, 2008; Rao *et al.*, 2009) (Table 1.4).

The HD-GYP domain

The HD-GYP domain is approximately 150 amino acids long and folds in seven α-helices (Figure 1.11). It is a c-di-GMP specialized hydrolase belonging to the widespread HD superfamily. Typically, the PDE activity is centred on the HD (histidine – aspartic acid) motif and dependent on binding to metal ions. The GYP (glycine – tyrosine – proline) motif is within a conserved region of residues located downstream of the HD motif. In the only three-dimensional structure of an HD-GYP domain available to date, at the same time that the GYP motif is positioned in close proximity to the A-site, it constitutes a surface exposed region (Table 1.4) (Lovering *et al.*, 2011). This suggests a role of the GYP motif on either assisting the specificity to c-di-GMP binding or mediating the interaction with other proteins.

Table 1.4 Structurally characterized proteins with a GGDEF, EAL or HD-GYP domain.

Protein	Species	Domains	Mode of action	References
GGDEF domain proteins				
PleD	*C. crescentus*	2xREC, GGDEF	Phosphorylation of the first REC domain promotes dimerization and DGC activity; binding of c-di-GMP to RxxD inhibits DGC activity.	Chan *et al.*, 2004; Paul *et al.*, 2007
WspR	*P. aeruginosa*	REC, GGDEF	Phosphorylation and oligomerization via the REC domain regulates DGC activity; binding of c-di-GMP to RxxD motif inhibits DGC activity.	De *et al.*, 2008; De *et al.*, 2009
XCC4471	*X. campestris*	TM[1], HAMP, GGDEF	Conserved GGDEF motif and degenerated RxxD motif; binding of 2 c-di-GMP molecules to the GGDEF motif inhibits its catalytic activity.	Yang *et al.*, 2011

Protein	Species	Domains	Mode of action	References
PelD	*P. aeruginosa*	TM, GAF, GGDEF	Degenerated GGDEF motif; c-di-GMP binds to the RxxD motif.	Li *et al.*, 2012
DgcZ	*E. coli*	CZB[2], GGDEF	Constitutively active dimer; binding of zinc to the CZB domain inhibits the DGC activity.	Zahringer *et al.*, 2013
EAL domain proteins				
YkuI	*B. subtilis*	EAL, PAS	c-di-GMP and one metal ion bind the EAL domain but catalytic PDE activity was not demonstrated.	Minasov *et al.*, 2009
BlrP1	*K. pneumoniae*	BLUF, EAL	Light and dimerization modulate PDE activity; EAL domain binds to c-di-GMP and to two catalytic metal ions.	Barends *et al.*, 2009
RocR	*P.aeruginosa*	REC, EAL	Phosphorylation and oligomerization modulate PDE activity; EAL domain binds to c-di-GMP and to one catalytic metal ion.	Chen *et al.*, 2012
GGDEF/EAL domain proteins				
TBD1265	*T. denitrificans*	PBPb, TM, GGDEF, EAL	Catalytic active PDE; the EAL domain forms dimers and binds to c-di-GMP and to two metal ions.	Tchigvintsev *et al.*, 2010
FimX	*P. aeruginosa* *X. axonopodis*	REC, PAS, GGDEF, EAL	Degenerated GGDEF and RxxD motifs; c-di-GMP binds to the catalytic inactive EAL domain. Forms dimers via N-terminal domains and interacts with c-di-GMP and other proteins via the EAL domain.	Guzzo *et al.*, 2013; Navarro *et al.*, 2009; Qi *et al.*, 2012
LapD	*P. fluorescens*	TM, HAMP, GGDEF, EAL	Degenerated GGDEF and RxxD motifs; forms dimers via the N-terminal domains; c-di-GMP binds to the catalytic inactive EAL domain and the protein adopts an active conformation.	Navarro *et al.*, 2011
DosP	*E. coli*	2xPAS, GGDEF, EAL	Catalytic active PDE; EAL domain binds c-di-GMP and two metal ions; presence of O_2, CO or NO sensed by the first PAS domain modulates PDE activity.	Tarnawski *et al.*, 2013
HD-GYP domain proteins				
Bd1817	*B. bacteriovorus*	NTD[3], HD-GYP	Catalytic PDE activity was not demonstrated; degenerated GYP motif.	Lovering *et al.*, 2011

[1]TM – transmembrane domain
[2]CZB – chemoreceptor zinc binding domain
[3]NTD – unknown N-terminal domain

The c-di-GMP receptors

The mechanisms by which c-di-GMP signalling translates into a molecular readout are various and only now they start to be understood. Reflecting the diversity of pathways where c-di-GMP takes part, the c-di-GMP receptors include (i) proteins with inactive GGDEF, EAL or HD-GYP domains, (ii) proteins with a PilZ domain, (iii) riboswitches and proteins involved in RNA processing and (iv) proteins that act as transcriptional factors (Ryan *et al.*, 2012b; Sondermann *et al.*, 2012). Interestingly, (v) c-di-GMP receptors in eukaryotic cells have also been identified (Krasteva *et al.*, 2012).

(i) Proteins with inactive GGDEF, EAL or HD-GYP domains

Sometimes, proteins with recognizable GGDEF, EAL or HD-GYP domains carry degenerated motifs that lose the catalytic function but maintain the ability to bind c-di-GMP. This is the case of the characterized PelD (c-di-GMP binds the conserved I-site), LapD (c-di-GMP binds to the EAL domain) and FimX (c-di-GMP binds to the EAL domain) proteins of *P. aeruginosa* which are described later.

(ii) Proteins with a PilZ domain

The PilZ domain is approximately 100 amino acids long and folds as six stranded β-barrels (Figure 1.11). Two conserved motifs have been implicated in c-di-GMP binding: the RxxxR (arginine – x – x – x – arginine; where *x* is any amino acid) motif and the DxSxxG (aspartic acid – x – serine – x – x – glycine; where *x* is any amino acid) motif (Amikam & Galperin, 2006). A well characterized PilZ domain protein from *P. aeruginosa* is Alg44 which is described later.

(iii) Riboswitches and proteins involved in RNA processing

Riboswitches are segments of RNA that work as receptors of small molecules in the 5' untranslated region of mRNAs. First in *Vibrio cholerae* and later in *Clostridium difficile*, it was shown that c-di-GMP can bind two different classes of riboswitches (class I and II) which exhibit a complex secondary and tertiary structure (Kulshina *et al.*, 2009; Lee *et al.*, 2010a; Smith & Strobel, 2011; Sudarsan *et al.*, 2008). Binding of c-di-GMP to these riboswitches was shown to exert a regulatory role upon the targeted mRNA, affecting transcription termination, translation or intron self-splicing (Hengge, 2010). No riboswitches capable of binding c-di-GMP have yet been found in *P. aeruginosa.*

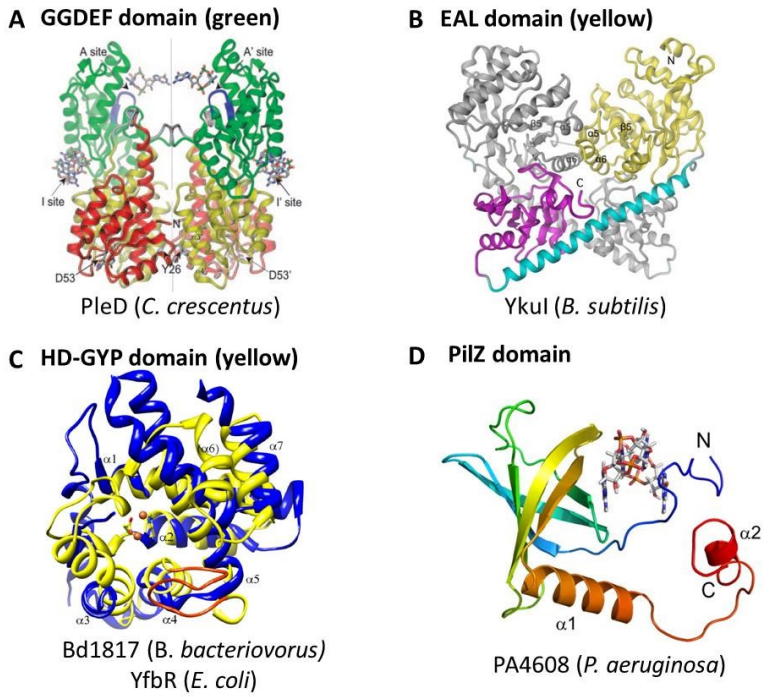

Figure 1.11 Three dimensional structure of c-di-GMP related proteins.

(A) Structure of a PleD homodimer. The two monomers form a two-fold dimer. The monomer consists of three domains: the first (residues 2–140, red) and second (residues 141–285, yellow) REC domains and the catalytic DGC domain (286–454, green). The first REC domain carries the phosphoacceptor D53. The GGEEF signature motif is located on the β-hairpin (blue) and constitutes part of the A-site to which a c-di-GMP molecule is bound. Two c-di-GMP molecules are found at the I-site and the figure represents a non-activated PleD dimer. The two chains interact via Y26 from the first REC domain. Figure retrieved from Chan et al. (2004). **(B)** Structure of a YkuI homodimer. The two monomers form a two-fold dimer. One chain is coloured and the other is grey. The monomer consists of two domains connected by a long helix (residues 246–289, blue). The N-terminal EAL domain is shown in yellow and the C-terminal PAS domain is shown in magenta. The interaction between the two EAL domains involves the loop between β5-α5 (loop 6) and the α6-helix. Figure retrieved from Minesov et al. (2009). **(C)** Superposition of the three dimensional structures of the HD-GYP fold with a representative of the wider HD family. Bd1817 is shown in yellow with the GYP motif in orange, the HD motif in stick form, and the active-site binuclear metal center in sphere form. The HD fold of YfbR from E. coli is shown in blue. All the α-helices of the HD-GYP fold have counterparts in the generalized HD fold, although α6 sits at a different angle. The GYP motif of Bd1817 replaces the short loop between α4 and α5 of HD proteins, with the 20 residues long loop of Bd1817 contrasting with the 5 residues long loop of YfbR. Figure retrieved from Lovering *et al.* (2011). **(D)** Structure of the PilZ domain protein PA4608. Cyclic di-GMP (in stick form) binds as an intercalated, symmetric dimer to one side of the β-barrel. Both termini of the protein undergo structural changes upon ligand binding. In particular, the C-terminal helix is displaced and the N-terminus containing the RxxxR motif wraps around the ligand. Figure retrieved from Habazettl et al. (2011).

In addition to the riboswitches, very recently a protein from *E. coli* involved in the degradation of RNA, PNPase (polynucleotide phosphorylase), has been shown to bind c-di-GMP, widening even more the range of c-di-GMP receptor proteins. In this case, c-di-GMP binding leads to the activation of PNPase in order to mediate RNA processing. This signaling pathway in *E. coli* is actually quite interesting as it seems to respond to the presence of oxygen and involves two other c-di-GMP related proteins (DosC, with a C-terminus active GGDEF domain; and DosP, with C-terminus active EAL domain, see Table 1.4) (Tuckerman *et al.*, 2011). An homologuous PNPase protein from *Xanthomonas campestris* has been shown to also bind c-di-GMP, indicating that this may be occurring in other bacterial species (Wang *et al.*, 2012).

(iv) Transcriptional factors

Transcriptional factors are proteins that control transcription of a gene by binding to their promoter region in response to signals around them. They are often abundant (*P. aeruginosa* has more than 500) and can act as repressors, activators or both (Galan-Vasquez *et al.*, 2011; Stover *et al.*, 2000). The function of transcriptional factors can be modulated in different ways: covalent modifications (like the phosphorylation of response regulators with DNA binding output domains), levels of the transcriptional factor (controlled by auto-regulation of their own expression or by proteolysis), competition for binding to other molecules (for example, titration of the transcriptional factor by binding to a regulatory protein) and binding to small ligands (like cAMP binding to Crp in the carbon catabolite control of *E. coli*) (Browning & Busby, 2004).

In a number of bacterial species – namely *P. aeruginosa* (FleQ), *V. cholerae* (VpsT), *B. cenocepacia* (Bcam1349), *X. campestris* (Clp) and *Klebsiella pneumonia* (MrkH) –, c-di-GMP has been shown to bind to transcriptional factors and modulate their activity. In *P. aeruginosa*, the mechanism of action of such transcriptional factor, FleQ, is described later.

(v) Eukaryotic c-di-GMP receptors

Despite the fact that eukaryotic cells are not recognized as being able to produce c-di-GMP, recently, two c-di-GMP receptors were identified in mammalian cells. One of them is DDX41, a cytosolic protein with two domains: a DEAD (aspartic acid – glutamic acid – alanine – aspartic acid) domain that binds DNA and cyclic nucleotides; and a helicase domain that hydrolysis ATP (Parvatiyar *et al.*, 2012). The other c-di-GMP receptor is STING, a membrane protein that locates to the endoplasmic reticulum and senses both DNA and cyclic nucleotides in the cytosol (Burdette *et al.*, 2011). DDX41

has a greater affinity for c-di-GMP than STING and is thought to act upstream of STING, sensing the presence of foreign molecules and, together with STING, eliciting the innate immune system with a type I interferon response (Bowie, 2012).

Because c-di-GMP stimulates the immune system, its use as a therapeutic molecule and vaccine adjuvant has been explored and is proving very promising. Several early studies have recognized the potential of c-di-GMP as an anticancer drug, as an immunomodulator of the burden caused by bacterial infections and as a vaccine adjuvant (Brouillette *et al.*, 2005; Chen *et al.*, 2010; Karaolis *et al.*, 2005; Karaolis *et al.*, 2007).

1.4 Proteins involved in cyclic di-GMP signalling in *P. aeruginosa*

Cyclic di-GMP signalling is widespread in bacteria and many species encode a large number of c-di-GMP related proteins – a few proteins with PilZ domains or HD-GYP domains, and many proteins with a GGDEF or EAL domain (2010; Galperin, 2010b). Usually, the domains involved in c-di-GMP metabolism (i.e., GGDEF, EAL and HD-GYP domains) are located at the C-terminus of the proteins and N-terminal transmembrane regions or input domains are present. All together, these proteins create an intricate c-di-GMP signaling network that specifically regulates many biological processes crucial for bacterial adaptation and virulence. Not surprisingly, *P. aeruginosa* encodes a vast array of c-di-GMP related proteins, summing up a total of 51 proteins: eighteen with a GGDEF domain, six with an EAL domain, sixteen with a GGDEF/EAL domain, three with an HD-GYP domain and eight with a PilZ domain (Kulasakara *et al.*, 2006; Merighi & Lory, 2010). A list of all these proteins is available in Table 1.5 and a description of the ones that have been characterized follows. Curiously, and as a mirror of the speed at which research in this field has progressed in the last years, when this work was initiated in 2009 only 9 out of the 51 proteins had been characterized. Today, publications on at least 20 of these proteins can be found.

Table 1.5 Proteins with a GGDEF, EAL, HD-GYP or PilZ domain in *P. aeruginosa*.

ORF (name)	Domain organization[1]	Additional Domains	Activity	References
GGDEF domain				
PA0169 (*siaD*)		--	DGC[2]	Irie *et al.*, 2012; Klebensberger *et al.*, 2009
PA0290		PAS	--	--
PA0338		PAS	--	--
PA0847		CHASE4, HAMP, PAS	DGC	Kulasakara *et al.*, 2006
PA1107 (*roeA*)		TM[2]	DGC	Merritt *et al.*, 2010
PA1120 (*yfiN*)		HAMP	DGC	Malone *et al.*, 2010; Malone *et al.*, 2012; Pu & Wood, 2010; Ueda & Wood, 2009; Ueda & Wood, 2010
PA1851		TM	--	--
PA2771		GAF	--	--
PA2870		TM	DGC	Kulasakara *et al.*, 2006
PA3061 (*pelD*)		TM, GAF	c-di-GMP receptor	Lee *et al.*, 2007; Li *et al.*, 2012; Whitney *et al.*, 2012
PA3177		--	DGC	J. Overhage, personal communication
PA3343		TM	DGC	Kulasakara *et al.*, 2006
PA3702 (*wspR*)		REC	DGC	De *et al.*, 2008; De *et al.*, 2009; Guvener & Harwood, 2007; Hickman *et al.*, 2005; Huangyutitham *et al.*, 2013; Malone *et al.*, 2007
PA4332 (*sadC*)		TM	DGC	Irie *et al.*, 2012; Kuchma *et al.*, 2010; Kuchma *et al.*, 2012; Merritt *et al.*, 2007;

ORF (name)	Domain organization[1]	Additional Domains	Activity	References
				Merritt *et al.*, 2010
PA4396		REC	--	--
PA4843 (*adcA*)		REC	DGC	C. J. Jones, personal communication
PA4929		7TMR_DISM	--	--
PA5487		--	DGC	Kulasakara *et al.*, 2006
GGEDF/EAL domain				
PA0285		TM, PAS	--	--
PA0575		PBPb, PAS	--	--
PA0861 (*rbdA*)		TM, PAS	PDE	An *et al.*, 2010
PA1181		MASE, PAS	--	--
PA1433 (*lapD*)		HAMP	c-di-GMP receptor[4]	Hinsa & O'Toole, 2006; Navarro *et al.*, 2011; Newell *et al.*, 2009; Newell *et al.*, 2011
PA1727 (*mucR*)		MHYT	DGC, PDE	Hay *et al.*, 2009; Li *et al.*, 2013
PA2072		Chase4, PAS	PDE	This study
PA2567		GAF	PDE	Rao *et al.*, 2009
PA3258		CBS	--	--
PA3311 (*nbdA*)		MHYT	PDE	Li *et al.*, 2013
PA4367 (*bifA*)		TM	PDE	Kuchma *et al.*, 2007
PA4601 (*morA*)		TM, PAS	--	Choy *et al.*, 2004
PA4959 (*fimX*)		PAS	c-di-GMP binding	Huang *et al.*, 2003; Jain *et al.*, 2012; Kazmierczak *et al.*, 2006; Navarro *et al.*, 2009; Qi *et al.*, 2012
PA5017		PAS, GAF	DGC	Roy *et al.*, 2012

ORF (name)	Domain organization[1]	Additional Domains	Activity	References
(*dipA*)				
PA5295		--	--	--
PA5442		TM, PAS	--	--
EAL domain				
PA0707 (*toxR*)		--	--	Walker *et al.*, 1994; Wozniak *et al.*, 1987
PA2133		--	PDE	Kulasakara *et al.*, 2006
PA2200		TM	PDE	Kulasakara *et al.*, 2006
PA2818 (*arr*)		TM	PDE	Gotoh *et al.*, 2008; Hoffman *et al.*, 2005
PA3825		--	PDE	Kulasakara *et al.*, 2006
PA3947 (*rocR*)		REC	PDE	Chen *et al.*, 2012; Kotaka *et al.*, 2009; Rao *et al.*, 2008; Rao *et al.*, 2009
HD-GYP domain				
PA2572		REC	--	Ryan *et al.*, 2009
PA4108		--	PDE	Ryan *et al.*, 2009
PA4781		REC	PDE	Ryan *et al.*, 2009
PilZ domain				
PA0012		--	c-di-GMP receptor	Merighi *et al.*, 2007
PA2799		--	c-di-GMP receptor	Merighi *et al.*, 2007
PA2960 (*pilZ*)		--	--	Merighi *et al.*, 2007
PA2989		--	c-di-GMP receptor	Merighi *et al.*, 2007
PA3353		--	c-di-GMP receptor	Merighi *et al.*, 2007
PA3542 (*alg44*)		TM	c-di-GMP receptor	Merighi *et al.*, 2007; Oglesby *et al.*, 2008

ORF (name)	Domain organization[1]	Additional Domains	Activity	References
PA4324		--	c-di-GMP receptor	Merighi *et al.*, 2007
PA4608		--	c-di-GMP receptor	Habazettl *et al.*, 2011; Shin *et al.*, 2011

[1] Blue boxes represent the GGDEF domain, pink boxes represent the EAL or HD-GYP domain, and purple boxes represent the PilZ domain. Grey shapes represent the additional domains as presented in the next column.
[2] Activity not confirmed biochemically.
[3] TM – transmembrane domain
[4] Based on the homologous protein found in *P. fluorescens.*

1.4.1 Proteins with DGC activity

WspR, a role in biofilm formation

WspR (PA3702) is a cytoplasmic protein with a REC domain at the N-terminus and a GGDEF domain with conserved I- and A-sites at the C-terminus. It is the response regulator of the chemotactic-like Wsp system and plays a role in biofilm formation.

Among all the DGC studied so far, the structure and function of WspR is one of the most well understood. Although the first reference to WspR in *P. aeruginosa* comes from D'Argenio *et al.* (2002) and links this protein to a CupA fimbriae dependent aggregative phenotype, subsequent studies come mainly from the groups of Caroline Harwood and Holger Sondermann and link WspR to biofilm formation and the production of Pel and Psl exopolysaccharide (Hickman *et al.*, 2005).

Several structural and microscopy studies of WspR have shown that the regulation of the DGC activity occurs by three different means: phosphorylation, feedback inhibition and cluster formation (Figure 1.9). Firstly, WspR forms homodimers via the interaction of the REC domain and DGC activity is stimulated by the formation of tetramers and phosphorylation of the REC domains (De *et al.*, 2008; De *et al.*, 2009; Malone *et al.*, 2007). Secondly, feedback inhibition of the DGC activity was shown to occur by binding of c-di-GMP to the I-sites of the tetramers, locking the complex in a conformation that prevents the encounter of the A-sites (De *et al.*, 2008; De *et al.*, 2009). Finally, the DGC activity is amplified by the formation of phosphorylated WspR subcellular clusters that are dynamic and locate to the periphery of the cells (Guvener & Harwood, 2007; Huangyutitham *et al.*, 2013).

Although there is a good insight on the structure and mode of action of WspR and this represents a great contribution to the field, the knowledge about the molecular pathway that operates downstream of WspR and leads to biofilm formation is very limited.

SadC, a role in swarming motility

SadC (PA4332) is a membrane associated DGC with a cytoplasmic GGDEF domain at the C-terminus that exhibits conserved I- and A-sites. Similar to WspR, SadC also forms subcellular clusters and this is indicative of the importance of oligomerization for its DGC catalytic activity (Merritt *et al.*, 2010).

In a screen of transposon mutants with a hypobiofilm phenotype, *sadC* was hit and shown to be arrested at the irreversible attachment stage (Merritt *et al.*, 2007). Further analyses showed that this phenotype was concomitant to a hyperswarming phenotype, verifying the overall concept about c-di-GMP regulation: high levels of c-di-GMP promote biofilm formation and low levels promote motility. Two other proteins are known to be in the same pathway as SadC. One of them is SadB (PA5346) which is required downstream of SadC in the induction of biofilm formation and repression of swarming motility. The other protein is BifA (PA4367) which is a PDE and antagonizes SadC (see section 1.4.2).

In addition to this, several components of the T4P (namely, PilY1, PilX and PilW) have been shown to repress swarming motility in a SadC dependent manner, and the same is true for biofilm induction through the production of Psl (Irie *et al.*, 2012; Kuchma *et al.*, 2010; Kuchma *et al.*, 2012).

RoeA, a role in exopolysaccharide production

RoeA (PA1107) has been shown to harbour DGC activity via its GGDEF domain (conserved I- and A-sites) and is predicted to have N-terminal transmembrane domains (Kulasakara *et al.*, 2006).

The characterization of RoeA came from a comparative study of the effects caused by deletion of *roeA* and deletion of *sadC* (Merritt *et al.*, 2010). Deletion of both proteins individually results in a hypobiofilm phenotype and simultaneous deletion has an additive effect. The same is true in relation to c-di-GMP: individual deletions cause an approximate 50% reduction in the levels of c-di-GMP when compared to the wild-type strain (PA14) but simultaneous deletion of both genes causes a greater reduction. However, the two mutants exhibit different phenotypes in relation to two features: swarming motility and Pel exopolysaccharide. A *sadC* mutant had a more pronounced effect on swarming motility whereas a *roeA* mutant was more affected for exopolysaccharide

production. The exact mechanism by which RoeA impacts Pel production is not known, although an attempt at elucidating this indicated that it is not at the transcriptional level.

Overall, this work from George O'Toole's laboratory is supportive of a signalling model where localized pools of c-di-GMP are formed to coordinate and execute their regulatory roles.

SiaD, a role in detergent stress aggregation

SiaD (PA0169) is encoded in the *sia* operon (PA0169-72) and consists of just a GGDEF domain. This protein was identified after a transposon screen for mutants that lost the ability to auto-aggregate in the presence of SDS stress. Two operons were hit, the *sia* operon (hit one time in SiaD, PA0172) and the *cupA* frimbriae operon (hit five times in the gene encoding the fimbrial unit CupA1, PA2128, or the usher protein CupA3, PA2130). This study suggested that production of CupA fimbriae is required for the SDS aggregative phenotype and that the products of the *sia* operon are involved in its regulation – in fact, it is shown that the SDS-induced up-regulation of *cupA* is dependent on functional SiaD and SiaA (PA0172) proteins (Klebensberger *et al.*, 2009). In accordance to this, a previous study that looked at transposon insertions leading to a CupA fimbriae dependent aggregative phenotype mapped to *siaB* (PA0171) and suggested that SiaB may act as an antagonist of SiaA (D'Argenio *et al.*, 2002).

Unfortunately, direct evidence of the ability of SiaD to produce c-di-GMP remains to be provided. However, a close look at its GGDEF domain shows that the protein harbours conserved I- and A-sites. Moreover, in the PA14 transposon collection screened by Kulasekara *et al.*, an insertion in *siaD* exhibited a hypobiofilm phenotype indicating a putative role in c-di-GMP synthesis (Kulasakara *et al.*, 2006). In addition to the work carried out by the group of Bodo Philipp, *siaD* was found to be up-regulated in the hyperbiofilm *wspF* deletion mutant, together with the up-regulation of the *pel* and *psl* exopolysaccharide operons (Hickman *et al.*, 2005). Interestingly, the SDS aggregative phenotype had previously been identified as dependent on Psl production and Psl, in turn, has been reported to act as a signal to SiaD to mediate c-di-GMP production (Irie *et al.*, 2012; Klebensberger *et al.*, 2007).

YfiN or TpbB, a role in small colony variant formation

YfiN (PA1120) is a membrane protein with a periplasmic PAS domain and C-terminal cytoplasmic HAMP and GGDEF (conserved I- and A-sites) domains. Its catalytic function is thought to be dependent on the dimerization of the cytoplasmic portion of the protein via the HAMP domain. Interestingly, *yfiN* belongs to the *yfiBNR* operon and is regulated by its neighbours: a small

periplasmic protein named YfiR (PA1121); and an outer membrane lipoprotein YfiB (PA1119) (Malone *et al.*, 2010).

The YfiR periplasmic protein is a constitutive repressor of YfiN that interacts with the YfiN PAS domain and inhibits c-di-GMP production, resulting in the prevention of the appearance of small colony variants. On the other hand, YfiB is an antagonist of YfiR that probably acts as both a sensor and sequester of YfiR to the outer membrane, releasing YfiN from its inhibition and inducing small colony variants (Malone *et al.*, 2012). The signal at which the Yfi system responds is not known but the system plays a role in the response to high salt concentrations and SDS stress. Transcriptional analysis of putative downstream targets of YfiN revealed that the *pel* exopolysaccharide operon is strongly up-regulated in a Δ*yfiR* mutant with the *psl* exopolysaccharide and *cupB* fimbriae genes (but not *cupA* or *cupC*) being only mildly affected (Malone *et al.*, 2010). Interestingly, both gain- and loss-of function mutations in the Yfi system were identified in small colony variants isolated from cystic fibrosis patients, highlighting the dynamic environment in the lung and the involvement of c-di-GMP signalling in disease progression.

As suggested by the double nomenclature, YfiN was characterized by two different research groups at the same time: Urs Jenal's group was working in the PAO1 background strain and named it YfiN; and Thomas Wood's group was working with PA14 and named it TpbB. Nevertheless, the signalling pathway identified by the latter was different to the one described above. In this case, YfiN (TpbB) was found to be under the negative control of a different periplasmic protein called TpbA (PA3885). This protein was then shown to be under the regulation of quorum sensing and to act as a phosphatase (Ueda & Wood, 2009). Intriguingly, the phosphatase activity of TpbA is towards the unusual STY phosphorylation which was observed in YfiN (Pu & Wood, 2010). Therefore, a mechanism whereby two different periplasmic proteins act to negatively control the DGC activity of YfiN (via TpbA dephosphorylation of YfiN or direct binding of YfiR to YfiN) seems to operate and culminates in the regulation of biological functions that prevention the formation of small colony variant. Whether these two pathways operate simultaneously, act synergistically or are active at different stages or upon different stimulation is not currently known.

Finally, YfiN was also identified, together with MorA (see section 1.4.5), in an identical study about small colony variants. This time, the phenotype was highly dependent on the expression of *cupA* fimbriae and deletion of *yfiN* or *morA* was accountable for the loss of phenotype (Meissner *et al.*, 2007). Nonetheless, the molecular details of this regulation need further validation and investigation. The same is true for a more recent study that establishes an inverse link between

extracellular DNA and c-di-GMP. In this study, the TpbA repressed YfiN was suggested to contribute to the pool of extracellular DNA in a biofilm via the induction of cell lysis (Ueda & Wood, 2010).

1.4.2 Proteins with PDE activity

RocR or SadR, a role in Cup fimbriae

RocR (PA3947) has an N-terminal REC domain and a C-terminal EAL domain which exhibits PDE activity (Rao *et al.*, 2008). It is encoded next to RocS1 (PA3946), an unorthodox sensor with a REC and Hpt domain after the H kinase domain, and RocA1 (PA3948), a response regulator with a C-terminus DNA binding domain.

The RocR response regulator was identified simultaneously by the groups of George O'Toole and Stephen Lory and therefore it can also be found referred to as SadR. Structural insights of the protein mode of action revealed that it forms a tetramer where only two A-sites seem to be available for c-di-GMP hydrolysis and that phosphorylation of the REC domain modulates PDE activity (Chen *et al.*, 2012). In terms of function, detailed molecular mechanisms are largely not understood, but it has been reported that RocR counteracts RocA1 positive effect on *cupC* fimbriae transcription (Kulasekara *et al.*, 2005). In a different study, RocR was shown to have a role in the repression of T3SS under a biofilm mode of growth (Kuchma *et al.*, 2005).

Interestingly, the involvement of PDEs in Cup fimbriae regulation seems to be broad. First of all, at the same time that RocS1 leads to transcriptional activation of *cupC* fimbriae via RocA1, it also leads to the up-regulation of *cupB* fimbriae expression via a yet unidentified protein, and to the repression of efflux pump genes involved in antibiotic resistance via the response regulator RocA2 (PA3045) (Sivaneson *et al.*, 2011). Secondly, although *cupA* expression is not altered by the Roc system, the catalytic active PA2133 protein with an EAL domain is encoded in the *cupA* operon. Thirdly, the CupD fimbriae produced by *P. aeruginosa* PA14 strain are under the regulation of two two-component systems (Rcs and Pvr, Table 1.2) and comprise a response regulator, PvrR, that has a C-terminal EAL domain with PDE activity and is associated with the conversion between antibiotic resistant/susceptible phenotypic variants (Drenkard & Ausubel, 2002; Mikkelsen *et al.*, 2011; Mikkelsen *et al.*, 2013).

BifA, a PDE that works with SadC and RoeA

BifA (PA4367) is a membrane protein with a C-terminal GGDEF (no conserved I- or A-sites) and EAL domain. It has been shown to have PDE activity and this is dependent on both domains (probably because the GGDEF domain is required for GTP binding and consequent stimulation of PDE activity, as it has been described for similar PDEs) (An *et al.*, 2010; Christen *et al.*, 2005; Kuchma *et al.*, 2007).

In contrast to a *sadC* or a *roeA* mutant which have hypobiofilm and hyperswarming phenotypes, a transposon insertion in *bifA* was identified for its ability to form hyperbiofilms and be a hyposwarmer (Kuchma *et al.*, 2007; Merritt *et al.*, 2007; Merritt *et al.*, 2010). Interestingly, when a *sadC* deletion is created in a *bifA* mutant, there is a reduction in the c-di-GMP levels compared to the wild-type and the hyperswarming phenotype is restored. When, in turn, a *roeA* deletion is inserted in Δ*bifA*, the same level of reduction in c-di-GMP is observed but it is the Pel exopolysaccharide phenotype that is restored and not the swarming phenotyoe. Finally, if the three genes are deleted at the same time, the strain has c-di-GMP levels close to the wild-type and shows a hypobiofilm/hyperswarming phenotype, indicating that BifA may function to specifically counteract the effect of SadC and RoeA.

Suppressors of a *bifA* mutant phenotype have been identified in *sadB* and *pilY1.* As mentioned before, SadB seems to be a downstream target of SadC, thus reinforcing the notion that BifA and SadC are in the same regulatory pathway. The latter, PilY1 (PA4554) is a protein related to T4P and is now linked to the inverse regulation of swarming and biofilm phenotypes via the SadC/RoeA/BifA pathway. Overexpression of PilY1 inhibits swarming motility in a way that is dependent on the SadC DGC activity, and the same seems to be true for the T4P minor pilins PilX and PilW (Kuchma *et al.*, 2010; Kuchma *et al.*, 2012). Together, PilY1, PilX and PilW seem to inversely coordinate T4P-mediated twitching motility and flagella-driven swarming motility. At the same time that they are required for normal twitching motility, they seem to act as a communication pathway that integrates signals associated with growth on surfaces and drives the repression of swarming motility, leading to irreversible attachment and allowing the progression of the biofilm developmental cycle.

NbdA, RbdA and DipA, a role in biofilm dispersal

NbdA (nitric oxide induced biofilm dispersal)

NbdA (PA3311) is a membrane protein with an N-terminal MHYT domain and C-terminal GGDEF (conserved I-site) and EAL domains.

In a very recent study, NbdA was shown to be specifically required for nitric oxide induced biofilm dispersal, a phenotypic behaviour that requires a decrease in c-di-GMP levels (Li *et al.*, 2013). Biochemical analysis showed that the protein carried PDE activity but had no detectable DGC activity. Furthermore, the presence of the GGDEF domain was required to stimulate the PDE activity of the protein by binding to GTP (Li *et al.*, 2013). Successful attempts in understanding how nitric oxide acts as a signal, i.e., transcriptionally, posttranscriptionally or posttranslationally, showed that addition of nitric oxide increases *nbdA* mRNA levels. It remains to be established however, if the MHYT domain plays a role in sensing nitric oxide to activate the PDE activity.

RbdA (regulator of biofilm dispersal)

RbdA (PA0861) is thought to be a membrane protein and has an N-terminal PAS domain and C-terminal GGDEF (conserved I- and A-sites) and EAL domains.

Similar to other PDEs that have a GGDEF domain, this domain is required to stimulate the PDE activity of RbdA by binding to GTP. In addition, critical residues within the PAS domain, thought to be involved in sensing oxygen, were also shown to be required for the function of RbdA (An *et al.*, 2010). A *rdbA* deletion mutant was shown to be unable to disperse like the wild-type strain, suggesting that this is a PDE that lowers the levels of c-di-GMP in biofilms so that biofilm dispersal is initiated.

RbdA is predicted to be encoded in an operon together with PA0860 and PA0862. However, this is missing experimental validation and there is currently no data supporting a relationship of RbdA with its neighbours.

DipA (dispersal induced phosphodiesterase)

DipA (PA5017) is a membrane associated PDE and has GAF and PAS domains at the N-terminus and GGDEF (no conserved I- or A-sites) and EAL domains at the C-terminus. *GAF domains* were named

after some of the proteins where it is found and have been shown to serve as an interface for protein interaction or binding to ligands (Aravind & Ponting, 1997; Cann, 2007; Heikaus *et al.*, 2009).

A first study on DipA suggested that this protein is required for flagella-driven motility and normal biofilm development (Li *et al.*, 2007). In agreement with this, DipA has recently been shown to be required for biofilm dispersal upon induction with nutrients, nitric oxide or inorganic compounds like ammonium chloride (Roy *et al.*, 2012). Overall, the expression of DipA was maximum in dispersed cells and its PDE activity was shown to contribute for the regulation of the c-di-GMP pool in planktonic cells rather than in biofilms. In addition, the PDE activity of the protein was found to be modulated by the GAF domain in such a way that it exerts an inhibitory effect that is released in the presence of cAMP (Roy *et al.*, 2012). This finding is very important because it suggests that DipA might serve as a component of a regulatory cascade where the cAMP and c-di-GMP signalling networks intersect, allowing the cell to inversely coordinate the levels of the two cyclic nucleotides.

Arr, a role in drug induced biofilm formation

Arr (PA2818) is a membrane associated PDE with a cytoplasmic C-terminal EAL domain. Curiously, while GTP is known to stimulate PDE activity when a GGDEF domain is present, in PDEs like Arr that lack a GGDEF domain, GTP seems to have an inhibitory role (Hoffman *et al.*, 2005; Ross *et al.*, 1990).

In two independent studies, Arr was shown to be involved in the biofilm formation response triggered by the presence of subinhibitory concentrations of different drugs. In one case, the drug used was an aminoglycoside antibiotic of clinical relevance in the treatment of cystic fibrosis patients, tobramycin, which works by inhibiting translation (Hoffman *et al.*, 2005). In the other case, two drugs that inhibit DNA replication, the antibiotic nalidixic acid and hydroxyrurea, were used (Gotoh *et al.*, 2008). In both cases, it was shown that induction of biofilm formation was dependent on the PDE activity of Arr. Since low levels of c-di-GMP are commonly known to induce a motile lifestyle and not the formation of biofilms, the role of Arr is an exception to the commonly accepted concept of c-di-GMP regulation. However, one should consider the following issues. Firstly, upon the addition of drugs, even if at very low concentrations, the bacterial cells are subjected to high selective pressure. Secondly, induction of the PDE activity of Arr results in the reduction of the already low levels of c-di-GMP of a classic wild-type strain (the levels of free c-di-GMP in *P. aeruginosa* does not reach 1 picomole per mg of cells (Simm *et al.*, 2009)), bringing it to a new low where the function of c-di-GMP signalling might be insignificant. Since the c-di-GMP network is not an essential bacterial trait, it is possible that under the conditions tested, Arr PDE activity suppresses the c-di-GMP signalling which then leads to an improved fitness.

PA4108 and PA4781, two HD-GYP that are catalytic active

PA4108 has only a recognizable HD-GYP domain and PA4781 has an N-terminal REC domain and a C-terminal HD-GYP domain.

Although deletion of each of these genes caused a similar increase in the c-di-GMP intracellular levels and a similar repression of twitching and swarming motility, they had different effects on other phenotypes (Ryan *et al.*, 2009). For instance, whereas a PA4108 mutant reduced pyocyanin production, a PA4781 mutant increased pyoverdine production. Also, the three dimensional structure of the biofilms was different and while a PA4108 mutant affected T3SS, a mutant in PA4781 had no effect.

Curiously, PA4781 and its neighbouring gene, PA4782, were identified in a microarray study to be positively regulated by magnesium via the two-component system PmrAB (Table 1.2) (McPhee *et al.*, 2006).

1.4.3 Protein with DGC and PDE activity

MucR, a role in alginate biogenesis and biofilm dispersal

MucR (PA1727) has a modular organization similar to NbdA, harbouring an N-terminal MHYT domain and C-terminal GGDEF (conserved I- and A-sites) and EAL domains. Curiously, MucR and NbdA are the only proteins found in *P. aeruginosa* that harbour a MHYT domain.

Initially described as an exclusive DGC, this protein has recently been shown to actually carry both DGC and PDE activities (Hay *et al.*, 2009; Kulasakara *et al.*, 2006; Li *et al.*, 2013). These two activities are thought to be activated *in vivo* at different stages so that in planktonic cells MucR works as a DGC and in biofilms, as a PDE. In accordance to this, MucR has been described as a positive regulator of exopolysaccharide alginate biogenesis in mucoid strains (correlating to DGC activity), and as a positive regulator of biofilm dispersal induced by nitric oxide or glutamate (correlating to PDE activity). Interestingly, in *in vitro* conditions, the presence of the EAL domain accelerated substantially the rate at which the GGDEF domain was able to convert GTP to c-di-GMP but the physiological relevance of this or the mechanisms of positive feedback is unknown (Li *et al.*, 2013).

1.4.4 Proteins that bind c-di-GMP

PelD, a role in Pel exopolysaccharide production

PelD (PA3061) is a membrane protein with a cytoplasmic GAF domain followed by a GGDEF domain (conserved I-site). It does not have DGC catalytic activity but it has been shown to be able to bind c-di-GMP via the I-site (Lee *et al.*, 2007; Li *et al.*, 2012; Whitney *et al.*, 2012).

The fact that the *pel* operon encodes a protein that is able to bind c-di-GMP and is required for the production of the Pel exopolysaccharide means that this biological trait is regulated by c-di-GMP at least at two different levels: transcriptional and posttranslational. Transcriptionally, it has been demonstrated that the Wsp system, for instance, increases the mRNA levels of the *pel* genes. Posttranslationally, it is known that the PelD protein binds c-di-GMP. Unfortunately, mechanistic details about both levels of regulation are missing.

FimX, a role inT4P-mediated twitching motility

FimX (PA4959) is a cytoplasmic protein with N-terminal REC and PAS domains and C-terminal GGDEF (no conserved I- or A-sites) and EAL domains. Notably, the REC domain lacks important conserved residues and is likely not able to accept phosphoryl groups.

Although FimX exhibits low PDE activity *in vitro*, *in vivo* this protein seems to work as a c-di-GMP binding protein that is required for surface assembly of T4P and hence, for twitching motility (Huang *et al.*, 2003; Kazmierczak *et al.*, 2006; Navarro *et al.*, 2009; Rao *et al.*, 2008; Robert-Paganin *et al.*, 2012). The exact signals at which FimX responds are not known but a *fimX* mutant was shown to not respond to two out of three twitiching motility inducers, namely tryptone and mucin (Huang *et al.*, 2003).

The c-di-GMP binding ability of FimX resides in the EAL domain but both GGDEF and EAL domains are required for the twitching phenotype. On the other hand, the REC domain seems to be required for protein dimerization and unipolar localization – FimX is seen to localize to the cell pole where pili are assembled (Kazmierczak *et al.*, 2006; Navarro *et al.*, 2009; Qi *et al.*, 2011). Interestingly, *fimX* expression is known to be positively controlled by the transcriptional regulator Vfr but the requirement of FimX in T4P assembly is bypassed at high levels of intracellular c-di-GMP (Wolfgang *et al.*, 2003). This last observation was made after a screen for *fimX* suppressors that restored T4P assembly (Jain *et al.*, 2012). The hits located to three genes belonging to operons that lead to c-di-GMP production, the *sia*, *yfi* and *wsp* operons. All together, this indicates that FimX is required for

T4P-mediated twitching motility at the early stages of biofilm formation and that as the c-di-GMP concentration in the cells increase, although T4P can still be assembled, other features intrinsic to biofilms mask the twitching phenotype.

Curiously, an homologous protein to FimX from *Xanthomonas axonopodis* has been shown to interact with a PilZ domain protein involved in T4P biogenesis (Chin *et al.*, 2012; Guzzo *et al.*, 2009; Guzzo *et al.*, 2013). In *P. aeruginosa*, although homologues of both proteins are present, this interaction is not conserved, thus the molecular mechanism by which FimX regulates twitching motility may vary from species to species (Qi *et al.*, 2012).

Alg44, a role in alginate production

Alg44 (PA3542) is a membrane protein with a cytoplasmic PilZ domain at the N-terminus and an uncharacterized periplasmic C-terminus domain (Merighi *et al.*, 2007; Oglesby *et al.*, 2008). It is able to bind c-di-GMP and this is required for the polymerization and transport of alginate. It is possible that Alg44 responds to the c-di-GMP produced via MucR but experimental data supporting this is missing (Hay *et al.*, 2009).

With the exception of one protein, all proteins involved in alginate biogenesis are encoded in the *alg* operon. Although transcription of this operon is complex and involves many proteins, the alternative σ^{22} sigma factor, also known as AlgT or AlgU (PA0762), is required for *alg* transcription. The availability of σ^{22} is known to be controlled by a membrane associated anti-sigma factor, MucA (PA0763), which is subjected to proteolysis to allow the release of σ^{22} (Damron & Goldberg, 2012; Ramsey & Wozniak, 2005).

FleQ, a c-di-GMP responsive transcriptional factor

Unlike the other proteins described so far, FleQ (PA1097) does not have an identifiable GGDEF, EAL, HD-GYP or PilZ domain. Instead, it has an N-terminal FleQ domain which is associated to transcriptional activators of σ^{54}-dependent flagellar genes; a central ATPase domain; and a C-terminus DNA-binding domain. Curiously, FleQ has been shown to be able to bind c-di-GMP, possibly via its central ATPase domain.

The *fleQ* gene is known to be constitutively expressed and to be subjected to negative regulation by the alginate related σ^{22} sigma factor and the cAMP-binding transcriptional regulator Vfr (Dasgupta *et al.*, 2002; Tart *et al.*, 2005). Once produced and at low levels of c-di-GMP, FleQ acts as an activator and a repressor of different genes: an activator of genes involved in flagella-driven swimming

motility (including the two-component system FleRS, Table 1.2); and a repressor of the *pel*, *psl* and *cdr* genes involved in exopolysaccharide and adhesin production (Arora *et al.*, 1997; Hickman & Harwood, 2008). To act as a repressor, FleQ binds to another protein, FleN (PA1454), and to the promoter region of the target genes (Dasgupta & Ramphal, 2001). Remarkably, at high levels of c-di-GMP, FleQ is able to bind c-di-GMP and switch from a repressor to an activator of transcription (Baraquet *et al.*, 2012). Possibly, FleQ responds to the c-di-GMP produced by WspR, as activation of this DGC has been shown to up-regulate the *pel*, *psl* and *cdr* operons. The impact of elevated levels of c-di-GMP on the ability of FleQ to activate the expression of flagellar genes is still unclear.

LapD, a role in attachment in P. fluorescens

LapD (PA1433) is a membrane protein with cytoplasmic HAMP, GGDEF (no conserved I- or A-sites) and EAL domains that does not have catalytic activity but binds c-di-GMP via the EAL domain (Newell *et al.*, 2009). Although this protein remains largely uncharacterized in *P. aeruginosa,* its mode of action and signaling pathway in *P. fluorescens* has been studied in detail by the group of George O'Toole and will be described here (Figure 1.12).

In *P. fluorescens,* LapD is known to be able to bind c-di-GMP and control the release of an adhesin, LapA, from the cell surface. The secretion of this adhesin is dependent on a T1SS encoded by the *lapBCE* operon located next to the large *lapA* gene. In *P. aeruginosa*, a similar operon is found, PA1875-PA1877, but the adjacent large PA1874 gene has not been recognized as being homologous to *lapA* (Hinsa *et al.*, 2003). In addition, while in *P. fluorescens* the c-di-GMP binding protein *lapD* is encoded in the same genetic locus as the adhesin and the ABC transporter, the same is not true in *P. aeruginosa*.

In terms of the signaling pathway, the secretion of the adhesin LapA is required for the irreversible attachment and is inhibited at low levels of phosphate. Under these conditions, a two-component system called PhoBR, is activated and leads to the up-regulation of a protein with PDE activity, RapA (which has 76% sequence identity to PA3258) (Monds *et al.*, 2007). By lowering the c-di-GMP levels in the cell, RapA prevents LapA secretion and further inhibits biofilm formation. At these low levels of c-di-GMP, the c-di-GMP binding protein LapD is inactive and a periplasmic calcium dependent protease, LapG, is free to cleave LapA from the cell surface, preventing attachment (Boyd *et al.*, 2012; Hinsa & O'Toole, 2006; Navarro *et al.*, 2011; Newell *et al.*, 2011). Conversely, at higher levels of phosphate in the medium, c-di-GMP is not degraded by RapA and is therefore available to bind LapD. Once c-di-GMP is bound to the EAL domain of LapD, a signal is translated via the HAMP domain to the periplasmic region of the protein. A conformational change in the periplasmic region

of LapD caused by the intracellular binding of c-di-GMP then increases the affinity of the protease LapG to LapD. Binding of LapG to LapD prevents the cleavage of LapA and consequently, allows the progression of biofilm development.

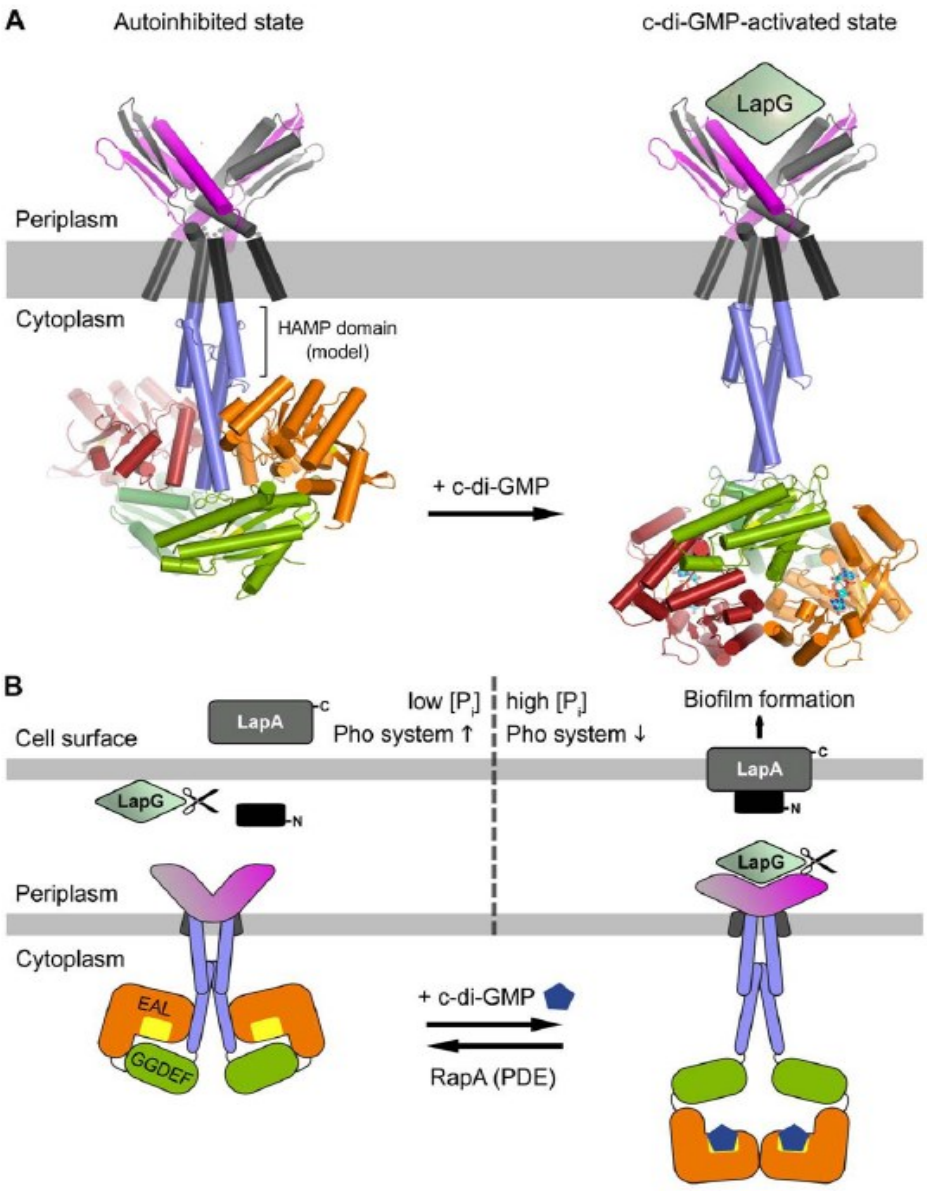

Figure 1.12 LapD inside-out signalling in *P. fluorescens*.

(A) Structural model of full-length LapD in the autoinhibited state and the c-di-GMP-activated state. LapD dimerizes via its EAL domain (green). Only the c-di-GMP-bound LapD is capable of binding LapG in the periplasm. The model is based on crystal structures obtained for the LapD periplasmic (purple) and cytoplasmic fragments (green and orange). The HAMP domain was modelled (blue). **(B)** Model for LapD-mediated control of biofilm formation. At low levels of phosphate, the PhoBR system is activated and the PDE RapA degrades c-di-GMP. In the absence of c-di-GMP, LapD is inactive and the

periplasmic protease LapG cleaves the LapA adhesin from the cell surface, preventing biofilm formation. At high levels of phosphate, the levels of c-di-GMP increase and LapD is activated. Binding of c-di-GMP to the cytoplasmic EAL domain increases the affinity of the periplasmic domain to LapG. The interaction between LapG and LapD prevents the cleavage of the adhesin and therefore biofilm formation is induced. Figure retrieved from Navarro et al. (2011).

In conclusion, in this signaling pathway LapD works as a modulator of cell surface exposure of the LapA adhesin. This control occurs by a c-di-GMP dependent inside-out signalling mechanism where intracellular high levels of c-di-GMP serve as a signal for the inhibition of LapA release, leading to biofilm formation.

Curiously, in *P. aeruginosa* the gene encoded next to the RapA homologue, Prc (PA3257), is a predicted protease that has been correlated to the mucoid phenotype and the modulation of the σ^{22} sigma factor that is involved in the transcription of the *alg* operon (Sautter *et al.*, 2012).

1.4.5 Other c-di-GMP related proteins

MorA, a role in biofilm development

MorA (PA4601) is a membrane protein with four PAS domains in tandem at the N-terminus and a GGDEF domain (conserved A-site) followed by an EAL domain at the C-terminus. Since this protein has both the GGDEF and EAL domains, it is unclear which catalytic activity it harbours, if any. The studies on MorA so far have implicated the protein in the regulation of CupA fimbriae and the early stages of biofilm development in *P. aeruginosa* (Choy *et al.*, 2004; Meissner *et al.*, 2007). In *P. putida*, deletion of *morA* not only impaired biofilm formation, but also led to an increase in flagella-driven swimming motility (Choy *et al.*, 2004). Overall, these results suggest a possible DGC function but this and the mechanism of action of the protein are still obscure.

ToxR or RegA, a role in exotoxin A production

ToxR (PA0707) has an EAL domain but it is not known if it has PDE activity. This protein was characterized before there was an established understanding of the c-di-GMP signalling so its enzymatic activity was not explored. An alignment of ToxR with other EAL domain proteins reveals that the EAL motif is not conserved, suggesting that it may be catalytic inactive.

This protein, also known as RegA, was identified for its role in the regulation of the production of exotoxin A, a virulence factor secreted by the T2SS (Wozniak *et al.*, 1987). The precise mechanism of this regulation is not known, but other proteins like the iron regulated sigma factor PvdS (PA2426) and the cAMP binding transcriptional regulator Vfr have been shown to also impact exotoxin A production (Davinic *et al.*, 2009; Gaines *et al.*, 2007).

PA2572, a degenerated HD-GYP domain protein

PA2572 has an N-terminal REC domain and a C-terminal HD-GYP domain followed by an uncharacterized extension.

The HD motif of PA2572 is degenerated and the protein has been shown not to be catalytically active (Ryan *et al.*, 2009). However, phenotypes related with pyoverdine, rhamnolipid and biofilm formation are observed when the protein is not produced. Furthermore, a truncated version of PA2572 where the C-terminal extension is missing revealed an ability of the protein to repress swarming motility. Overall, despite the fact that PA2572 lacks PDE activity, its function as a regulatory protein seems obvious. Recently, the neighbouring putative MCP PA2573 was shown to bind PA2572 and the regulon of both deletion mutants overlaps in the regulation of at least 49 genes (McLaughlin *et al.*, 2012).

PilZ, a PilZ domain protein that does not bind c-di-GMP

PilZ (PA2960) has only a PilZ domain and is required for normal pilus assembly (Alm *et al.*, 1996). Tragically, although the c-di-GMP binding PilZ domains were named after this protein, PilZ does not bind c-di-GMP because it lacks certain key residues (Merighi *et al.*, 2007). It also does not interact with the c-di-GMP binding protein FimX which was shown to interact with PilZ in other species (Chin *et al.*, 2012; Qi *et al.*, 2012). Therefore, the precise role of PilZ in T4P biogenesis and its possible link to c-di-GMP remain unclear.

Uncharacterized proteins

Although significant amounts of knowledge have been produced in relation to the proteins that are involved in c-di-GMP signalling, in many cases very incomplete information is available and there is still a lot to do. Besides the proteins that were detailed here, a significant number of other c-di-GMP related proteins remain uncharacterized despite an early effort by Kulasekara *et al.* in performing a

comprehensive analysis of a collection of PA14 transposon mutantos in genes encoding GGDEF and EAL domain proteins (Kulasakara *et al.*, 2006).

Proteins with a C-terminal GGDEF domain that remain uncharacterized are: PA0290, PA0338, PA0847, PA1851, PA2771, PA2870, PA3177, PA3343, PA4396, PA4843, PA4929 and PA5487. Among these, PA0847, PA2870, PA3343 and PA5487 are known to have DGC activity (Kulasakara *et al.*, 2006).

Proteins with both GGDEF and EAL domains that have not been characterized are: PA0285, PA0575, PA1181, PA2072, PA2567, PA3258, PA5295 and PA5442. Of these, the EAL domain of PA2567 is known to have PDE activity (Rao *et al.*, 2009). Noteworthy, PA0575 has an N-terminal PBPb domain followed by four PAS domains in tandem; and PA3258 has an N-terminal EAL domain and a C-terminal GGDEF domain separated by a CBS domain. *PBPb domains* (also referred to as SBP_bac_3 domains) are periplasmic solute-binding protein domains found in bacteria (Table 1.1) (Bermejo *et al.*, 2010; Tam & Saier, 1993). *CBS domains* are found in cystathionine beta-synthase and other proteins in all domains of life. They are small modules that can sense intracellular metabolites, like ATP, or mediate protein interactions (Table 1.1) (Bateman, 1997).

Proteins with a C-terminal EAL domain that are not yet characterized are: PA2133, PA2200, PA3825. However, all of them are known to have PDE activity and PA2133 is used as a model.

Finally, proteins with a PilZ domain that are uncharacterized are: PA0012, PA2799, PA2989, PA3353, PA4608 and PA4324. All of them have been shown to bind c-di-GMP and a three dimensional structure of PA4608 is available (Habazettl *et al.*, 2011; Merighi *et al.*, 2007; Shin *et al.*, 2011).

1.4 Cyclic di-GMP signalling pathways in other bacteria

The ubiquity of c-di-GMP in bacteria means that this signalling molecule has been in the forefront of research in different species during the past years. In a few cases, the signalling pathways that involve c-di-GMP are well established and represent milestones in the understanding of its signalling mechanisms. Generally, a c-di-GMP dependent signalling cascade involves the perception of a signal by a sensor that responds by modulating the activity of DGCs or PDEs to control a pool of c-di-GMP. Then, the availability of c-di-GMP determines the binding of c-di-GMP to receptors proteins, creating a molecular output that ultimately translates into a change in bacterial behaviour. Below, famous regulatory cascades involving c-di-GMP in species other than *P. aeruginosa* are described.

The control of virulence and T4P motility in X. campestris

In this phytopathogen, a cluster of 8 genes, the *rpf(A-H)* genes, is known to be involved in the regulation of pathogenicity factors and the production of a cell-to-cell signalling molecule. This signalling molecule is referred to as DSF, for diffusible signalling factor, and is a *cis*-unsaturated fatty acid that can be extracted with organic solvents from the supernatant of *X. campestris* cultures.

DSF is produced by two genes of the *rpf* cluster, RpfB and RpfF, and sensed by a two-component system also encoded in the cluster, RpfC and RpfG (Barber *et al.*, 1997; Slater *et al.*, 2000). Upon sensing of DSF, the sensor RpfC acts via its response regulator RpfG to positively control the production of virulent extracellular enzymes and T4P-mediated motility (Figure 1.13).

The response regulator RpfG harbours a C-terminal HD-GYP domain whose PDE activity is dependent on a functional HD motif. By degrading c-di-GMP, RpfG positively impacts the production of extracellular enzymes by modulation of Clp activity (Chin *et al.*, 2010; He *et al.*, 2007; Tao *et al.*, 2010). Clp is transcriptional regulator with homology to Crp from *E. coli* or Vfr from *P. aeruginosa*, but in *X. campestris* rather than binding to cAMP, it binds c-di-GMP via an E residue at the N-terminus (Beatson *et al.*, 2002; Gorshkova *et al.*, 1995). Binding of c-di-GMP to Clp inhibits the transcription of virulence factors meaning that the PDE activity of RpfG is required for their production.

In addition to this, RpfG can interact with GGDEF domain proteins via its GYP motif to modulate motility (Ryan & Dow, 2010; Ryan *et al.*, 2010). The interaction between RpfG and two GGDEF domain proteins (with additional N-terminal domains) is dependent on a DxD (aspartic acid – x – aspartic acid) motif located upstream of the GGDEF motif, but independent of DGC activity. Formation of this HD-GYP/GGDEF protein complex was then shown to be able to bind to a PilZ domain protein and interact with PilT, an ATPase protein of the T4P machinery required for pili retraction. Despite the fact that the PilZ domain protein was able to bind c-di-GMP, the formation of the multiprotein complex was independent of this ability (Ryan *et al.*, 2012a).

In summary, this signalling pathway shows how c-di-GMP related proteins can coordinate the regulation of more than one bacterial phenotype upon sensing of one particular signal. It also shows how the same protein can lead, at the same time, to the regulation of gene expression at different levels (transcriptional level via Clp and allosteric level via the formation of the multiprotein complex).

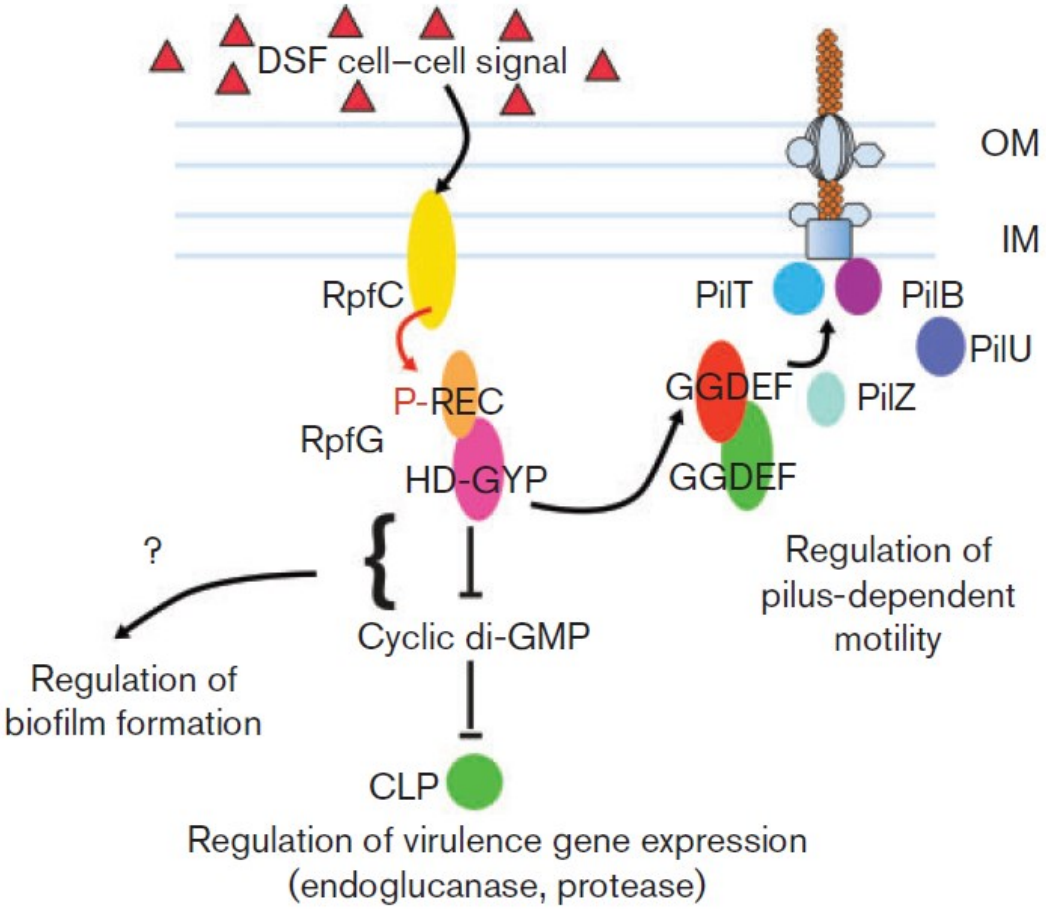

Figure 1.13 Model for the control of virulence and T4P motility in *X. campestris*.

The DSF signal is perceived by the two-component system RpfCG. Upon sensing of DSF, the sensor RpfC autophosphorylates and transfers the phosphoryl group to the response regulator RpfG. The output domain of RpfG is a HD-GYP domain with PDE activity. The activation of RpfG catalytic activity by phosphorylation decreases the levels of c-di-GMP in the cell and leads to the production of extracellular enzymes like endoglucanase and protease via Clp. Clp is a transcriptional activator that is inhibited by binding to c-di-GMP. In parallel, the phosphorylation of RpfG also promotes the interaction of RpfG with two GGDEF domain proteins, XC_0249 and XC_0420, to regulate T4P-mediated motility. The interaction between RpfG and the proteins containing the GGDEF domain requires the GYP motif of the HD-GYP domain and a DxD motif in the GGDEF domain but is independent of the DGC activity of the proteins. This complex goes on to recruit a PilZ domain protein, XC_2249, which interacts with the PilT ATPase involved in the depolymerisation of the pilus. The recruitment of the protein containing the PilZ domain is independent of c-di-GMP binding and the RpfG influence in pilus function is exerted at the posttranslational level. The RpfCG system also regulates negatively biofilm formation but the mechanism is unknown. OM, outer membrane; IM, inner membrane. Figure retrieved from Ryan, R. (2013).

The Csr regulatory cascade and exopolysaccharide production in E. coli

In *E. coli*, the *pga(A-D)* operon is responsible for the production and secretion of the exopolysaccharide PGA (poly-β-1,6-*N*-acetylglucosamine), one of the major components of its biofilm extracellular matrix (Romeo & Babitzke, 2010). While PgaC and PgaD are involved in PGA synthesis, PgaA and PgaB are required for its secretion. Both the expression of the *pga* operon and the production of PGA are regulated by c-di-GMP and depend on the RsmA *E. coli* homologue, CsrA (Fig 1.14).

CsrA is a downstream target of the Gac system (in *E. coli* referred to as BarA/UvrY system, where BarA is the sensor and UvrY is the response regulator) and acts as a translational repressor of *pga* expression (Lapouge *et al.*, 2008). Like in *P. aeruginosa,* the CsrA repressing activity is counteracted by two sRNAs, CsrB and CsrC. In this case, the transcription of these sRNAs is not only controlled by BarA/UvrY, but it also requires CsrA which, via a yet unknown mechanism, positively regulates BarA activity.

Once transcribed, the levels of the sRNAs CsrB and CsrC are regulated posttranscriptionally by a membrane protein called CsrD with a cytoplasmic HAMP domain followed by a GGDEF and EAL domain (Jonas *et al.*, 2008). Interestingly, both of these domains have no catalytic activity and instead, the protein works as a RNA-binding protein to mediate the degradation of CsrB and CsrC. Along with other ribonucleases, the c-di-GMP binding PNPase described earlier is involved in this RNA processing (Tuckerman *et al.*, 2011).

When CsrA is sequestered by the two sRNAs, translation of its mRNA targets can occur. These are the *pga* operon and many other genes, including those encoding two GGDEF domain proteins, YdeH and YcdT. Whereas YcdT is a membrane protein with DGC activity whose specific function is not clear, YdeH was shown to be specifically involved in the production of PGA (Boehm *et al.*, 2009).

YdeH is a cytoplasmic DGC whose gene expression is transcriptionally regulated by a two-component system and posttranscriptionally regulated by CsrA. Once produced, its DGC activity is thought to control a pool of c-di-GMP that is required for the allosteric activation of PGA synthesis. The two proteins involved in the PGA catalytic process are the membrane proteins PgaC and PgaD. In the absence of the YdeH DGC activity, PgaD is unstable and unable to interact with PgaC. However, when YdeH is present, the produced c-di-GMP binds both PgaC and PgaD and stabilizes their interaction therefore promoting the synthesis of PGA (Steiner *et al.*, 2013).

In conclusion, PGA biogenesis is CsrA-dependent but is subjected to c-di-GMP control at various levels: indirectly at the posttranscriptional level through c-di-GMP regulation of the sRNAs CsrB and

CsrC metabolism; and directly at the posttranslational level by the participation of c-di-GMP in the formation of a protein complex that is required for PGA synthesis.

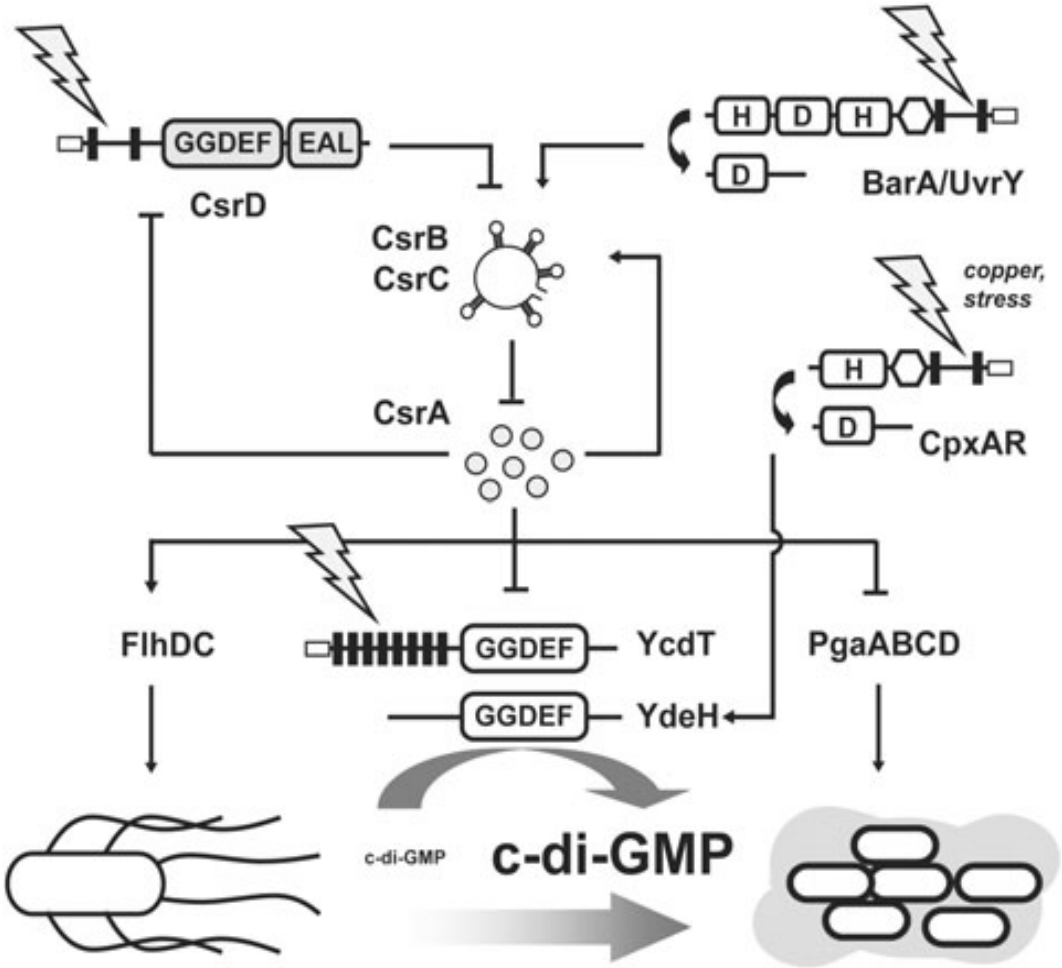

Figure 1.14 Cyclic di-GMP and the Gac system in *E. coli*.

The activity of the RsmA homologue, CsrA is controlled by the sRNAs CsrB and CsrC. The expression of the sRNAs is controlled by the GacAS homologue system where the sensor is referred to as BarA and the response regulator as UvrY, and by CsrA. Posttranscriptionally, the production of the sRNAs is controlled by the RNA-binding protein CsrD which in turn is also under the control of CsrA. CsrA modulates the switch between motility and sessility by controlling directly the production of flagella, c-di-GMP and exopolysaccharides. CsrA has a positive impact on the mRNA levels of flagella genes (*flhDC*) and a negative impact on PGA exopolysaccharide genes (*pgaABDC*) and genes encoding proteins with a GGDEF domain (*ycdT* and *ydeH*). In this network, extracellular signals can be integrated via BarA/UvrY, CsrD and YcdT. In addition, the transcription of *ydeH* is also known to be controlled by the CpxAR two-component system which responds to cell envelope stress and external copper. Although CsrD does not bind c-di-GMP or have a c-di-GMP associated catalytic function, the negative impact upon the sRNAs is dependent on the ribonucleases RNase E and PNPase and the latter has been shown to bind c-di-GMP. In relation to PGA biogenesis, c-di-GMP also acts at the allosteric level. Figure retrieved from Jonas et al. (2008).

The production of cellulose in S. enterica

In *S. enterica*, the *csg(A-G)* genetic locus encodes genes required for the production of curli fimbriae and is involved in the regulation of the rdar morphotype (Romling *et al.*, 2010). This morphotype is frequent in clinical isolates of *S. enterica* and correlates with both the expression of curli frimbriae and the production of the exopolysaccharide cellulose. One of the genes of the *csg* cluster, *csgD*, is a transcriptional regulator and is central for the occurrence of the rdar morphotype.

In this signalling pathway, CsgD production is positively regulated, posttranscriptionally or posttranslationally, by two membrane associated DGCs, STM2123 and STM3388. Although these proteins individually do not contribute greatly to the global pool of intracellular c-di-GMP and have in tandem GGDEF and EAL domains at the C-terminus, their ability to function as DGCs and synergistically influence *csgD* expression was demonstrated. In addition, the specific PDE activity of two other proteins, STM4264 and STM1703, was shown to counteract the effect of the mentioned DGCs (Romling *et al.*, 2010).

Once produced, the transcriptional regulator CsgD acts an activator of AdrA, a membrane protein with a cytoplasmic GGDEF domain. AdrA was shown to be a potent DGC and production of c-di-GMP activates cellulose biogenesis. This occurs posttranslationally, by binding of c-di-GMP to the C-terminal PilZ domain of the cellulose synthase BcsA protein (Romling *et al.*, 2010).

So, in cellulose biogenesis in *S. enterica*, c-di-GMP regulation seems to be spatially and temporarily separated: firstly, a specific pool of c-di-GMP modulates CsgD; secondly, the c-di-GMP produced by a target of CsgD, activates the synthesis of cellulose.

The control of the cell cycle in Caulobacter crescentus

In the aquatic bacterium *C. crescentus*, cell division yields two different cells: a motile cell with polar flagellum and pili, and a sessile cell that is attached to the surface by a stalk and a holdfast. While the motile cell cannot undergo cell division and needs to swarm away from the sessile cell to later attach to a surface, cell division always occurs in the attached cells. In these, the stalk and holdfast develop at the same pole of the flagellum and pili, and it is the opposite pole that drives cell division. The coordination of this process is accomplished by a dynamic network that involves phosphorylation, proteolysis and c-di-GMP signalling (Abel & Jenal, 2010).

Very briefly, at least five c-di-GMP related proteins are required for progression of this *C. crescentus* cell cycle and the temporal activation and spatial localization of these proteins is crucial for the process (Abel & Jenal, 2010). Generally, the inability of the motile cell to divide is due to the

presence of the phosphorylated response regulator CtrA which blocks the initiation of DNA replication. When a motile cell attaches to a surface to start differentiating into a sessile cell, the levels of c-di-GMP increase at the swarmer-to-stalked cell pole by three means: (i) DGC activity of phosphorylated PleD, (ii) DGC activity of DgcB, and (iii) proteolysis of the PDE PdeA. This increased level of c-di-GMP is required for the formation of the holdfast and the ejection of the flagellum. At the same time, c-di-GMP binds a catalytic inactive GGDEF domain protein, PopA, which recruits CtrA to the same pole. Here, CtrA suffers proteolysis, allowing the initiation of replication. Interestingly, when PopA is not recruited to the swarmer-to-stalked cell pole by binding to c-di-GMP, it interacts with another protein and localizes to the opposite cell pole. At this cell pole, where the new swarmer cell is then formed, an EAL domain protein, TipF, is present and its PDE activity is known to be required for the assembly of the flagellum in the swarmer cell (Abel & Jenal, 2010).

The swimming motility velocity in E. coli

A role of c-di-GMP in the control of the speed of flagella-driven swimming motility has elegantly been elucidated recently. Briefly, during exponential growth, *E. coli* cells are motile and c-di-GMP levels are kept low due to the action of a PDE, YjhH. Upon depletion of nutrients, the activity of YjhH is repressed whereas DGCs are active and therefore, the levels of c-di-GMP increase. This leads to the binding of c-di-GMP to a protein, YcgR, which has a C-terminal PilZ domain. Binding of c-di-GMP to the PilZ domain of YcgR enables the protein to interact with components of the flagella motor, acting as a brake and reducing swimming motility. There is no consensus yet on what protein or proteins of the flagella motor interact with YcgR, but interactions with the stator protein MotA and two rotor proteins (FliG and FliM) have been demonstrated (Boehm *et al.*, 2010; Fang & Gomelsky, 2010; Paul *et al.*, 2010). In conclusion, this is a signaling pathway where c-di-GMP modulates the swimming phenotype of the bacterial cells by promoting protein-protein interactions. At high levels of c-di-GMP, motility is reduced via a c-di-GMP-dependent braking system and at the same time, biofilm formation is induced.

1.6 Aims of this study

In order to adapt to an ever-changing environment, bacteria need to integrate signals into molecular readouts that modulate their biological behaviour. Many of the biological functions that define the mode of infection of *P. aeruginosa* depend on regulatory cascades that involve the second messenger c-di-GMP. To explore the molecular mechanisms employed by these cascades, the aims of the present study were:

1. To understand the level of specificity at which the c-di-GMP network operates. *P. aeruginosa* encodes many putative DGCs and PDEs but they are thought to specifically regulate a wide variety of functions. In Chapter 3, I look at different GGDEF and EAL domain proteins and to their role in biofilm formation and dispersal;

2. To investigate how c-di-GMP relates to other signalling cascades. Like c-di-GMP, the Gac system is also known to regulate the transition between the motile and sessile bacterial lifestyles. In addition, the Gac system modulates the switch between two different secretion machineries and therefore, the impact of c-di-GMP in this switch is explored in Chapter 4;

3. To elucidate the molecular basis of the link between the Gac signalling cascade and the c-di-GMP network. In other words, to find out which c-di-GMP related proteins are under regulation of the Gac signalling cascade (Chapter 5).

2) Materials and Methods

Chapter 2 – Materials and Methods

2.1 Strains, Plasmids and Growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1.

Bacteria were routinely cultured at 37°C with shaking in LB medium or at 37°C on LB agar plates.

When appropriate, culture media were supplemented with antibiotics at the following concentrations. For *E. coli*: 50 μg/mL ampicilin (Ap), 50 μg/mL kanamycin (Km), 50 μg/mL streptomycin (Sm), 50 μg/mL gentamycin (Gm), 15 μg/mL tetracyclin (Tc). For *P. aeruginosa*: 500 μg/mL carbenicillin (Cb) for selection and 300 μg/mL Cb for maintenance; 150 μg/mL Gm for selection and 100 μg/mL Gm for maintenance; 2000 μg/mL Sm for selection; 200 μg/mL Tc for selection and 100 μg/mL for maintenance.

When indicated, calcium chelation was used to induce the T3SS by adding 5 mM EGTA (ethylene glycol tetra-acetic acid, Sigma) followed by 20 mM $MgCl_2$ to the growth medium.

The M9 minimal medium was prepared according to Sambrook & Russell (2001) and supplemented with 01% or 0.4% glucose, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$.

Table 2.1 Strains and plasmids used in this study.

Strain or Plasmid	Genotype/Description	Source
Escherichia coli		
TOP10	Strain used for transformation of pCR2.1 and pCR-Blunt plasmids; F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ^-	Invitrogen
OmniMAX	F' {*pro*AB^+ *lac*I^q *lac*ZΔM15 Tn10(Tet^R) Δ(*ccd*AB)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D	Invitrogen
CC118λpir	Host strain for pKNG101; D (*ara-leu*) *araD DlacX74 galE galK-phoA20 thi-1 rpsE rpoB argE* (Am) *recA1 Rf*^{r} *(lpir)*	Herrero *et al.*, 1990
Sm10λpir	Host strain for miniCTX1; *F*$^-$ *thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44 (MuC*$^+$*)* I^- *[RP4-2(Tc::Mu)] Km*^{r} *Tra*$^+$	de Lorenzo & Timmis, 1994

Strain or Plasmid	Genotype/Description	Source
XL1-Blue	Strain used for transformation of plasmids subjected to the site-directed mutagenesis protocol; *rec*A1 *end*A1 *gyr*A96 *thi-1 hsd*R17 *sup*E44 *rel*A1 lac [F′ *pro*AB *lacI*^{q}*lacZ*ΔM15 Tn10 (Tet^r)]	Stratagene
DHM1	Strain used for transformation of the bacterial two-hybrid plasmids; F$^-$, *cya-854*, *recA1, endA1, gyrA96 (Nal^r)*, *thi1, hsdR17*, *spoT1*, *rfbD1, glnV44(AS)*	Karimova *et al.*, 1998
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdS$_B$(r$_B^-$ m$_B^-$) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Stratagene
BL21(DE3) C41	Spontaneous BL21 (DE3) mutant that has a mutated promoter that slows down transcription of the T7 RNA polymerase; F$^-$ ompT gal dcm hsdS$_B$(r$_B^-$ m$_B^-$)(DE3)	Lucigen
Pseudomonas aeruginosa		
PAO1	Wild-type	Stover *et al.*, 2000
PAO1*ΔPA0847*	Deletion of PA0847 in PAO1	This study
PA14	Wild-type	Liberati *et al.*, 2006
PA14*ΔwspR*	Deletion of *wspR* in PA14	This study
PAK	Wild-type	Goodman *et al.*, 2004
PAK*ΔretS*	Deletion of *retS* in PAK	Goodman *et al.*, 2004
PAK*ΔpelG*	Deletion of *pelG* in PAK	Vasseur *et al.*, 2005
PAK*ΔretSΔpelF*	Deletion of *retS* and *pelF* in PAK	Sadovskaya *et al.*, 2010
PAK*ΔrsmYΔrsmZ*	Deletions of *rsmY* and *rsmZ* in PAK	Bordi *et al.*, 2010
PAK*ΔladS*	Deletions of *ladS* in PAK	Ventre *et al.*, 2006
PAK*ΔgacS*	Deletion of *gacS* in PAK	Bordi *et al.*, 2010
PAK*ΔgacA*	Deletions of *gacA* in PAK	Bordi *et al.*, 2010
PAK*ΔretSΔrsmYΔrsmZ*	Deletions of *retS, rsmY* and *rsmZ* in PAK	Bordi *et al.*, 2010
PAK*ΔretSΔrsmY*	Deletion of *retS* and *rsmY* in PAK	Bordi *et al.*, 2010
PAK*ΔretSΔrsmZ*	Deletion of *retS* and *rsmZ* in PAK	Bordi *et al.*, 2010
PAK*ΔretSΔPA0338*	Deletion of *retS* andPA0338 in PAK	This study
PAK*ΔretSΔsadC*	Deletion of *retS* and *sadC* in PAK	This study

Strain or Plasmid	Genotype/Description	Source
PAK*ΔhptB*	Deletion of *hptB* in PAK	Bordi *et al.*, 2010
PAK*ΔhptBΔsadC*	Deletions of *hptB* and *sadC* in PAK	This study
PAK*ΔsadC*	Deletion of *sadC* in PAK	This study
PAK*ΔrsmA*	Deletion of *rsmA* in PAK	Ventre *et al.*, 2006
PAK*ΔrsmAΔsadC*	Deletion of *rsmA* and *sadC* in PAK	This study
PAK*ΔretSΔsadB*	Deletion of *retS* and *sadB* in PAK	This study
PAK*ΔhptBΔsadB*	Deletion of *hptB* and *sadB* in PAK	This study
Plasmids*		
pRK2013	Tra^+ Mob^+ helper plasmid for mobilization of non-self-transmissible plasmids, Km^R	Figurski & Helinski, 1979
pCR2.1	Cloning plasmid, Ap^R, Km^R	Invitrogen
pCR-Blunt	Cloning plasmid, Km^R	Invitrogen
pBBR1-MCS4	Broad host range vector, Ap^R	Kovach *et al.*, 1995
pJN105	Plasmid allowing protein expression under the arabinose inducible P_{BAD} promoter, Gm^R	Newman & Fuqua, 1999
pKNG101	Suicide vector carrying *sacB*, Sm^R	Kaniga *et al.*, 1991
miniCTX1	Plasmid for the integration of genes into the *att* site of the *P. aeruginosa* chromosome, Tc^R	Hoang *et al.*, 2000
pKT25	Plasmid allowing construction of in-frame fusions at the C-terminus of the T25 polypeptide, Km^R	Karimova *et al.*, 1998
pKNT25	Plasmid allowing construction of in-frame fusions at the N-terminus of the T25 polypeptide, Km^R	Euromedex
pUT18C	Plasmid allowing construction of in-frame fusions at the C-terminus of the T18 polypeptide, Ap^R	Euromedex
pUT18	Plasmid allowing construction of in-frame fusions at the N-terminus of the T18 polypeptide, Ap^R	Karimova *et al.*, 1998
pKT25-zip	Fusion of *zip* encoding leucine zipper from GCN4 to *cyaA* gene T25 fragment in pKT25, Km^R	Karimova *et al.*, 1998
pUT18C-zip	Fusion of *zip* encoding leucine zipper from GCN4 to *cyaA* gene T18 fragment in pUT18C, Ap^R	Karimova *et al.*, 1998

Strain or Plasmid	Genotype/Description	Source
pKNG101-ΔPA0847	Mutator fragment for deletion of PA0847 (from amino acid 61 to 733), Sm^R	This study
pKNG101-ΔwspR	Mutator fragment for deletion of *wspR* (from amino acid 4 to 347), Sm^R	This study
pKNG101-ΔPA0338	Mutator fragment for deletion of PA0338 (from amino acid 2 to 367), Sm^R	This study
pKNG101-ΔsadC	Mutator fragment for deletion of *sadC* (from amino acid 87 to 414), Sm^R	This study
pKNG101-ΔsadB	Mutator fragment for deletion of *sadB* (from amino acid 4 to 467), Sm^R	This study
pJN105-*wspR*	*wspR* cloned into pJN105 (SacI/XbaI), Gm^R	This study
pJN105-*wspR***	*wspR*** encoding mutations of the I-site and the phosphorylation residue, cloned into pJN105 (SacI/XbaI), Gm^R	This study
pJN105-PA2133	PA2133 cloned into pJN105 (SacI/XbaI), Gm^R	This study
pBBR1-MCS4-*wspR*	*wspR* cloned into pBBR1-MCS4 (SacI/XbaI), Ap^R	This study
pBBR1-MCS4-$wspR^{R242A}$	*wspR(R252A)* cloned into pBBR1-MCS4 (SacI/XbaI), Ap^R	This study
pBBR1-MCS4-PA2133	PA2133 cloned into pBBR1-MCS4 (SacI/XbaI), Ap^R	This study
pBBR1-MCS4-PA2072	PA2072 cloned into pBBR1-MCS4 (BamHI/XbaI), Ap^R	This study (E. Musial)
pBBR1-MCS4-$PA2072^{GGAAF}$	PA2072(GGAAF) cloned into pBBR1-MCS4 (BamHI/XbaI), Ap^R	This study (E. Musial)
pBBR1-MCS4-$PA2072^{AAL}$	PA2072(AAL) cloned into pBBR1-MCS4 (BamHI/XbaI), Ap^R	This study (E. Musial)
pBBR1-MCS4-yfiN	*yfiN* (PA1120) cloned into pBBR1-MCS4 (SacI/XbaI), Ap^R	This study
pBBR1-MCS4-*ladS*	Plasmid expressing *ladS* (PA3974), Ap^R	Ventre *et al.*, 2006
pBBR1-MCS4-*hsbR*	Plasmid expressing *hsbR* (PA3346), Ap^R	Bordi *et al.*, 2010
pVL847 (MBP)	Plasmid expressing MBP (maltose-binding protein), Cb^R	Roelofs *et al.*, 2011

Strain or Plasmid	Genotype/Description	Source
pVL882 (MBP-Alg44PilZ)	Plasmid expressing MBP-Alg44PilZ, Cb^R	Roelofs *et al.*, 2011
pJB3	Broad host range vector, Ap^R, Cb^R	Blatny *et al.*, 1997
pJB3-PA5487	PA5487 cloned into pJB3, Ap^R, Cb^R	Isabel Cortés
pET28a	Plasmid allowing protein expression under the T7 promoter, Km^R	Novagen
pET28a-FLAG-*retS*	FLAG-*retS* cloned into pET28a (NdeI/HindIII), Km^R	This study
pET28a-FLAG-*ladS*	FLAG-*ladS* cloned into pET28a (NdeI/HindIII), Km^R	This study
pET28a-FLAG-*gacS*	FLAG-*gacS* cloned into pET28a (NdeI/HindIII), Km^R	This study
pET28a-FLAG-*gacA*	FLAG-*gacA* cloned into pET28a (NdeI/HindIII), Km^R	This study
pET28a-FLAG-*rsmA*	FLAG-*rsmA* cloned into pET28a (EcoRI/HindIII), Km^R	This study
pET42b-HIS-*dgrA*	Plasmid expressing HIS-*dgrA,* Km^R	Christen *et al.*, 2007; Nesper *et al.*, 2012
pET42b-HIS-*yfiN*	Plasmid expressing HIS-*yfiN*, Km^R	T. Jaeger
pKNT25-$sadC^{fl}$	$sadC^{fl}$ cloned into pKNT25, Km^R	This study
pKT25-$sadC^{ct}$	$sadC^{ct}$ cloned into pKT25, Km^R	This study
pKT25-*sadB*	*sadB* cloned into pKT25, Km^R	This study
pKT25-PA4379	*PA4379* cloned into pKT25, Km^R	This study
pUT18-$sadC^{fl}$	$sadC^{fl}$ cloned into pUT18, Ap^R	This study
pUT18C-$sadC^{ct}$	$sadC^{ct}$ cloned into pUT18C, Ap^R	This study
pUT18C-*sadB*	*sadB* cloned into pUT18C, Ap^R	This study
pUT18C-PA4379	*PA4379* cloned into pUT18C, Ap^R	This study
miniCTX1-P_{BAD}	*araC*-P_{BAD} cloned into miniCTX1 (SacI/SpeI)	This study
miniCTX1-P_{BAD}-$sadC^{R392A}$	SadC(R392A) cloned into miniCTX1-P_{BAD} (SmaI/KpnI)	This study (Q. Pan)
P_{cdrA}-*gfp*	Plasmid expressing *gfp* from the promoter of *cdrA*, Ap^R, Gm^R	Rybtke *et al.*, 2012

Strain or Plasmid	Genotype/Description	Source
PAO1 transposon mutants – all come from Jacobs *et al.* (2003)		
PW1288	PA0169 (*siaD*), Tc^R	
PW1531	PA0290, Tc^R	
PW1627	PA0338, Tc^R	
PW2543	PA0847, Tc^R	
PW2999	PA1107 (*roeA*), Tc^R	
PW3023	PA1120 (*yfiN*), Tc^R	
PW4248	PA1851, Tc^R	
PW5641	PA2771, Tc^R	
PW5818	PA2870, Tc^R	
PW6315	PA3177, Tc^R	
PW6631	PA3343, Tc^R	
PW7263	PA3702 (*wspR*), Tc^R	
PW8315	PA4332 (*sadC*), Tc^R	
PW8423	PA4396, Tc^R	
PW9146	PA4843, Tc^R	
PW9303	PA4929, Tc^R	
PW10280	PA5487, Tc^R	
PW1520	PA0285, Tc^R	
PW2060	PA0575, Tc^R	
PW2569	PA0861 (*rbdA*), Tc^R	
PW3133	PA1181, Tc^R	
PW3602	PA1433, Tc^R	
PW4043	PA1727 (*mucR*), Tc^R	
PW4568	PA2072, Tc^R	
PW5308	PA2567, Tc^R	
PW6465	PA3258, Tc^R	
PW6567	PA3311, Tc^R	
PW8371	PA4367 (*bifA*), Tc^R	
PW8754	PA4601 (*morA*), Tc^R	
PW9347	PA4959 (*fimX*), Tc^R	
PW9424	PA5017 (*dipA*), Tc^R	
PW9918	PA5295, Tc^R	
PW10193	PA5442, Tc^R	
PW2282	PA0707 (*toxR*), Tc^R	
PW4665	PA2133, Tc^R	
PW4747	PA2200, Tc^R	
PW5718	PA2818 (*arr*), Tc^R	
PW7455	PA3825, Tc^R	
PW7674	PA3947 (*rocR*), Tc^R	
PW5317	PA2572, Tc^R	
PW7948	PA4108, Tc^R	
PW9032	PA4781, Tc^R	
PA14 transposon mutants – all come from Liberati *et al.* (2006)		
55715	PA14_02110 (PA0169), Gm^R	
41840	PA14_03790 (PA0290), Gm^R	
55979	PA14_04420 PA0338), Gm^R	
53886	PA14_53310 (PA0847), Gm^R	
35398	PA14_50060 (PA1107), Gm^R	
37761	PA14_49890 (PA1120), Gm^R	

Strain or Plasmid	Genotype/Description	Source
41347	PA14_40570 (PA1851), Gm^R	
29609	PA14_23130 (PA3177), Gm^R	
48657	PA14_20820 (PA3343), Gm^R	
28622	PA14_16500 (PA3702), Gm^R	
26451	PA14_56280 (PA4332), Gm^R	
30693	PA14_57140 (PA4396), Gm^R	
44678	PA14_64050 (PA4843), Gm^R	
48450	PA14_65090 (PA4929), Gm^R	
33256	PA14_72420 (PA5487), Gm^R	
30208	PA14_03720 (PA0285), Gm^R	
54913	PA14_07500 (PA0575), Gm^R	
43246	PA14_53140 (PA0861), Gm^R	
41487	PA14_49160 (PA1181), Gm^R	
55781	PA14_45930 (PA1433), Gm^R	
42074	PA14_42220 (PA1727), Gm^R	
56016	PA14_37690 (PA2072), Gm^R	
27009	PA14_12430 (PA2567), Gm^R	
23726	PA14_21870 (PA3258), Gm^R	
32733	PA14_21190 (PA3311), Gm^R	
52637	PA14_56790 (PA4367), Gm^R	
26307	PA14_60870 (PA4601), Gm^R	
27822	PA14_65540 (PA4959), Gm^R	
45771	PA14_66320 (PA5017), Gm^R	
23485	PA14_69900 (PA5295), Gm^R	
30226	PA14_71850 (PA5442), Gm^R	
26651	PA14_36990 (PA2133), Gm^R	
23393	PA14_36260 (PA2200), Gm^R	
32363	PA14_14530 (PA3825), Gm^R	
46879	PA14_12810 (PA3947), Gm^R	
37812	PA14_59790 (*pvrR*), Gm^R	
53749	PA14_30830 (PA2572), Gm^R	
56340	PA14_10820 (PA4108), Gm^R	
55086	PA14_63210 (PA4781), Gm^R	
56214	PA14_27930 (PA2799), Gm^R	
44270	PA14_25770 (PA2960), Gm^R	
48398	PA14_25420 (PA2989), Gm^R	
54293	PA14_20700 (PA3353), Gm^R	
23859	PA14_18550 (PA3542), Gm^R	
27409	PA14_56180 (PA4324), Gm^R	
44974	PA14_60970 (PA4608), Gm^R	

* All constructs were confirmed to be error-free by sequencing at GATC Biotech (Germany).

2.2 Molecular Biology Techniques

Standard methods were performed, if not otherwise indicated, according to the Sambrook & Russel books (Sambrook & Russell, 2001a; Sambrook & Russell, 2001b; Sambrook & Russell, 2001c).

2.2.1 Primers

The primers used in this study are listed in Table 2.2.

Table 2.2 Oligonucleotides used in this study.

Name	Sequence	Comments
Primers for clean deletion mutants		
1PA0847F	GGTCGATCAGGAACGAAGCAC	Mutator for PA0847
2PA0847R	TCAGGCTGGGTGCGAATTGAAATTCGACATTTC	Mutator for PA0847
3PA0847F	AATTCGCACCCAGCCTGAAATGCGCGG	Mutator for PA0847
4PA0847R	GGCAGGAAGTAGGCGAGCTTGC	Mutator for PA0847
5PA0847F	CACCATCTCGTCCACCTCAC	Mutator for PA0847
6PA0847R	ATCATCCAGATGCCGAAGAG	Mutator for PA0847
1wspRF	GCAGCATGTCGACGAGGTAT	Mutator for *wspR*
2wspRR	TCAGGCCCAGTTGTGCATGTTTCTCTCCGG	Mutator for *wspR*
3WspRF	ATGCACAACTGGGCCTGATGGAACAGCCG	Mutator for *wspR*
4WspRR	GATGTCCAGGTAGGCGTTGT	Mutator for *wspR*
5wspRF	GAGGATCGGAATCGTCAATG	Mutator for *wspR*
6wspRR	CTGCATTTCCAGCTCGTACA	Mutator for *wspR*
1PA0338F	GACTTCCTTTCGGTCGGTTC	Mutator for PA0338
2PA0338R	ATCGCGACGCACGACGAAGCGTCC	Mutator for PA0338
3PA0338F	TTCGTCGTGCGTCGCGATTGCGTG	Mutator for PA0338
4PA0338R	CCATCAGCTCTGGCACTACA	Mutator for PA0338
5PA0338F	ATCCTGCTGCCGATGATCT	Mutator for PA0338
6PA0338R	ACTATTCCGGCTTCAACCTG	Mutator for PA0338
1sadCF	TATCGTCGACGCTCATGGTA	Mutator for *sadC*
2sadCR	TTCCAGCTGAGCGGACCGCGGATTTATC	Mutator for *sadC*
3sadCF	CGGTCCGCTCAGCTGGAAAGCTGTTGCGA	Mutator for *sadC*
4sadCR	TCAGGATCGAAAGGCTGCAA	Mutator for *sadC*
5sadCF	ATGGTCGGTACGGTTTTCAG	Mutator for *sadC*
6sadCR	CTCCACAGCAGCAGGTGCT	Mutator for *sadC*
1SadBF	ACTGCGTGCTGCTCTACCAC	Mutator for *sadB*
2SadBR	TCACCCCGGTTCTGTCATGACGAGACCATG	Mutator for *sadB*
3SadBF	ATGACAGAACCGGGGTGACCGGGTAG	Mutator for *sadB*
4SadBR	GGCAAGGACCTGGTGTTCCAGTT	Mutator for *sadB*
5SadBF	CAACGCCAGCCTGATGAT	Mutator for *sadB*
6SadBR	CGTCTTCTCCTGTGGATGCT	Mutator for *sadB*

Name	Sequence	Comments
Primers for expression vectors		
WspRF	CGTGGAACAGCATTCAATTT	*wspR*
WspRR	AAAGATACCCCCGAATGGTC	*wspR*
PA2133F	TTCACACAGGAAACTACAGTGAACGGTTCCCCAC	PA2133
PA2133R	GGAAGGCTGATTGCTCTGTT	PA2134
PA2072F	GCCCCCTGGATGCTTAACTA	PA2072
PA2072R	AAGACGGGAAGGGAATCAGT	PA2072
PA1120F	TTCACACAGGAAACCGTGATGAACCGTCGTC	*yfiN*
PA1120R	AACAGCACCTTGCTCGACAT	*yfiN*
sadCF	TTCACACAGGAAAAGGAGATGAACTGCAGGGC	*sadC*
sadCR	GATCGAAAGGCTGCAACAC	*sadC*
F*araC*-P_{BAD}	AACATATGCGTCAATTGTCTGATTCGTTACCAAT	NdeI, *araC*-P_{BAD}
R*araC*-P_{BAD}	AATCGCTAGCCCAAAAAAACGG	NheI, *araC*-P_{BAD}
pETretSFow	GATAACATATGGACTACAAAGACCATGACGGTGATTATAA AGATCATGATATCGACTACAAAGATGACGACGATAAAGTA CGGCTTCGGATCGCCATAGG	NdeI, 3xFLAG-RetS
pETretSRev	CTATTAAGCTTTCAGGAGGGCAGGGCGT GATAACATATGGACTACAAAGACCATGACGGTGATTATAA	HindIII, 3xFLAG-RetS
pETladSFow	AGATCATGATATCGACTACAAAGATGACGACGATAAACGG CACTGGCTGATTCTCTTTC	NdeI, 3xFLAG-LadS
pETladSRev	CTATTAAGCTTTCAGGCGGACTTGGTGA GATAACATATGGACTACAAAGACCATGACGGTGATTATAA	HindIII, 3xFLAG-LadS
pETgacSFow	AGATCATGATATCGACTACAAAGATGACGACGATAAATTC AAGGATCTCGGCATCAAGGGG	NdeI, 3xFLAG-GacA
pETgacSRev	CTATTAAGCTTTCAGAGTTCGCTGGAGTCGA GATAACATATGGACTACAAAGACCATGACGGTGATTATAA	HindIII, 3xFLAG-GacA
pETgacAFow	AGATCATGATATCGACTACAAAGATGACGACGATAAAATT AAGGTGCTGGTGGTCGACGA	NdeI, 3xFLAG-GacS
pETgacARev	CTATTAAGCTTCTAGCTGGCGGCATCGA CATATGGACTACAAAGACCATGACGGTGATTATAAAGATC	HindIII, 3xFLAG-GacS
pETrsmAFow	ATGATATCGACTACAAAGATGACGACGATAAACTGATTCT GACTCGTCGGGTCG	NdeI, 3xFLAG-RsmA
pETrsmARev	CTATTAAGCTTTTAATGGTTTGGCTCTTGATC	HindIII, 3xFLAG-RsmA
THflPA4332Fxba	*GCTCTAGAG*ATGAACTGCAGGGCATC	XbaI, $sadC^{fl}$
THctPA4332Fxba	*GCTCTAGAG*CAGGGGGCAGGGTACATT	XbaI, $sadC^{ct}$
THPA4332Reco	GGTGAATTCGAGGCACTGGTGACCTCCC	EcoRI, sadC
THsadBFxba	*GCTCTAGAG*ATGACAGAAGCCGCCCTG	XbaI, sadB
THsadBRkpn	AAGGTACCTCACCCCGGCAAGCG	KpnI, sadB
THPA4379Fxba	GCTCTAGAGATGAACAATCCGCCTGT	XbaI, PA4379
THPA4379Rkpn	AAGGTACCTTATTCCTTTCGCAAGATATAGAC	KpnI, PA4379
Primers for site-directed mutagenesis		
WspRmutD70EF	GTGATCCTCCAGGAACTGGTGATGCCCGG	Phosphorylation site
WspRmutD70ER	CCGGGCATCACCAGTTCCTGGAGGATCAC	Phosphorylation site
WspRmutR242AF	CGAGGGCTGCAGTGCCTCCTCGGACCTG	I-site, R242A
WspRmutR242AR	CAGGTCCGAGGAGGCACTGCAGCCCTCG	I-site, R242A
PA2072EF	TCGCCTCGGCGGCAGCGTTGATCCG	EAL, E631A
PA2072ER	CGGATCAACGCTGCCGCCGAGGCGA	EAL, E631A

Name	Sequence	Comments
PA2072GF	GGCTCGGCGGCGCCGCGTTCGTGGTGGT	GGDEF, D505A and E506A
PA2072GR	ACCACCACGAACGCGGCGCCGCCGAGCC	GGDEF, D505A and E506A
sadCmutRxxDF	GCGTTCCTGTCTGGCCGATGGCGACGTC	I-site, R392A
sadCmutRxxDR	GACGTCGCCATCGGCCAGACAGGAACGC	I-site, R392A
Primers for RT-PCR		
IG1PA3348-47F	CGAATACGACACCCTGGCGATG	PA3348/PA3347
IG1PA3348-47R	CCTGGATCTGGATGGTCAAC	PA3348/PA3348
IG2PA3347-46F	GATCCTCGCCATCTCCAACTTCG	PA3347/PA3346
IG2PA3347-46R	CCTGGCGGCTGACGATGGTCGA	PA3347/PA3346
IG3PA3346-45F	GTCTGCTGGTGCGGGTCGAGGA	PA3346/*hptB*
IG3PA3346-45R	AGCACGAAGGTATCCAGCAGG	PA3346/*hptB*
IG4PA3345-44F	AACAGGAACGTCAGCGCTAT	*hptB/recQ*
IG4PA3345-44R	CCTGGAAACACAGGGACTTG	*hptB*/*recQ*
IG5PA3344-43F	AGGAACTCCTCGGCGAAAT	*recQ*/PA3343
IG5PA3344-43R	ATTGGTCGGCATAGAGGTTG	*recQ*/PA3343
IG6PA3343-42F	CAACGACCTGAAGCGTCTCT	PA3343/PA3342
IG6PA3343-42R	GATCAGGCCGAAGTACTGGA	PA3343/PA3342
IG7PA3342-41F	GCGAGCAACTTCAAGCAGAT	PA3342/PA3341
IG7PA3342-41R	AGCAAGGTGCCGAAATAGTG	PA3342/PA3341
IG8PA3341-40F	GTTCGCAACGACGTACTGAC	PA3341/PA3340
IG8PA3341-40R	AGTTCGATATCCGCATCCAG	PA3341/PA3340
Primers for qRT-PCR		
qGyrAF	AACGACTGGAACAAGCCCTACA	*gyrA*
qGyrAR	GCGCACGATGGTGTCGTA	*gyrA*
qPcrVF	AGTGGGATCTGCGCGAGTT	*pcrV*
qPcrVR	TGGGTCTGCAGGACATCCTT	*pcrV*
qHsiA1F	ATCGCCTGCTCGAGTATTACG	*hsiA1*
qHsiA1R	GGGATGAGATTCCTCACGATTT	*hsiA1*
qSiaDF	GCTGGCAATGCTCGATGTG	PA0169
qSiaDR	TGTCGTGGCCCCAGGTAT	PA0169
qPA0290F	GGATACCGTTGGCGTTCCT	PA0290
qPA0290R	AGCGGTCGTTGATCTTCTTGA	PA0290
qPA0338F	GAGCGTGGAGACCTGGAAGA	PA0338
qPA0338R	GCAGTATTCCGGGCTTTCG	PA0338
qPAKPA0847F	TCGGCGGTATCTGGCTCTT	PA0847
qPAKPA0847R	CTGGTCCTCTCGGTCGAAAC	PA0847
qPA1107F	AACGCAAGCTGGAGTTGCA	PA1107
qPA1107R	CAGGTACTCCAACTGGCGATTC	PA1107
qPA1120F	CGCCTGGCGGAACTCA	PA1120
qPA1120R	GGGTAGCACTGTCTATCTCCTTTTCT	PA1120
qPA1851F	GGCTGGTCTGCCGGATAAT	PA1851
qPA1851R	ACCGCAACCAACGAAAGC	PA1851
qPA2771F	GCACCAGCATGAACTGGAGTT	PA2771
qPA2771R	TTGAAATGGTCGATATCGCTGTA	PA2771
qPAKPA2870F	CCAGGCGGAGCTCATGCT	PA2870

Name	Sequence	Comments
qPAKPA2870R	GATGTTCTTGAAGTGGTCGATGTC	PA2870
qPelDF	ACCGATGGGCGGATCCT	PA3061
qPelDR	CAGGCTGAAGGTACGTTCGTT	PA3061
qPA3177F	CGTGGCGGAAACCATCA	PA3177
qPA3177R	CGAAGCGGAACACCATGTC	PA3177
qPA3343F	TGCTACCTGCTGGAGTTCAAGTC	PA3343
qPA3343R	GCCATCACCCGCAACAG	PA3343
qWspRF	GCGGTCATGGTACTGCTTGTC	PA3702
qWspRR	CGGAACAGAAATGGAAGTCGAT	PA3702
qPAKSadCF	GCAGCAGCGTCTCGAGCTA	*sadC*
qPAKSadCR	CCAGACCATTACGATGAACATCA	*sadC*
qPA4396F	ATGGACGGCCTGGAACTG	PA4396
qPA4396R	GGTGTAGTGGTTGATGCTTTCG	PA4396
qPA4843F	CGAGCCACTGTCGGTGATG	PA4843
qPA4843R	AGGCATTCGGGCATGTACAT	PA4843
qPA4929F	TTCAAGCCGATCAACGATACC	PA4929
qPA4929R	CCACGTTCTGCAGGACGAA	PA4929
qPA5487F	GACCACTTCAAGCGGATCAAC	PA5487
qPA5487R	AACCGTTTGCGCAATTCG	PA5487
qPA0285F	CGCCTGAGCCTGGCTTATC	PA0285
qPA0285R	TGTAACAAATGATCGCTCAGGAA	PA0285
qPA0575F	CAAGACCGGCGAGCTGTAC	PA0575
qPA0575R	GGTGGAGATATCGGCGAAGA	PA0575
qPA0861F	GCCGGTGGATTACCTGAAGA	PA0861
qPA0861R	GATGGGATCCTCGAGCATGT	PA0861
qPA1181F	CCGGCCTGAGTTCCTTCA	PA1181
qPAKPA1181R	AGCTACCGTCGATCTTGATC	PA1181
qPA1433F	CCGTTGATCGCGGAACA	*lapD*
qPA1433R	AAACCCATTTCCCGCAGTACT	*lapD*
qPA1727F	CGGAACGGCTACTGCTTCTT	*mucR*
qPA1727R	GCAGGTCATGCAGTAGTTGCA	*mucR*
qPA2072F	TCCTGAAACGCAGCAACCA	PA2072
qPA2072R	CGAAATGCCCGTCGACTT	PA2072
qPA2567F	CGTATCGGCATCGGACATG	PA2567
qPA2567R	GCGAGTCTGACGGCCTCTT	PA2567
qPA3258F	AACTGGAACTACTCTGCCGACAA	PA3258
qPAKPA3258R	GGGAGACGTTGAGGAACAGCAT	PA3258
qPA3311F	AGGGCCTGGCGGTGAT	PA3311
qPA3311R	GCCGAGGGAATCGTTGATC	PA3311
qPA4367F	CATCTTCATCTCCGGCATTCTC	*bifA*
qPA4367R	TTGGTCAGCATCCAGTGGTAGA	*bifA*
qPA4601F	GAAGGCCGTGAATTCTTCGT	*morA*
qPA4601R	TGGAAGTTGTTCTTGCCCATCT	*morA*
qPA4959F	CCTTCGTCCAGGACCTCAAC	*fimX*
qPA4959R	GCTTCTGCTGTTCGTGCAGTT	*fimX*

Name	Sequence	Comments
qPA5017F	CAGCCGCCGCAAGAAC	*dipA*
qPA5017R	ATGTCTTCGTAGATGCCGATGTAG	*dipA*
qPA5295F	CCGGTTTCTCGTCCTTCGT	PA5295
qPA5295R	CTGCGGTCGATCTTCAACAG	PA5295
qPA5442F	CGATCCCTATCCCCAATGG	PA5442
qPA5442R	GGGCACTGAGATCGGTGAA	PA5442
Sequencing/screening primers		
M13F	TGTAAAACGACGGCCAGT	GATC Biotech
M13R	CAGGAAACAGCTATGACC	GATC Biotech
pUT18CUp	CGGATGTACTGGAAACGGTG	(Houot *et al.*, 2012)
pUT18Dn	TGCGGAACGGGCGCCGGCGCGAGCG	(Houot *et al.*, 2012)

2.2.2 DNA purification, extraction and quantification

Genomic DNA was isolated using the PureLink Miniprep kit (Invitrogen). Plasmid DNA was isolated using the QIAprep Spin Miniprep and Midiprep Kits (Qiagen). PCR fragments were purified using QIAquick PCR Purification Kit (Qiagen). Digested DNA fragments were extracted from agarose gels using QIAquick Gel Extraction Kit (Qiagen). DNA concentrations were determined by using the ND-1000 Spectrophotometer (NanoDrop).

2.2.3 Polymerase chain reaction (PCR)

PCR was performed using primers purchased from Eurogentec.

For cloning purposes, fragments were amplified from PAO1 gDNA (genomic DNA). For up to 2kb, the Expand High Fidelity^{PLUS} DNA polymerase from Roche was used. For bigger fragments, the KOD Hot Start DNA Polymerase (Merck) was used. For site-directed mutagenesis, the *Pfu*UltraII Fusion HS DNA Polymerase from Agilent Technologies was used as indicated in the QuickChange Site-directed Mutagnesis Kit (Stratagene). In each case, the PCR reactions and thermal cycling conditions used were according to the manufacturer's instructions. In all cases, 1.2 M betaine (Sigma) was added to the PCR reaction because of the high GC content of the *P. aeruginosa* genome.

Colony PCR was used for screening purposes using the *Taq* polymerase from New England BioLabs, Inc. Single *E. coli* and *P. aeruginosa* colonies were resuspended in 100 µL of molecular biology water and lysed by one or two cycles, respectively, of freeze (-80^{o}C, 10 min) and thaw (95^{o}C, 10 min). Cell debris were pelleted and 5 µL of the supernatant were used in each PCR reaction (30 µL of final volume). DMSO was added to a final concentration of 3.3%. The fragments were amplified in 30 cycles (95^{o}C for 30 sec, 55^{o}C for 30 sec, 72^{o}C for 1 min/kb) after an initiation step of 5 min at 95^{o}C.

RT-PCR (reverse transcription PCR) was performed using SuperScript III Rnase H-Reverse Transcriptase (Invitrogen) and according to instructions. cDNA (complementary DNA) was synthesized from 200 ng of RNA template (extracted as described below) adding 20 Units of Protector RNase Inhibitor from Roche, 10 pmol of $Pd(N)_6$ random hexamer oligonucleotides from Amersham and 10 pmol dNTPs from Bioline to the reaction mix.

2.2.4 Sequencing

Sequencing was performed at GATC Biotech, Germany.

2.2.5 Restriction endonuclease digestion and DNA ligation

Restriction enzymes from Roche and T4 DNA Ligase (Roche) were used as recommended by the manufacturer. PCR fragments were ligated into pCR2.1 or pCR-Blunt using the TA Cloning and Zero Blunt Cloning kits (Life Technologies).

2.2.6 Bacterial transformation

E. coli TOP10 and OmniMAX chemically competent cells were used for standard genetic manipulations. TOP10 cells were purchased from Invitrogen and used according to the specifications. OmniMAX, CC118λpir, Sm10λpir, XL1-Blue, DHM1, BL21 (DE3) and BL21 (DE3) C41 competent cells were home-made and prepared using a solution of 50 mM $CaCl_2$ with 15% glycerol. When appropriate, IPTG (iso-propanyl-β-D-thiogalactopyranoside, Sigma) and X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside, Invitrogen) were added to the medium and blue-white screening was performed.

P. aeruginosa electrocompetent cells were used for incorporation of non-conjugative plasmids. Cells were prepared at room temperature with 300 mM sucrose and the electric pulse was applied at constant 2.5 kV.

2.2.7 Agarose gel electrophoresis

DNA was visualized in a blue light transilluminator (Peqlab) after electrophoresis on an agarose gel supplemented with SYBR Safe (Invitrogen). 1% gels were used to separate DNA fragments between 500bp and 3kb. Fragments bigger than 3kb were separated in 0.8% gels whereas fragments smaller than 500bp were separated in 1.2% gels.

2.2.8 RNA extraction

Overnight PAK and PAKΔ*retS* cultures were subcultured in LB medium with a starting OD_{600nm} of 0.1 and incubated at 37°C with shaking for 6 h. Cells were then harvested into RNA*later* (Ambion) and RNA was extracted using the RNeasy extraction kit (Qiagen). To remove DNA the Turbo DNA-free kit (Applied Biosystems) was used and the RNA was re-purified using the RNeasy kit, following the supplier's indications.

2.2.9 qRT-PCR (real time reverse transcription PCR)

qRT-PCR was performed on the ABI 7300 Real Time PCR System using the ABI SYBR Green PCR Master Mix. cDNA was synthesized by RT-PCR as described above.

2.2.10 SDS-PAGE and Western blot

For immunoblot analysis, overnight cultures were subcultured in LB medium with a starting OD_{600nm} of 0.1 and incubated at 37°C with shaking for 6 h unless otherwise specified. To standardize samples, the volume of culture harvested was equal to $1.5/OD_{600}$ and cells were resuspended in loading buffer containing 4% glycerol, 5% β-mercaptoethanol, 0.06 M Tris pH 6.8, 2% SDS and 0.002% bromophenol blue. Whole cell lysates were separated by SDS-PAGE using a 12% or 8% resolving gel. Proteins were electrophoretically transferred to nitrocellulose membranes (Whatman) and blocked overnight in 1x TBST (0.5 M Tris pH 8.0, 1.5 M NaCl, 0.1% Tween-20) containing 5% skim milk powder. Primary antibodies, α-Hcp1 (Hachani *et al.*, 2011), α-LasB (Bleves *et al.*, 1999) and α-VgrG1 (Hachani *et al.*, 2011) were used at dilutions of 1:1000, and α-PcrV (Lee *et al.*, 2010b), α-FLAG (Sigma) and α-HIS (Sigma) were used at 1:10000. Secondary antibodies horseradish peroxidise-conjugated goat anti-rabbit IgG (Sigma) or anti-mouse IgG (Sigma) were used at a 1:5000 dilution. Visualization was achieved using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce) and a LAS3000 Imaging System (Fuji).

2.3 Three-partner conjugation

Transfer of plasmids into *P. aeruginosa* strains was carried out by triparental mating using the mobilizing plasmid pRK2013. Prior to the addition of the receiver strain to a co-culture of the donor and helper strains, the receiver strain was incubated for 2 h at 43°C to inactive its restriction

modification system and prevent the degradation of the foreign plasmid. Conjugation was then allowed for at least 4 h at 37^{o}C by co-culturing the three strains on an agar plate. Transconjugants were isolated on Pseudomonas Isolation Agar (PIA, Difco) plates supplemented with appropriate antibiotics.

2.4 Construction of *P. aeruginosa* chromosomal mutants

Clean deletion mutants were engineered by using the suicide vector pKNG101 which contains the *sacB* gene conferring sensitivity to sucrose (Kaniga *et al.*, 1991). Mutator fragments were constructed by PCR amplification of upstream and downstream fragments of approximately 500bp flanking the chromosomal region to be mutated. The two fragments were then joined together using overlap extension PCR (Warrens *et al.*, 1997) and the product was cloned into pCR2.1 to check sequence before being sub-cloned into pKNG101. The first crossover event occurred upon conjugation and was selected for using Sm. The second crossover event occurred upon growth on LB agar plates supplemented with 5% sucrose after 3 days incubation at room temperature. Deletions were confirmed using external primers designed to anneal to the flanking region of the mutator fragment.

2.5 Swimming motility

Swimming motility was assessed in freshly poured 0.3% LB agar plates incubated at 30^{o}C. Plates were prepared by diluting the commercial 1.2% LB agar with warm LB. Plates were inoculated by injecting 0.5 µL of overnight liquid cultures below the surface of the agar.

2.6 Biofilm assays

The formation and dispersal of biofilms was evaluated using microtiter plates and microfermentors. For a robust and quick visualization of biofilms, glass test tubes were used.

2.6.1 Microfermentors

M9 medium with 0.1% glucose was used to grow biofilms in microfermentors (Pasteur Institute). This method is described in more detail in Appendix 3 ("Methods for studying biofilm dispersal in Pseudomonas aeruginosa" in *Methods in Pseudomonas*, in press). The amount of cells of overnight cultures equivalent to an OD_{600} of 2 was used as the inoculum. After an initial attachment period of 1 h, strains were incubated for a minimum of 20h at 37°C under a continuous flow of medium (peristaltic pump: 4 rpm) and air (air pressure: 0.4 bar). Biofilm dispersal was induced by changing from growth medium to medium supplemented with 10 mM $NaNO_2$ or prepared without glucose. The optical density of the effluent was monitored every 10 min during the dispersal treatment.

2.6.1 Microtiter plates (for screening and time-course)

Quantification of biofilm formation was performed in 24-well polystyrene microtiter plates. M9 medium (1 mL/well) with 0.4% glucose and antibiotics when appropriate was inoculated to a final OD_{600nm} of 0.01. The plates were incubated for different time periods at 37°C under shaking conditions. Biofilms were stained with 100 µL of crystal violet (CV) and washed twice with water before being solubilized in 96% ethanol. CV staining was measured by reading the optical density at 600 nm.

For screening purposes, strains were inoculated in 48-well polystyrene microtiter plates (600 µL/well, starting OD_{600} of 0.01) for 6 h at 37°C under shaking conditions. Biofilm dispersal was induced by the addition of freshly made 10 mM $NaNO_2$. After 30 min the supernatant was collected in a disposable cuvette and the optical density of the planktonic biomass was measured. Biofilms were stained with 0.1% CV for 10 min and washed and dissolved as above. The optical density of the CV staining biofilm biomass was measured at 600 nm. Three replicates were used per condition (treated versus untreated) and for each strain at least three independent experiments were carried out. The calculation of the biofilm remaining after dispersal induction corresponds to the ratio between the biofilm present in treated wells and the biofilm present in untreated wells at the end of the experiment.

In both cases, microtiter plates were wrapped in cling film and aluminium foil to avoid spatial variations on biofilm formation.

2.6.2 Glass test tubes (for visualization)

Visualization of biofilms was carried out in 14 mL borosilicate tubes. 3 mL of LB supplemented with antibiotics when appropriate were inoculated with 1 µL of overnight cultures and incubated at 37°C for different time periods, under static or shaking conditions, as specified. Biofilms were stained with 300 µL of CV and tubes were washed with water twice to remove unbound dye before being photographed.

2.7 Congo Red binding

To assess exopolysaccharide production, Congo Red binding assays were performed at 30^{o}C using plates with 1% agar and 1% tryptone. The dyes Congo Red and Coomassie Brilliant Blue were used at the following concentrations: 40 µg/mL and 20 µg/mL, respectively. Plates were inoculated by spotting 5-10 µL of overnight liquid cultures. After the first day of incubation at 37^{o}C, plates were left at room temperature for at least 5 days.

2.8 c-di-GMP detection

The detection of c-di-GMP was performed using two methods: nucleotide extraction followed by LC-MS/MS, and measurement of fluorescence produced by a c-di-GMP reporter.

2.9.1 Extraction and measurement by LC-MS/MS

Nucleotide extraction was performed as previously described (Simm *et al.*, 2009), with some modifications. Bacteria from 100 mL of overnight liquid cultures grown in M9 medium supplemented with nutrients and appropriate antibiotics were resuspended in ice-cold 0.19 % formaldehyde to a final concentration of 300 mg wet weight per mL. This suspension was divided into 4 tubes and incubated on ice for 10 min before centrifugation (5000x *g* for 15min). The pellet was then resuspended in 300 µL of water and when required, c-di-GMP (BioLog) was spiked at a concentration of 1.7 µM. After the ethanol extraction of nucleotides, samples were dried under vacuum and sent to the University of Nottingham at -20^{o}C. The pellets were reconstituted in 50 µL of water by vigorous vortexing and sonication for 5 min, centrifuged for 15 min at 10,000× *g* and the

supernatants were analyzed using an Agilent 1200 HPLC system with aC18 ACE 3 AQ 150×2.1 mm column with a matching guard maintained at 55°C. Themobile phase consisted of 0.1% formic acid run in an H_2O-acetonitrile gradient at a flow rate of 300 µl/min, reaching 50% acetonitrile in 14 min. Mass spectrometry analysis was then performed by negative ion electrospray from the Agilent HPLC system into a Bruker HCT Plus ion trap in multiple reaction mode. Using SmartFrag (HyStar software), the trap was set to isolate from full scan and then fragment ions at m/z 688.9, with a monitored mass range of 450-700 m/z. DataAnalysis v3.3 was used to interrogate the acquisitions, and extracted ion chromatograms of m/z 538 were produced from the negative ion MS/MS of m/z 688.9. Retention times and peak spectra were matched to the standard injected at intervals throughout the run. Peak areas and relative c-di-GMP levels were normalized to the levels obtained with the reference strains.

2.9.2 c-di-GMP reporter

The reporter P_{cdrA}-*gfp* plasmid received from Matthew Parsek (Rybtke *et al.*, 2012) was introduced in the *P. aeruginosa* strains of interest by electroporation and overnight cultures were subcultured in LB medium supplemented with the appropriate antibiotic to a starting OD_{600nm} of 0.1. After shaking incubation at 37°C for 6 h or other specified time, 1mL of culture was harvested in a microcentrifuge at maximum speed and cells were resuspended in 1xPBS (phosphate buffered saline) before the optical density (600nm) and fluorescence (excitation 485nm, emission 520nm) was measured in a black 96-well plate with see-through bottom (Falcon) using a FLUOstarOptima plate reader (BMG Labtech). Quantifications were performed in triplicate and data are presented as relative fluorescent units (RFU) which are arbitrary fluorescent units corrected for cell density.

2.9 c-di-GMP binding assays

The ability of proteins of interest to bind c-di-GMP was assessed from whole cell lysates using two methods: the c-di-GMP capture compound and the DRaCALA assay.

2.9.1 c-di-GMP capture compound

This assay was carried out at the Biozentrum (University of Basel) applying the methods developed by Jutta Nesper (Nesper *et al.*, 2012). Overnight liquid cultures grown in LB with appropriate

antibiotics were diluted 1:100 and grown to an OD_{600} of 0.5 before a 3 h induction with 0.5mM IPTG at 37°C. Cells were pelleted by centrifugation (5000x *g* for 20min) and resuspended in lysis buffer [6.7 mM MES (2-(*N*-morpholino)ethanesulfonic acid), 6.7 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), 200 mM NaCl, 6.7 mM KAc, 10 mM β-mercaptoethanol] supplemented with DNaseI (Roche) and protease inhibitor (Roche). Cells were lysed using a French press at 20 000 psi. After ultracentrifugation (10 000x *g* for 60min) the protein concentration in the supernatant was quantified using a ND-1000 Spectrophotometer (NanoDrop). The incubation with the capture compound was performed in 12-tube PCR strips for 1 h at 4°C on a rotatory wheel. The final volume was 100 µL and 100 µg of the extract plus 20 µL of 5x capture buffer (100 mM HEPES, 250 mM KAc, 50 mM MgAc, 50% glycerol) were used. The c-di-GMP capture compound (Caprotec Bioanalytics GmbH) was added at a concentration of 10 µM. Cross-linking was induced with UV light for 4 min in a caproBox (Caprotec Bioanalytics GmbH) and subsequently, 30 µL of magnetic streptavidin beads (Dynabeads MyOne Streptavidin C1, Invitrogen) together with 25 µL of 5x washing buffer (250 mM Tris pH 7.5, 5 M NaCl, 0.1% n-ocyl-β-glucopyranoside) were added. After incubation for 30 min at 4°C on a rotatory wheel the beads were collected with a caproMag (Caprotec Bioanalytics GmbH) and washed six times with 1x washing buffer. For immunoblot analysis, the beads were resuspended in 30 µL of SDS-PAGE loading buffer and the experiment proceeded as described in section 2.2.10. For membrane proteins, the following modifications applied: the IPTG induction was performed at 30°C and the collected cells were resuspended in 1x PBS with protease inhibitor and then lysed. After ultracentrifugation (10 000x *g* for 60 min), the pellet was resuspended in lysis buffer supplemented with n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) and the proteins were solubilized for 3 h at 4°C on a rotatory wheel. In addition, the incubation with the capture compound was performed overnight using 200 µg of extract and the n-ocyl-β-glucopyranoside in the washing buffer was replaced by 0.1% DDM.

2.9.2 DRaCALA

DRaCALA assays were performed as previously described (Roelofs *et al.*, 2011). Briefly, radioactive labeled c-di-GMP was synthesized using purified $WspR^{D70E}$ and α-^{32}P-GTP (PerkinElmer) and isolated using a Nanosep column (Pall). Overnight liquid cultures grown in LB with appropriate antibiotics were diluted 1:50 and grown for 6 h or to an OD_{600} of 0.5 before a 3 h induction with 0.5mM IPTG. OmniMAX cells were used to express pBBR1-MCS4 derivatives, BL21 (DE3) cells were used for soluble proteins expressed from pET28a, and BL21 (DE3) C41 cells were used for membrane proteins expressed from pET28a. Whole-cell lysates were prepared in 1x c-di-GMP binding buffer prepared with 100 mM KCl, 5 mM $MgCl_2$, 100 mM Tris (pH 8.0), 100 µM PMSF, 500 µg/mL lysozyme and 20

µg/mL of DNaseI. 10 µL of lysate were mixed with 4 nM radioactive labelled c-di-GMP and incubated for 10 min at room temperature. The mixtures were then spotted (~2.5 µL) in triplicate on dry untreated nitrocellulose membranes (GE Healthcare) and allowed to dry. An FLA7100 Fujifilm Life Science PhosphorImager was used to detect luminescence following a 5 min exposure of blotted nitrocellulose to phosphorimager film. Data were quantified using Fujifilm Multi Gauge software v3.0.

2.10 Bacterial two-hybrid assay

Protein interactions were assessed by using a bacterial two-hybrid approach following the BACTH System Kit's methodology (Euromedex).

2.10.1 Interactions between target proteins

DNA fragments encoding the protein of interest were cloned into pKT25 or pUT18C vectors for N-terminal fusions to the T25 or T18 fragments, respectively, of the CyaA protein of *Bordetella pertussis*. For C-terminal fusions, the plasmids pKNT25 and pUT18 were used. The AC deficient *E. coli* strain DHM1 was used to screen for positive interactions and the interaction between the leucine zipper domains expressed from the pKT25-zip and pUT18C-zip plasmids was used as control. DHM1 competent cells were transformed simultaneously with the pKT25/pKNT25 and pUT18C/pUT18 derivatives and co-transformants were selected on LB agar plates supplemented with Ap and Km. Interactions were identified by spotting 5 µL of overnight cultures onto MacConkey (Difco) agar plates supplemented with antibiotics (100 µg/mL Ap and 50 µg/mL Km) and with 1% of maltose and 1 mM of IPTG. Positive interactions resulted in the appearance of red colonies due to the acidification of the medium by the cAMP-dependent degradation of maltose. The colour was checked after incubation for 48 h at 30^{o}C.

2.10.2 Screen of the two hybrid genome fragment PAO1 library

This assay was carried out at the CNRS in Marseille with the guidance of Christopher Bordi. The library prepared by Houot *et al.* (2012) was tested four times by transforming 10 µL of the library into 100 mL of chemically competent DMH1 cells carrying the pKT25-SadC^{fl} bait plasmid. The transformations were plated on MacConkey agar plates supplemented as above and the colour was

monitored during incubation for 48h at 30^{o}C followed by 5 days at room temperature. Red colonies were picked up and re-streaked to confirm interaction prior to plasmid isolation and sequencing.

2.11 Infection of *Galleria mellonella* with *P. aeruginosa*

Infections were performed as previously described (Miyata *et al.*, 2003), with some modifications. Overnight liquid cultures grown in LB were diluted 1:100 and grown to an OD_{600} of 0.3 to 0.4. Cells were harvested in a microcentrifuge and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.1. Six serial 10-fold dilutions were made in 10 mM $MgSO_4$ and 5 µL of each were injected into *G*. *mellonella* larvae (www.livefoods.co.uk) with a Hamilton 802N syringe (Sigma), in the right proleg of the first pair of prolegs. Ten larvae were injected per dilution and then incubated at 37°C in standard Petri dishes covered with Whatman paper. The syringe was cleaned with 96% ethanol for 10 min and rinsed three times with water when changing strains. The number of dead larvae, i.e. larvae that did not respond to touch, was counted 1 to 2 days after infection. Mock inoculations were performed to monitor killing due to physical injury or infection by other pathogens.

2.12 Confocal microscopy

The LSM510 Confocal microscope (Zeiss) was used to visualize strains expressing the P_{cdrA}-*gfp* reporter. Bacteria were grown as described above for the c-di-GMP reporter and washed once with 1xPBS before spotting 5 µL on a glass slide. Samples were dried for 1 h at 37^{o}C prior to visualization. The image is 20x amplified.

2.13 Bioinformatic tools

PAO1 and PA14 sequences were retrieved from the Pseudomonas database (www.pseudomonas.com) (Winsor *et al.*, 2009). The PAK sequence is unpublished and was obtained from Marvin Whiteley and manipulated using the Artemis genome browser (Rutherford *et al.*, 2000).

Primers were designed with the help of Primer3 (Untergasser *et al.*, 2012), OligoEvaluator (Sigma) and QuikChange Primer Design Program from Agilent Technologies. Sequence alignments were performed using NCBI-BLAST (Altschul *et al.*, 1990). Sequencing results were analysed using Chromas Lite (Technelysium Pty Ltd).

Mfold web server (Zuker, 2003) was used to predict RNA secondary structures and Phyre2 (Kelley & Sternberg, 2009) to predict protein three dimensional structures and performe the BackPhyre analysis. PyMOL (Schrödinger, LLC) was used to visualize three dimensional structures.

2.14 Statistical analysis

The Students *t*-test and ANOVA with Tukey's posthoc test were used throughout and unless otherwise indicated. The software used was Microsoft Excel 2010 and SPSS (Statistical Package for Social Sciences, IBM), respectively.

3) Cyclic di-GMP modulation of biofilm formation and dispersal

Chapter 3 – Cyclic di-GMP modulation of biofilm formation and dispersal

In the past decade, c-di-GMP has emerged as a signalling molecule that modulates the switch between motile and sessile lifestyles in bacteria (Hengge, 2009). In *P. aeruginosa*, which has been used as a model organism to study c-di-GMP regulation, a link between c-di-GMP and biofilm formation or dispersal is now well established, but the environmental cues or signalling cascades controlling these phenomena are still poorly understood.

In *P. aeruginosa*, the c-di-GMP network is composed of many components including proteins with a GGDEF, EAL or HD-GYP domain. In order to understand the complexity and specificity of this c-di-GMP signalling network, I started by setting up a microtiter plate (MTP) assay suitable for the study of both biofilm formation and dispersal. Subsequently, I used this assay to screen a collection of 42 transposon mutants for altered biofilm formation or dispersal phenotypes.

3.1 c-di-GMP influences *P. aeruginosa* lifestyle

The paradigm of c-di-GMP regulation is that high levels of c-di-GMP repress motility and promote biofilm formation whereas low levels of c-di-GMP do the opposite (Jenal & Malone, 2006). To confirm this assumption, two genes involved in the production or degradation of c-di-GMP were cloned into the plasmids pJN105 or pBBR1-MCS-4. The two cloned genes were *wspR,* encoding a known DGC that synthesizes c-di-GMP (Figure 1.9, Table 1.5) (De *et al.*, 2009; Hickman *et al.*, 2005); and PA2133, encoding a known PDE that hydrolyzes c-di-GMP (Table 1.5) (Kulasakara *et al.*, 2006). The pJN105 plasmid contains an arabinose inducible promoter that allows for tight regulation of the expression of the cloned genes by the addition of the repressor D-glucose or the inducer L-arabinose (Newman & Fuqua, 1999). The pBBR1-MCS4 plasmid allows for constitutive expression of the cloned gene from the promoter of the *lac* operon (Kovach *et al.*, 1995). To investigate the impact of c-di-GMP in biofilms, the constructed plasmids were conjugated into the *P. aeruginosa* strains PAO1 and PAK. Biofilms were visualized and quantified by measuring crystal violet (CV) staining of the attached biomass. CV is one of the dyes used in the Gram technique and is able to bind to cell surface molecules and extracellular matrix components.

As shown in Figure 3.1A, the production of WspR (induced by the addition of increasing concentrations of L-arabinose) resulted in enhanced biofilm formation when compared to the strain carrying the pJN105 empty vector. In contrast, overexpression of PA2133 displayed a hypobiofilm phenotype. This hypobiofilm phenotype was independent of the addition of D-glucose or L-arabinose suggesting that the expression of PA2133 is leaky. Nonetheless, these results are according to the established paradigm that low levels of c-di-GMP prevent biofilm formation whereas high levels of c-di-GMP promote the sessile lifestyle.

To further investigate the impact of c-di-GMP in the lifestyle switch, the gene encoding for WspR was subjected to site-directed mutagenesis. Firstly, the I-site of WspR was mutated in order to release the catalytic activity of WspR from c-di-GMP inhibition. This corresponded to an A mutation of the R residue located in the RxxD motif, yielding $WspR^{R242A}$. Secondly, and in addition to the I-site mutation, the phosphorylation site of WspR was also mutated in order to render the protein constitutively active. This corresponded to an E mutation of the D residue in the N-terminal REC domain, yielding WspR**.

As expected, when the plasmid encoding for the wild-type WspR was conjugated into PAK, the resulting strain displayed a hyperbiofilm phenotype and this phenotype was more pronounced in the strain overproducing $WspR^{R242A}$, suggesting that the higher the levels of c-di-GMP are, the stronger the biofilm phenotype is (Figure 3.1B). In agreement to this induction of the sessile lifestyle, the strains also displayed increased Congo Red binding which is indicative of exopolysaccharide production; and reduced swimming motility which is indicative of reduced flagella rotation (Figure 3.1C).

In relation to the constitutively active WspR** which has the phosphorylation and I- sites mutated, conjugation of p*wspR*** was only possible using the pJN105 derivative when the repressor D-glucose was added to the medium. No bacterial growth was observed when the production of WspR** was induced by arabinose, suggesting that there is a threshold when c-di-GMP becomes deleterious to the cells and it is possible that this is due to depletion of the precursor GTP from the cell (Figure 3.1D).

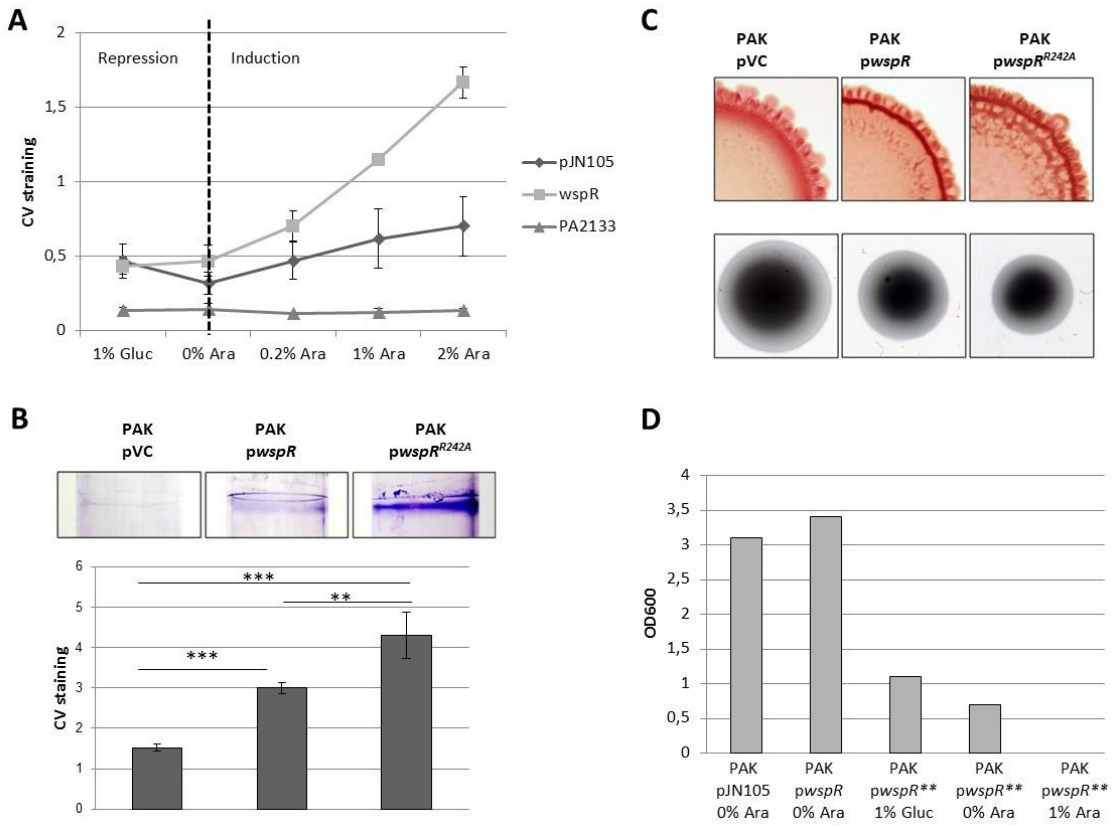

Figure 3.1 Production of the DGC WspR promotes biofilm formation whereas production of the PDE PA2133 inhibits the sessile lifestyle.

(A) Expression of *wspR* or PA2133 has antagonist effects on biofilm formation. PAO1 was conjugated with the control plasmid pJN105 and its derivatives encoding for WspR and PA2133. Biofilm formation was quantified by CV staining after growth in LB for 8 h under shaking conditions in a MTP. Expression was controlled by adding D-glucose (Gluc) or increasing concentrations of L-arabinose (Ara) at the time of inoculation. Results are the average of 6 technical replicates and error bars represent standard deviation. Two independent experiments were performed. **(B)** Disruption of the I-site (R242A) enhances biofilm formation. PAK was conjugated with the vector control pBBR1-MCS4 (pVC) and its derivatives p*wspR* and p*wspR*^{R242A} encoding for WspR and WspR^{R242A}, respectively. In the top, biofilms formed in glass tubes at the air-liquid interface under static conditions are visualized by crystal violet staining. In the chart, biofilms formed in a MTP under shaking conditions were quantified by crystal violet staining. An ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes. At least three independent experiments were performed. **(C)** Enhanced biofilm formation is concomitant with enhanced Congo Red binding (top images) and reduced swimming motility (bottom images). **(D)** Expression of the constitutively active WspR** inhibits bacterial growth. WspR** produced from pJN105-*wspR*** harbours the I-site mutation (R242A) and a D70E mutation that mimics the phosphorylated stated of the D residue in the REC domain. In this case, optical density was measured from overnight liquid cultures grown at 37^{o}C. The experiment was repeated three times with one technical replicate in each case.

3.2 Nitric oxide and glucose starvation induce biofilm dispersal

In the biofilm developmental cycle, biofilms disperse so that motile cells are released from the biofilm to colonize new areas. In the same way that high levels of c-di-GMP are required for motile cells to form biofilms, it is thought that low levels of c-di-GMP are required for biofilm cells to disperse. However, compared to biofilm formation, the molecular mechanisms underlying biofilm dispersal are not well understood.

Several studies have identified specific growth conditions and environmental cues that can induce biofilm dispersal including (i) up- or down-shifts in carbon sources (Sauer *et al.*, 2004; Schleheck *et al.*, 2009), (ii) addition of the chelating agent EDTA (Banin *et al.*, 2006), (iii) oxygen limitation (An *et al.*, 2010), (iv) addition of the *P. aeruginosa* fatty acid cis-2-decenoic acid (Davies & Marques, 2009), (v) addition of nitric oxide donors (Barraud *et al.*, 2006; Barraud *et al.*, 2009), (vi) availability of iron (Glick *et al.*, 2010; Musk *et al.*, 2005), (vii) production of the rhamnolipid surfactant (Boles *et al.*, 2005; Schooling *et al.*, 2004), (viii) addition of a reverse amide oroidin analogue (Richards *et al.*, 2008) and (ix) addition of D-aminoacids (Kolodkin-Gal *et al.*, 2010).

Herein, two treatments were initially considered for the study of biofilm dispersal: glucose starvation, induced by changing the growth medium to medium without any carbon source; and exposure to nitric oxide, achieved by addition of nontoxic concentrations of sodium nitrite which spontaneously generate nitric oxide in solution. Both of these treatments are thought to mimic conditionsthat are not favourable to the bacteria, i.e. nutrient and oxygen depletion respectively. Before setting up the MTP assay, the dispersal response to the two treatments was studied in microfermentors (MFs) (Figure 3.2A).

MFs were developed by the group of Jean-Marc Ghigo and consist of glass vessels where biofilms grow on a spatula under a continuous flow of medium and air (see Appendix 3) (Barraud *et al.*, 2013; Garcia-Sanchez *et al.*, 2004; Ghigo, 2001). For *P. aeruginosa* PAO1 strain, macroscopic growth of biofilms in the MFs was visible on the spatula after 1 day of incubation while for PA14, biofilms were visible only after 3 days but covered the spatula and the walls of the glass vessel. To induce biofilm dispersal, the glucose supplemented M9 minimal medium was changed to medium without glucose (no carbon source) or medium containing sodium nitrite (nitric oxide donor). To monitor biofilm dispersal, i.e. the release of cells from the biofilm, the effluent was collected in Falcon tubes every 10 min and readings of the optical density were taken.

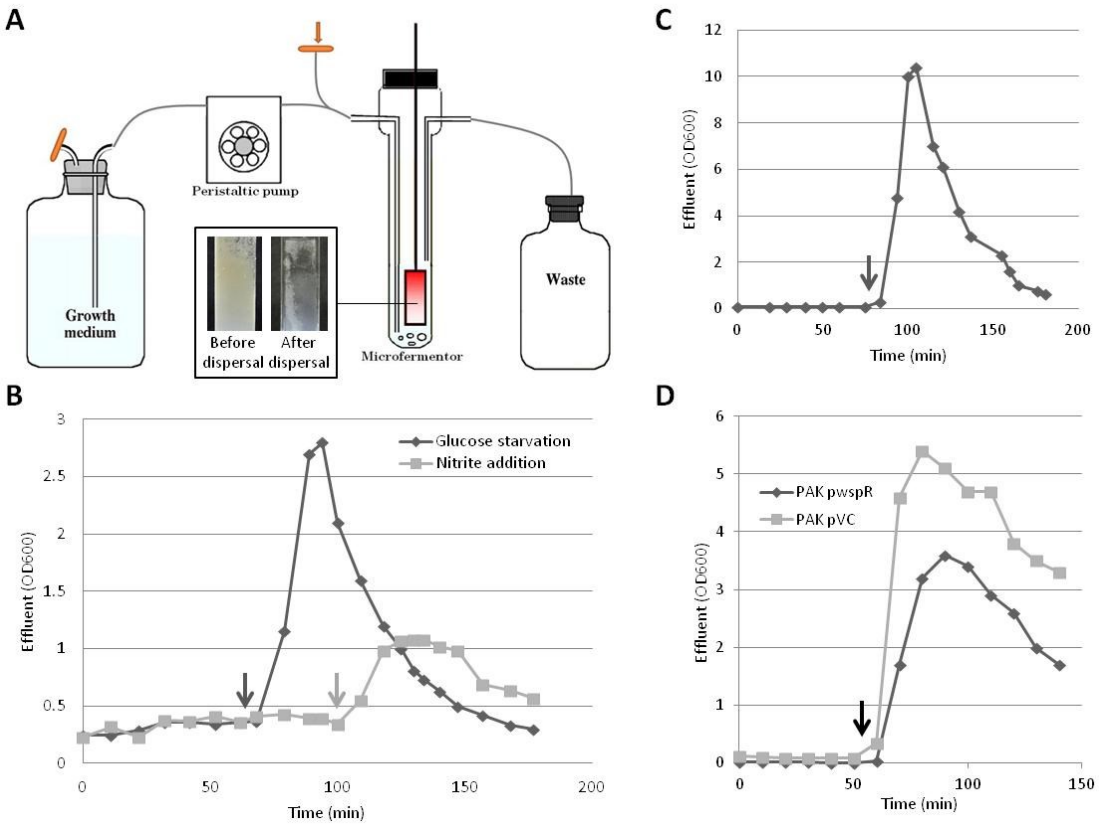

Figure 3.2 Biofilms disperse upon glucose starvation or exposure to nitric oxide in MFs.

(A) Schematic illustration of the MF set up and visualization of a spatula before and after a dispersal treatment. Biofilms grow on the spatula (red) which is placed inside the glass vessel. This system is under a continuous flow of air and medium provided by an air compressor (symbolized by an orange arrow) and peristaltic pump, respectively. The effluent is collected in a waste bottle until the dispersal treatment is initiated. **(B)** Biofilm dispersal occurs upon glucose starvation or addition of 10 mM sodium nitrite (arrows). PAO1 biofilms were grown for 24 h and dispersal was seen as an increase of the optical density of the biofilm effluent. **(C)** Biofilm dispersal upon addition of sodium nitrite (arrow) in PA14 biofilms grown for 3 days. **(D)** Constitutive production of WspR makes biofilms less responsive to biofilm dispersal upon addition of sodium nitrite (arrow). The pBBR1-MCS4 vector control (pVC) and its derivative p*wspR* were introduced in PAK by conjugation and biofilms were grown for 24 h in minimal medium supplemented with the appropriate antibiotic. The levels of biofilm formed by the two strains at the time of the dispersal treatment were considered equivalent after 24 h based on a visual inspection of the spatula.

For both dispersal treatments in PAO1, the dispersal response was almost immediate and the optical density of the effluent increased rapidly until a maximum that was reached after approximately 30 min of the treatment initiation (Figure 3.2B). Curiously, for glucose starvation the amplitude of the dispersal response was higher than the sodium nitrite addition (~8-fold increase compared to ~3-fold), but for the latter, the dispersal peak appeared wider (30 min versus 10 min). In the case of

PA14 which forms prominent biofilms after 3 days, the amplitude of the dispersal response upon addition of sodium nitrite corresponded to a 34-fold increase of bacterial cells released in the effluent (Figure 3.2C) and resulted in a dramatic clearance of the biofilms from the spatula and glass vessel.

To confirm that the biofilm dispersal response is related to c-di-GMP, the impact of sodium nitrite addition to a PAK strain with constitutive high levels of c-di-GMP (achieved by means of *wspR* overexpression) was compared to dispersal of a PAK strain carrying the pBBR1-MCS4 vector control. After growth for 24 h in the MFs, the resulting strains formed visible biofilms on the spatula and dispersal was induced. As shown in Figure 3.2D, the strain overproducing WspR was less able to disperse than the empty vector control, supporting the notions that the observed nitric oxide dispersal involves c-di-GMP signalling and reduced levels of c-di-GMP are required for biofilm dispersal to occur.

3.3 Setting up a MTP assay to monitor biofilm formation and dispersal

Traditionally, the biofilm developmental cycle has been studied using flow cells and microscopy (Weiss Nielsen *et al.*, 2011). However, this set up is labour-intensive and inappropriate for screening purposes. Therefore, in order to characterise biofilm formation in a range of mutants, assays that combine the use of MTPs with CV staining have been preferred (Christensen *et al.*, 1985; Peeters *et al.*, 2008).

For the purposes of this study, a MTP assay that allows a quick evaluation of not only biofilm formation but also biofilm dispersal was developed. To set it up, PAO1 was used because it can form biofilms faster and dispersal was induced by nitric oxide treatment because it is less invasive (performed by simple addition of sodium nitrite without requiring the removal of the supernatant). Briefly, in this MTP assay the strains of interest are inoculated in duplicate (for treated versus untreated conditions) and incubated for a defined period of time to allow biofilm formation. After, the dispersal treatment is applied and biofilm dispersal is assessed through the quantification of the optical density of the supernatant (planktonic biomass) and CV staining (biofilm biomass). Upon treatment, dispersal can be observed as a reduction of the biofilm biomass and an increase in the planktonic biomass by comparing the values for the treated and untreated conditions (Fig 3.3A).

For PAO1, the kinetic profiles of planktonic growth and biofilm formation in a MTP showed that the biofilm biomass after 6 h of incubation under shaking conditions corresponded to a CV staining of

approximately 2.5 OD units (Figure 3.3B). This time point was chosen to study the dispersal response and as shown in Figure 3.3C, addition of sodium nitrite ($NaNO_2$) resulted in a decrease in the biofilm biomass and an increase in the planktonic biomass. In fact, although concentrations were kept low to avoid the toxic effects of nitric oxide, the response to sodium nitrite was dose-dependent and statistically significant changes in planktonic and biofilm biomasses occurred at a concentration of 10 mM. Based on the dispersal response obtained in the MFs, in the MTP assay the incubation with the dispersal agent was performed for 30 min. To ensure that the effect of sodium nitrite addition was mainly due to the production of nitric oxide and not due to the addition of sodium cations, sodium nitrate ($NaNO_3$) and sodium chloride (NaCl) were also tested. Unlike NO_2^- which is readily converted to nitric oxide in the periplasm, NO_3^- needs to be incorporated into the cells to be reduced to NO_2^- (Ye *et al.*, 1994). Only addition of $NaNO_2$ caused a significant biofilm dispersal response (Figure 3.3D) confirming that the observed dispersal phenotypes are due to nitric oxide production.

Based on these data, the developed MTP assay was considered suitable for the assessment of both biofilm formation and dispersal and was used for screening purposes.

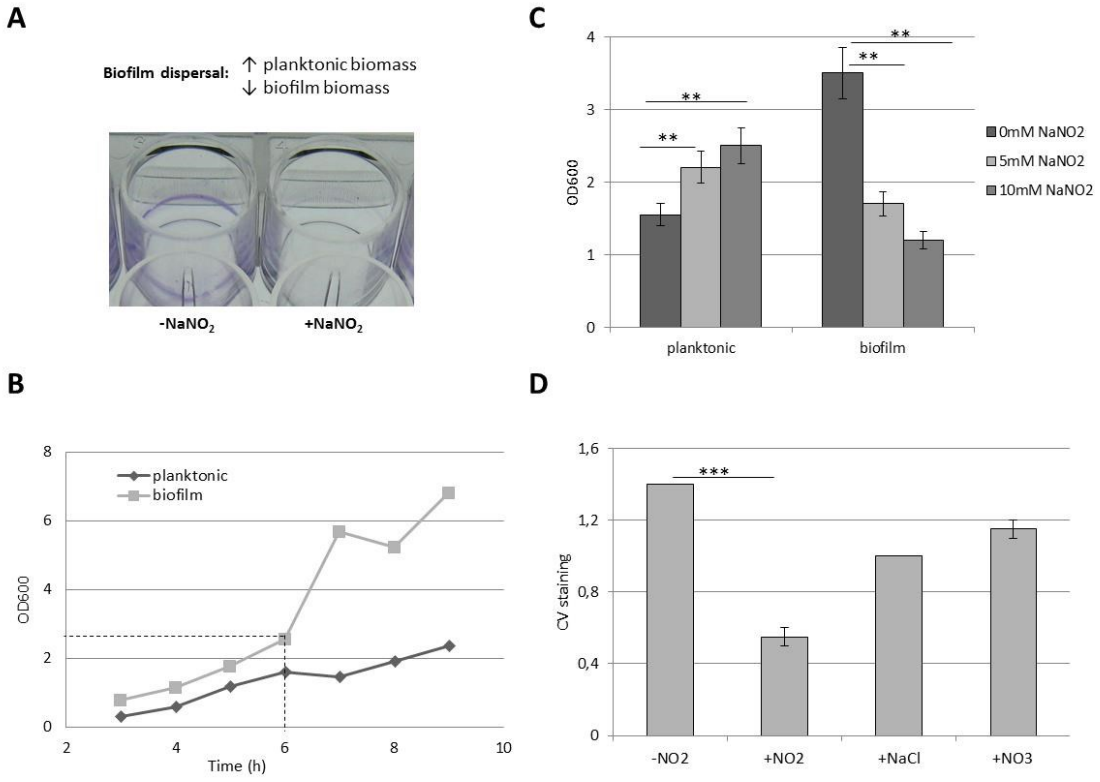

Figure 3.3 Assessment of biofilm formation and dispersal in the MTP assay.

(A) Biofilm dispersal is monitored by a reduction in the biofilm biomass and an increase in the planktonic biomass. A photograph of two CV stained MTP wells, one treated with the PBS control (-$NaNO_2$) and the other treated with sodium nitrite (+$NaNO_2$), is shown. Biofilms were stained with CV.

(B) Planktonic and biofilm growth curves of PAO1 grown at 37°C under shaking conditions in a MTP. The 6 h time-point was chosen for subsequent assays. **(C)** PAO1 biofilms disperse upon addition of 10 mM of sodium nitrite in the MTP assay for 30 min. The data indicates measurements of OD_{600} x 10 for the supernatant (planktonic) and OD_{600} of the CV staining (biofilm), and are the average of 3 techincal replicates ± standard deviation. An ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes. Two independent experiments were performed. **(D)** Statistically significant biofilm dispersal in MTPs occurs upon addition of 10 mM $NaNO_2$ (+NO2) but not 1x PBS (-NO2), 10 mM NaCl or 10 mM $NaNO_3$ (+NO3). An ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes. Two independent experiments were performed.

3.4 Biofilm formation phenotypes of transposon mutants of genes encoding proteins with a GGDEF, EAL or HD-GYP domain

A collection of 42 *P. aeruginosa* PAO1 transposon mutants was screened using the developed MTP assay (Jacobs *et al.*, 2003). This collection included all the known genes that encode for proteins with a GGDEF, EAL or HD-GYP domain, with the exception of the transposon mutant in the gene encoding for PelD which contains a catalytic inactive GGDEF domain (Table 2.1). In each MTP, four strains were assessed and the performance of mutant strains was compared to the performance of the wild-type strain.

In relation to biofilm formation, six hypobiofilm formers with at least 1.5-fold reduction in the biofilm biomass and three hyperbiofilm formers with at least 1.5-fold increase in the biofilm biomass, were identified (Figure 3.4A).

The hypobiofilm mutants corresponded to insertions in the genes *siaD*, PA0338 and PA4843, encoding proteins with a GGDEF domain; and in the genes PA1181, PA2072 and *fimX*, encoding proteins with a GGDEF/EAL domain. The hyperbiofilm mutants corresponded to insertions in *rbdA*, *bifA* and *dipA* which encode proteins with a GGDEF/EAL domain. Mutants in genes encoding proteins with an EAL or a HD-GYP domain showed a biofilm formation phenotype similar to wild-type.

3.5 PA2072 seems to have PDE activity

Among the hypobiofilm phenotypes, the PA2072 transposon mutant had the most severe phenotype (~7-fold reduction) and has never been described before. The PA2072 gene is predicted to encode a

protein with N-terminal CHASE4 and PAS domains followed by GGDEF (conserved I- and A-sites) and EAL domains (Figure 3.5A).

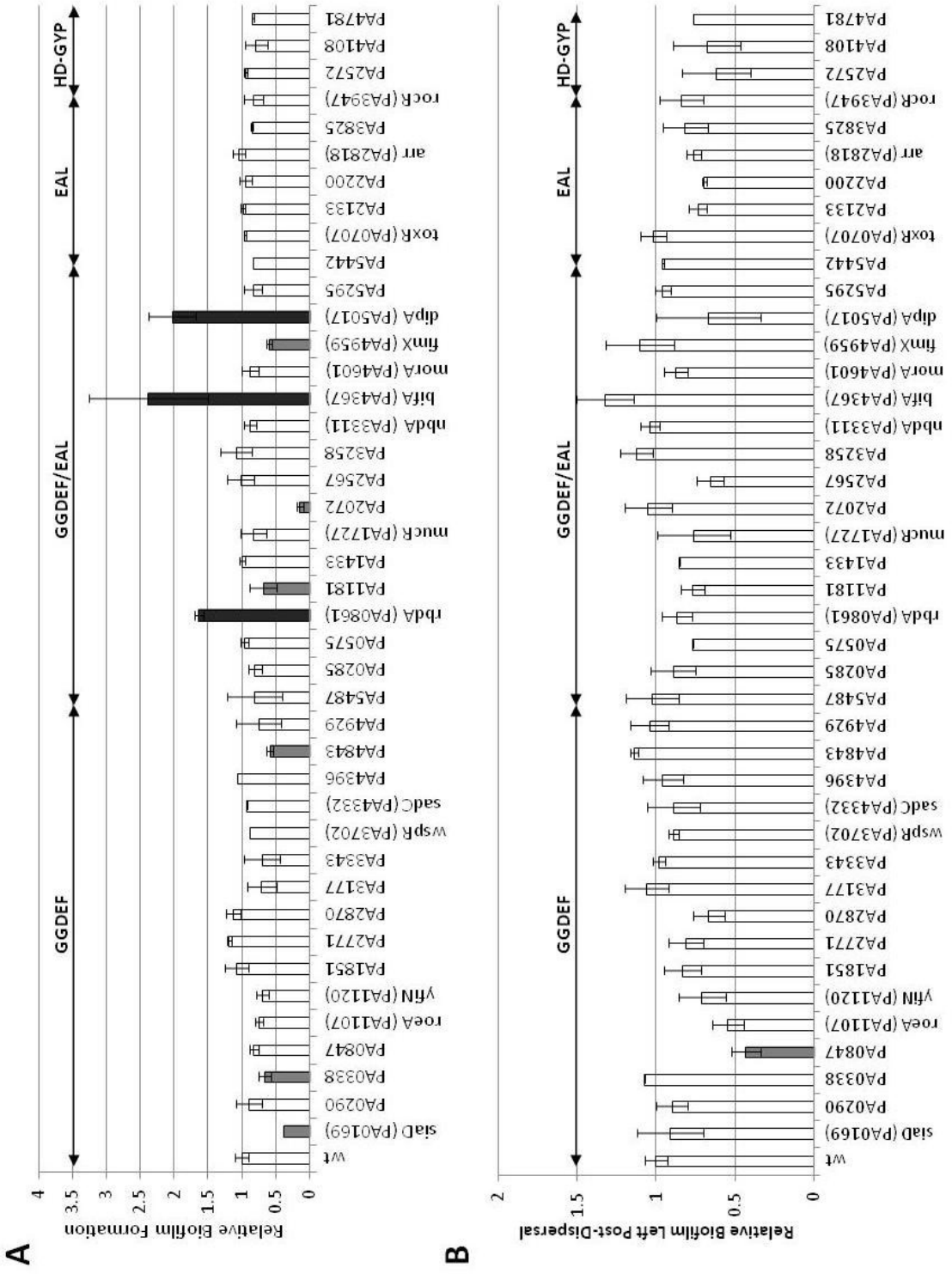

Figure 3.4 Screen of the *P. aeruginosa* PAO1 transposon collection of genes encoding GGDEF, EAL or HD-GYP domain proteins. (Figure legend continues on the next page.)

The mutant collection was obtained from the University of Washington Genome Center (Jacobs et al., 2003). Data are the average of a total of 6 technical replicates from two independent experiments ± standard deviation, normalized by setting the CV staining of *P. aeruginosa* PAO1 strain (wt) as 1. **(A)** Relative biofilm formation. Strains with at least 1.5-fold impairment on biofilm formation are represented by grey bars while black bars represent strains with at least 1.5-fold enhancement on biofilm formation. **(B)** Relative biofilm biomass remaining on the well surfaces after dispersal upon addition of sodium nitrite. Grey bars represent hyperdispersive strains, i. e. strains with at least 2-fold decrease in the biofilm biomass. The amount of biofilm remaing after dispersal was calculated as the ratio between the biofilm present in treated wells and the biofilm present in untreated wells.

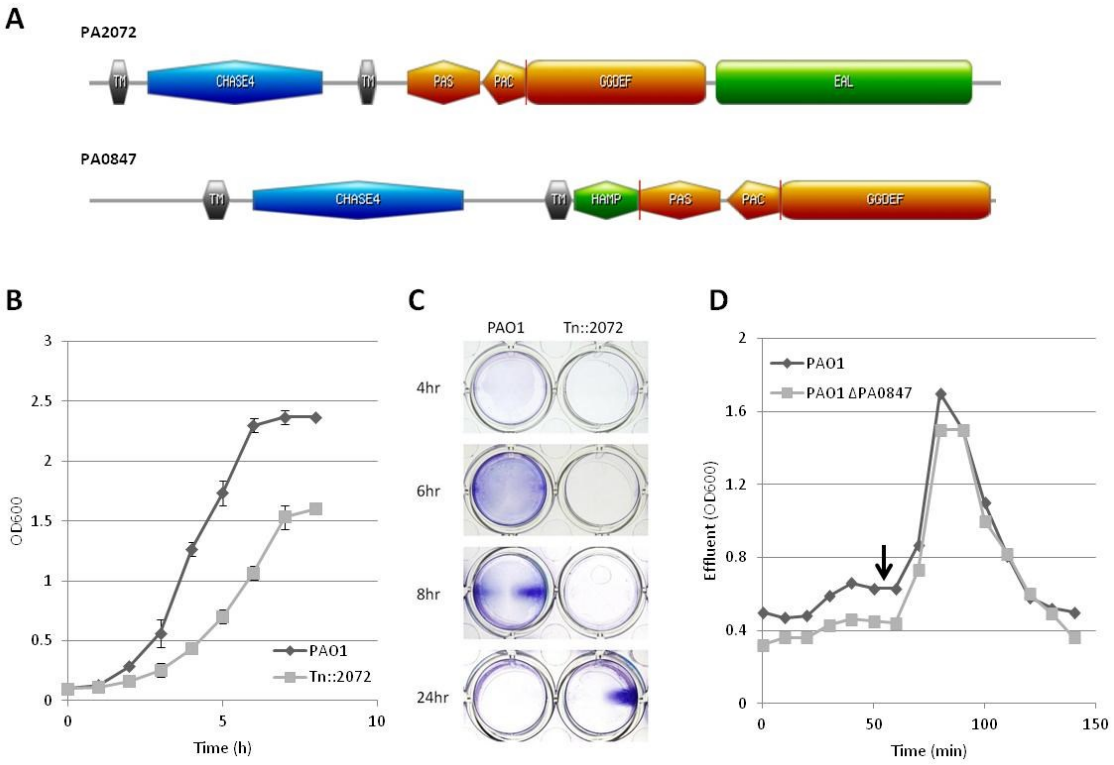

Figure 3.5 Further characterization of two mutant strains reveals that the PA2072 transposon mutant has a growth defect, and a PA0847 clean deletion mutant is not hyperdispersive.

(A) Predicted domain organization of PA2072 and PA0847. The Pfam database (Punta *et al.*, 2012) was used to identify the domains and the diagram was produced in MyDomains (www.expasy.ch/tools/mydomains). **(B)** Planktonic growth curves of *P. aeruginosa* PAO1 and the PA2072 transposon mutant grown in M9 medium with 0.4% glucose at 37°C under shaking conditions. Data are the average of 3 technical replicates ± standard deviation. **(C)** Time-course assessment of biofilm formation in a MTP. Photograph of the bottom of the microtiter plate is shown. Biofilms grown in M9 medium with 0.4% glucose were stained with 0.1% CV and the wells were washed twice with water. **(D)** Biofilm dispersal of PAO1 and PAO1ΔPA0847 in the MFs. Biofilms were grown for 24 h until the dispersal agent sodium nitrite was added to the medium (arrow).

To investigate the hypobiofilm phenotype of the PA2072 transposon mutant, the kinetics of the planktonic growth and biofilm formation were investigated. Strikingly, the mutant showed a growth defect (Figure 3.5B) and biofilms were visible after 24 h of incubation (Figure 3.5C). Therefore, it was concluded that the observed hypobiofilm phenotype is likely due to the slow growth rate of the mutant rather than an innate inability of the mutant to produce biofilms.

Nevertheless, the catalytic activity of PA2072 was investigated. For that purpose, the gene was cloned into pBBR1-MCS4 and the resulting plasmid was conjugated into PAK. Overexpression of PA2072 in PAK led to a reduced biofilm formation when compared to the strain carrying the vector control (Figure 3.6A). This impairment in biofilm formation was also visible when PA2072 was overexpressed in the hyperbiofilm strain PAKΔ*retS*, suggesting that PA2072 may work as a PDE under the conditions tested (Figure 3.6A).

To further confirm this hypothesis, site-directed mutagenesis was used to disrupt the putative DGC and PDE catalytic sites. To test the DGC activity, the GGDEF motif was mutated to GGAAF and to test the PDE activity, the EAL motif was mutated to AAL. Interestingly, overexpression of both PA2072 versions, PA2072^{GGAAF} or PA2072^{AAL}, was unable to cause a reduction in biofilm formation to the same extent of the wild type PA2072 (Figure 3.6B). Therefore, these results support the idea that both the EAL and GGDEF motifs are required for a fully functional PA2072. Like for other GGDEF/EAL domain proteins, it is possible that the GGDEF motif works by binding to GTP and stimulating the PDE activity of the EAL domain.

To elucidate the relation of PA2072 with c-di-GMP, whole cell lysates of strains overexpressing the wild-type PA2072 or its mutated derivatives, were tested for c-di-GMP binding (Figure 3.6C). This work was done in collaboration with the group of Vincent Lee who developed an assay, the DRaCALA assay (differential radial capillary action of ligand assay) (Table 4.1), that allows for quick assessments of the ability of proteins to bind c-di-GMP (Roelofs *et al.*, 2011). Basically, this assay measures the ability of dry nitrocellulose membranes to separate unbound radioactive labelled c-di-GMP (c-di-GMP*) from protein bound c-di-GMP* complexes. Consistent with the hypothesis of PA2072 being a PDE whose activity is stimulated by the GGDEF domain, the wild-type PA2072 was able to bind c-di-GMP* and mutation of the GGDEF motif positively affected this binding. In contrast, PA2072^{AAL} abolished c-di-GMP* binding, underpinning the role of the EAL domain in binding c-di-GMP to drive its degradation.

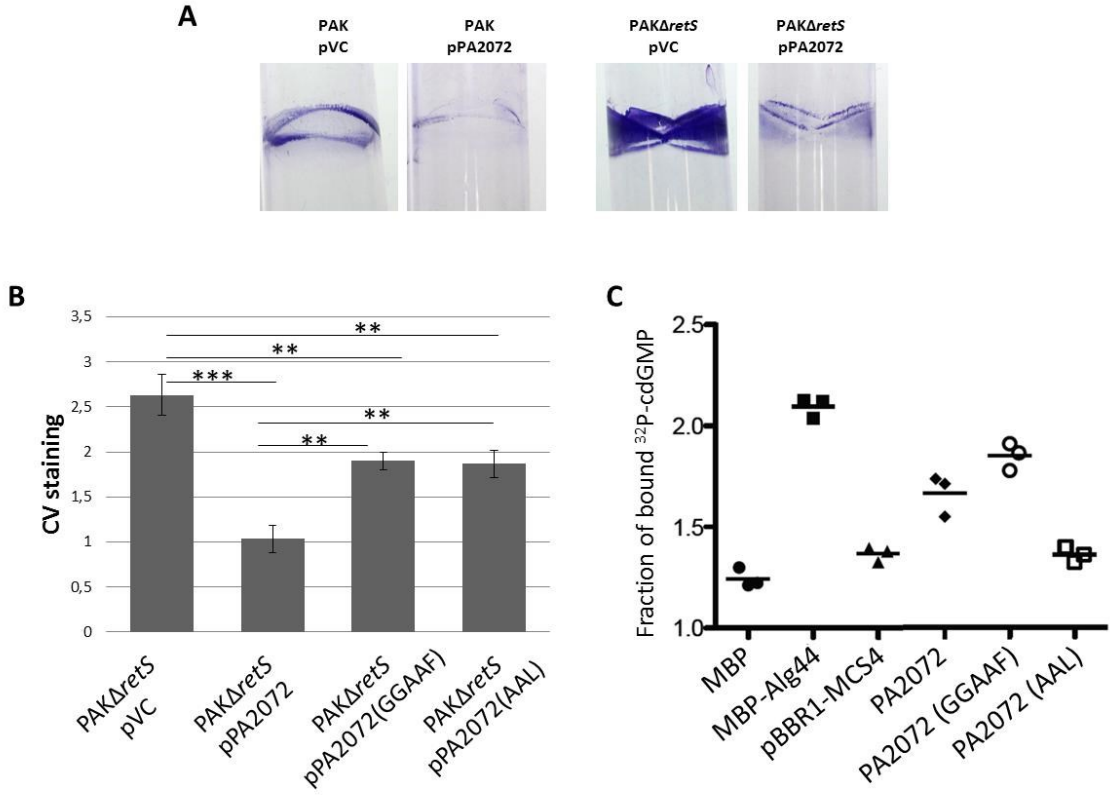

Figure 3.6 Overexpression of PA2072 prevents biofilm formation.

(A) PAK and PAKΔ*retS* were conjugated with the vector control pBBR1-MCS4 (pVC) and its derivative pPA2072 encoding wild-type PA2072. Biofilms formed at the air-liquid interface under shaking conditions are visualized by CV staining. **(B)** Biofilms formed in a MTP under shaking conditions for 6 h in M9 medium with 0.4% glucose were quantified by CV staining. The levels of CV staining in PAK conjugated with pVC were normalized to 1. An ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes. At least three independent experiments were performed. **(C)** Specific c-di-GMP binding assessed by DRaCALA. Graph of ^{32}P-c-di-GMP binding by *E. coli* whole-cell lysates overproducing the indicated proteins. The average is indicated by a horizontal bar. The lysate of a strain overproducing MBP-Alg44PilZ was used as positive control and the lysates of strains harbouring the empty vectors were used as negative control. Figure provided by Vincent Lee (unpublished).

3.6 Biofilm dispersal phenotypes of transposon mutants of genes encoding proteins with a GGDEF, EAL or HD-GYP domain

In relation to biofilm dispersal, the MTP assay identified one hyperdispersive transposon mutant, the PA0847 mutant strain. As illustrated in Figure 3.4B, besides the transposon mutant in PA0847, all the

mutant strains displayed a dispersive phenotype similar to wild-type. For the PA0847 mutant, the biofilm biomass remaining after dispersal was approximately 2-fold lower than the wild-type. No non- or hypodispersive phenotypes were observed.

3.7 The phenotypes of the PA0847 mutant

In relation to the hyperdispersive phenotype of the PA0847 transposon mutant (~2-fold increase), the phenotype had never been reported before and thus was considered for further studies.

The PA0847 gene is predicted to be located within an operon with PA0846 which encodes a protein with similarity to a sulfate uptake protein (Winsor *et al.*, 2009). Like PA2072, PA0847 has an N-terminal CHASE4 domain but in PA0847 this is followed by an HAMP, a PAS and a GGDEF domain (conserved I- and A-sites) (Figure 3.5A). In order to confirm the hyperdispersive phenotype, a clean deletion mutant of PA0847 was engineered in PAO1 and tested in the MTP and MF assays. Surprisingly, the hyperdispersive phenotype was not observed for the clean deletion mutant (Figure 3.5D) and therefore research on PA0847 was not carried further.

3.8 Discussion

Since its discovery in 1987 for its role in the regulation of cellulose synthesis in *G. xylinus*, c-di-GMP has emerged as a bacterial second messenger that regulates a variety of cellular processes in a very specific way. However, for many years the proteins involved in the turnover of c-di-GMP remained poorly understood and later it was unclear how the c-di-GMP network was operating to specifically regulate different cellular processes. This uncertainty was primarily due to two main observations. On the one hand, the occurrence of a large number of genes encoding putative DGCs and PDEs in a single bacterium; on the other hand, the presence of additional N-terminal domains in these proteins. Today, although the *modus operandi* of many of the putative DGCs and PDEs is still unknown, the debate about the specificity of the c-di-GMP network seems to be settled. In a recent report from the laboratory of Christopher Waters, it was demonstrated for *V. cholerae* that there is no correlation between the total level of c-di-GMP in a cell and the induction of biofilm formation (Massie *et al.*, 2012). Instead, only a fraction of all the DGCs found in *V.cholerae* was active for biofilm formation under the conditions tested (18 out of 40 proteins with a GGDEF domain) and

induction of biofilm formation was correlated to the expression of individual DGCs rather than the intracellular concentration of c-di-GMP.

In light of this, the 43 GGDEF, EAL and HD-GYP domain proteins found in *P. aeruginosa* (Table 1.5) are thought not to be functionally redundant and they have been associated with the regulation of different biological attributes related to the motile/sessile lifestyle switch. In order to investigate which c-di-GMP related proteins could be specifically involved in the modulation of either biofilm formation or dispersal, a screen using a MTP assay was performed.

From the 42 PAO1 transposon mutants tested, a total of six hypobiofilm formers, three hyperbiofilm formers and one hyperdispersive mutant were identified (Figure 3.4).

In relation to biofilm formation, 33 out of the 42 mutants tested did not display an altered phenotype. This high rate of wild type phenotypes is not surprising and denotes the high specificity of the c-di-GMP network. For these mutants, it is possible that (i) the genes were not expressed or the proteins inactive under the conditions tested and therefore the phenotype was not visible, (ii) the encoded proteins are involved in the regulation of attributes that do not affect biofilm formation, or (iii) the biofilm phenotype is dominant and the impact of single gene mutants is masked by the expression of other genes.

In fact, the GGDEF domain protein WspR is known to have DGC activity and to affect biofilm formation but the *wspR* transposon mutant in this study or the clean deletion mutant in the study by Hickman *et al.* (2005) (Hickman *et al.*, 2005) show an insignificant impact of the mutation in biofilm formation. The observation that WspR is involved in biofilm formation comes from studies where WspR is overexpressed or constitutively active due to deletion of *wspF* (Figure 1.9). It is known that the activation of the Wsp system is somehow surface contact dependent, so under the conditions tested it is possible that the system was not active (Guvener & Harwood, 2007).

Also, the transposon mutant in the gene encoding the PA5487 protein with a GGDEF domain did not show an altered biofilm formation phenotype in this study in which the biofilm assay is performed under shaking conditions. However, in a collaborative work with Isabel Cortés from the group of Cayo Ramos Rodríguez at the University of Málaga, a PA5487 clean deletion mutant showed a hypobiofilm phenotype under static conditions (Figure 3.7). Like for WspR, this observation reflects how c-di-GMP forms an intricate intracellular signalling network that responds to specific stimuli. Interestingly, the PA5487 mutant was also shown to be attenuated in an acute lung injury mice model and overexpression of PA5487 led to enhanced biofilm formation suggesting that the protein has DGC activity (manuscript under preparation).

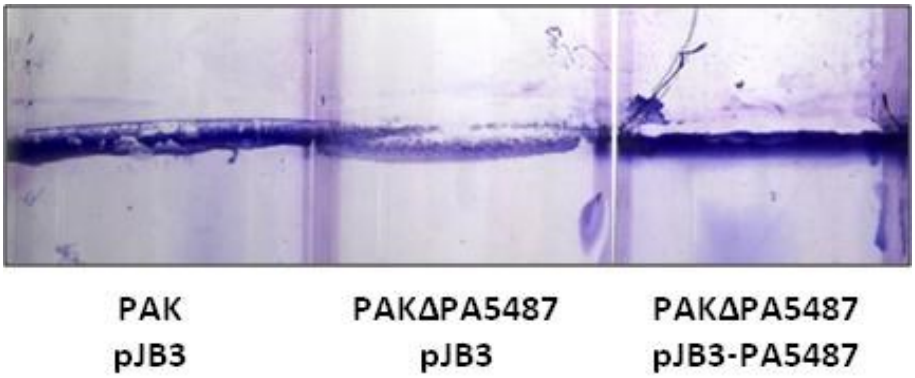

Figure 3.7 Deletion of PA5487 causes a hypobiofilm phenotype under static growth conditions.

This experiment was performed together with Isabel Cortés. The empty plasmid pJB3 and its derivative pJB3-PA5487 were conjugated into *P. aeruginosa* PAK strain and biofilms were grown for 24 h at 37^{o}C.

In *E. coli*, 21 out of the 28 GGDEF and EAL domain proteins have been shown to be expressed at some point during the exponential or stationary phases of growth in batch cultures and a subset of genes were differentially regulated at 28^{o}C and 37^{o}C (Sommerfeldt *et al.*, 2009). In *P. aeruginosa*, and based on a microarray study performed by Helga Mikkelsen in the laboratory of Martin Welch (Mikkelsen *et al.*, 2009b), approximately 1/3 of the genes encoding proteins with a GGDEF, EAL or HD-GYP domain were not expressed in any of the conditions tested, i.e. planktonic exponential or stationary phase, colonies grown on a agar plate and assayed at 15h or 40h post-incubation, and biofilms grown in flow cells for 3 days (Table 3.1). The expression of the other 2/3 was detected in at least one of the conditions tested but a high proportion of these genes (25 out of 29) were expressed at the early stages of colony formation (including *wspR*). Only the expression of two genes, PA3177 encoding a putative DGC and PA4801 encoding a known PDE, was detected in all the conditions tested. All together, this and the relatively low number of hits in the MTP screen are supportive of a model whereby c-di-GMP constitutes a highly regulated signalling network that operates with a high degree of specificity.

From the subset of transposon mutants in genes encoding proteins with a C-terminal GGDEF domain, mutants in *siaD*, PA0338 and PA4843 had a hypobiofilm phenotype. This is consistent with a putative DGC catalytic activity of the proteins under the conditions tested. For *siaD* the observed phenotype is in agreement with a previous report where the protein was shown to be involved in a stress dependent auto-aggregative phenotype (Klebensberger *et al.*, 2009).

From the subset of mutants in genes with C-terminal GGDEF and EAL domains, strains harbouring transposon insertions in PA1181, PA2072 and *fimX* exhibited a hypobiofilm phenotype whereas insertions in *rbdA*, *bifA* and *dipA* led to hyperbiofilm formation. These results suggest that in the hypobiofilm formers the GGDEF domain is likely to have DGC catalytic activity while in the hyperbiofilm formers, it is the EAL domain that may be enzymatically functional.

Indeed, for RbdA, BifA and DipA, a PDE activity has already been confirmed (An *et al.*, 2010; Kuchma *et al.*, 2007; Roy *et al.*, 2012). In the case of RbdA and DipA, the PDE activity was shown to be required for biofilm dispersal indicating that the observed hyperbiofilm phenotype is due to an inability of the mutant to disperse properly. In the case of BifA, the PDE activity was shown to counteract the effect of two DGCs in the regulation of biofilm formation and swarming motility.

Table 3.1 Expression of the GGDEF, EAL and HD-GYP domain proteins in *P. aeruginosa* at different phases and growth conditions.*

Gene	Protein[1]	Planktonic exponential	Planktonic stationary	Colony 15h	Colony 40h	Biofilm 3 days
PA0338		--	--	--	--	--
PA0847		--	--	--	--	--
roeA		--	--	--	--	--
PA1851		--	--	--	--	--
PA2072		--	--	--	--	--
PA2133		--	--	--	--	--
PA2771		--	--	--	--	--
arr		--	--	--	--	--
PA2870		--	--	--	--	--
pelD		--	--	--	--	--
PA3258		--	--	--	--	--
nbdA		--	--	--	--	--
PA3343		--	--	--	--	--
PA3825		--	--	--	--	--

Gene	Protein[1]	Planktonic exponential	Planktonic stationary	Colony 15h	Colony 40h	Biofilm 3 days
PA5442		--	--	--	--	--
siaD		✓	--	--	--	--
PA4843		✓	--	--	--	--
PA4929		--	✓	--	--	--
PA0285		--	--	✓	--	--
PA0575		--	--	✓	--	--
yfiN		--	--	✓	--	--
PA1181		--	--	✓	--	--
lapD		--	--	✓	--	--
PA2200		--	--	✓	--	--
PA2567		--	--	✓	--	--
wspR		--	--	✓	--	--
toxR		--	--	✓	✓	--
mucR		✓	--	✓	--	--
rocR		✓	--	✓	--	--
morA		✓	--	✓	--	--
sadC		✓	--	✓	--	--
fimX		✓	--	✓	--	--
PA5295		✓	--	✓	--	--
PA4396		✓	--	✓	--	✓
bifA		✓	--	✓	✓	✓
PA5487		✓	--	✓	✓	✓
rbdA		✓	✓	✓	--	--
dipA		✓	✓	✓	--	--
PA0290		--	✓	✓	✓	--
PA2572		--	✓	✓	✓	--

Gene	Protein[1]	Planktonic exponential	Planktonic stationary	Colony 15h	Colony 40h	Biofilm 3 days
PA4781		--	✓	✓	✓	✓
PA3177		✓	✓	✓	✓	✓
PA4108		✓	✓	✓	✓	✓
Total		15	8	25	8	6

*Based on the microarray data provided by Helga Mikkelsen.
[1] Blue boxes represent the GGDEF domain and pink boxes the EAL domain. Grey boxes indicate presence of additional domains as specified in Table 1.5.

Similar hypobiofilm phenotypes for PA1181 and *fimX* have previously been identified in the PA14 screen performed by of Kulasekara *et al.* (2006). Interestingly, it has been shown that instead of being catalytically active, FimX works as a c-di-GMP binding protein and is required for twitching motility at the early stages of biofilm formation (Jain *et al.*, 2012).

The severe hypobiofilm phenotype of the PA2072 transposon mutant was chosen for further analysis but subsequent experiments showed that the mutant had a growth defect and biofilm formation was visible at a later time point (Figure 3.5). Nevertheless, experiments where PA2072 was overexpressed suggested that it is the EAL domain of the protein that is catalytic active. This hypothesis was further supported by the inability of PA2072^{AAL} to cause the same reduction in biofilm formation as the wild-type PA2072 and to be unable to bind c-di-GMP (Figure 3.6). However, the physiological role of this PDE remains unknown.

To screen the transposon collection for mutants with a dispersal phenotype, addition of sodium nitrite as a nitric oxide donor was used. Only one hyperdispersive mutant affected in the PA0847 gene was identified but a clean deletion of PA0847 failed to confirm the phenotype. This could have been because of polar effects caused by the transposon insertion and the hit was not studied further.

The fact that the developed MTP assay failed to identify more hypo- or hyperdispersive mutants, prompted the question about its suitability. Addition of sodium nitrite was used as a dispersal trigger because it acts as a nitric oxide donor and this has been shown here (Figure 3.2 and 3.3) and in the work of Nicolas Barraud from the University of New South Wales, to induce biofilm dispersal (Barraud *et al.*, 2006; Barraud *et al.*, 2009). However, other unpredicted dispersal triggers such as oxygen availability may have been present during the execution of the experiments. Since the MTP

assay was performed under shaking conditions in an incubator of communal use and the dispersal treatment required a transient withdraw of the MTP from the incubator, it is quite possible that other uncontrolled dispersal triggers were being applied. Subsequent discussions and experiments conducted by Nicolas Barraud, have indeed shown that biofilm dispersal in MTPs may be induced by limiting oxygen availability through simple reduction of the rotation speed of a shaking incubator (Figure 3.8), questioning the reliability of the developed MTP assay in assessing biofilm dispersal.

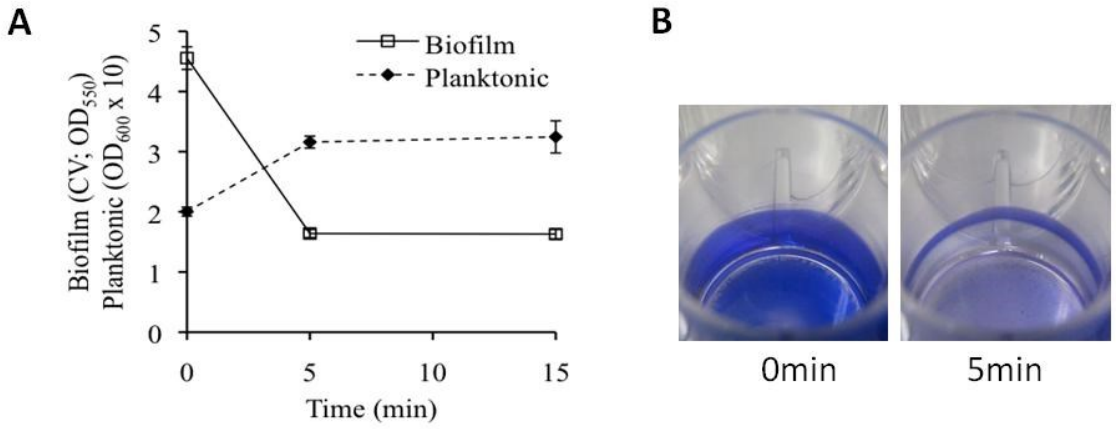

Figure 3.8 Oxygen-induced biofilm dispersal using MTPs.

(A) PAO1 planktonic and biofilm biomasses after oxygen limitation induced by reducing the shaking speed of the incubator (200rpm to 60rpm). The assay was performed at 37^{o}C for 6 h in M9 medium with 0.4% glucose. **(B)** Photograph showing CV staining of biofilms at the time when dispersal was induced (0 min) and after the dispersal treatment (5 min). Figure provided by Nicolas Barraud (unpublished).

Recently, three proteins with PDE activity have been shown to be involved in biofilm dispersal in studies mainly conducted using flow tube reactor systems. These proteins were NbdA, RbdA and DipA. For the recently described NbdA the biofilm dispersal phenotype was shown to be induced by nitric oxide and it was postulated that nitric oxide can act at both the posttranscriptional and the posttranslational level (Li *et al.*, 2013).

Besides the MTP screen, the work presented here demonstrates that c-di-GMP is associated with the switch between a planktonic and a biofilm lifestyle and confirms the paradigm that high levels of c-di-GMP promote biofilms whereas low levels promote motility. Notwithstanding the high specificity at which intracellular c-di-GMP signalling seems to operate under natural conditions, overexpression

of genes encoding active DGCs or PDEs seems to have a more general impact that saturates or depletes, respectively, the signalling mechanisms of c-di-GMP and dictates the bacterial lifestyle. Hence, when the active DGCs WspR or $WspR^{R242A}$ were produced, an increment of the biofilm biomass was observed and when the active PDE PA2133 was produced, the appearance of biofilms was inhibited (Figure 3.1).

Strikingly, the constitutively active WspR** which had the I-site and the phosphorylation site mutated, inhibited bacterial growth. This suggests that c-di-GMP at high levels can be toxic to the cells. A similar observation has previously been reported by Ryjenkov and co-workers who were unable to overexpress a wild-type DGC in a pET23a vector using an *E. coli* BL21 (DE3) strain (Ryjenkov *et al.*, 2005). Interestingly, also for PDEs a toxic effect has been postulated when Lacey and co-workers were unable to overexpress the *E. coli* gene *yfgF* from a plasmid which drives the constitutive expression of the gene (Lacey *et al.*, 2010).

In summary, these results highlight the importance of the c-di-GMP regulatory network in controlling bacterial lifestyle and further emphasize the high specificity at which it operates to modulate an array of different biological attributes. In theory, this specificity may be achieve by many means, including (i) the temporal expression of specific genes, (ii) the activation of DGCs and PDEs under certain conditions, (iii) the subcellular co-localization of proteins with the effectors, or (iv) the differences in the c-di-GMP binding affinities of the effector proteins. For the majority of the c-di-GMP related proteins or c-di-GMP regulated functions, the precise mechanism by which their regulatory role is achieved remains to be elucidated.

4) Cyclic di-GMP control on Type III and Type VI secretion systems

Chapter 4 – Cyclic di-GMP control on Type III and Type VI secretion systems

Bacteria can cause both acute and chronic infections. In the versatile *P. aeruginosa* pathogen, it is now well documented that the bacterium lifestyle strongly correlates to its mode of infection. In the motile lifestyle, bacteria exhibit flagella-driven swimming motility and produce toxins via the T2SS and T3SS that damage the host tissues and subvert host cells, causing an acute infection. In the sessile lifestyle, bacteria establish as a biofilm and express the T6SS, causing a chronic infection that is prevalent in the lungs of cystic fibrosis patients.

A key determinant in the *P. aeruginosa* course of infection is the regulatory cascade comprising the central two-component system GacAS (Figure 1.6). Major findings in my work have resulted from the investigation of a link between the Gac regulatory cascade and c-di-GMP signalling, and subsequently, on how this link goes beyond the modulation of biofilm formation to also regulate the expression of T2SS, T3SS and T6SS. Part of the work presented here has been published and a reprint of the paper can be found in the appendices (Appendix 1 and 2).

4.1 The *retS* hyperbiofilm mutant has high levels of c-di-GMP

The sensor RetS is known to form heterodimers with GacS (Goodman *et al.*, 2009) thus preventing the expression of the two sRNAs, RsmY and RsmZ, and favouring the motile lifestyle. A *retS* deletion mutant releases the central GacAS cascade from the RetS inhibition and displays a hyperbiofilm phenotype (Figure 4.1A).

Considering that high levels of c-di-GMP are associated with hyperbiofilm formation, the levels of c-di-GMP in the *retS* mutant were investigated. Two methods were employed to achieve this. Firstly, the intracellular c-di-GMP levels were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) using nucleotide extracts and in collaboration with Stephan Heeb and Paul Williams at the University of Nottingham. Secondly, c-di-GMP level was qualitatively assessed by using a transcriptional reporter fusion between a promoter that positively responds to an increase in c-di-GMP (the promoter of *cdrA* which encodes an adhesin) and the *gfp* gene (encoding for green fluorescent protein) (Rybtke *et al.*, 2012). As shown in Figure 4.1B and C, when compared to the wild-type strain, the hyperbiofilm phenotype of the *retS* mutant coincided with an increase in the

intracellular levels of c-di-GMP (Moscoso *et al.*, 2011), both using LC-MS/MS and the c-di-GMP reporter.

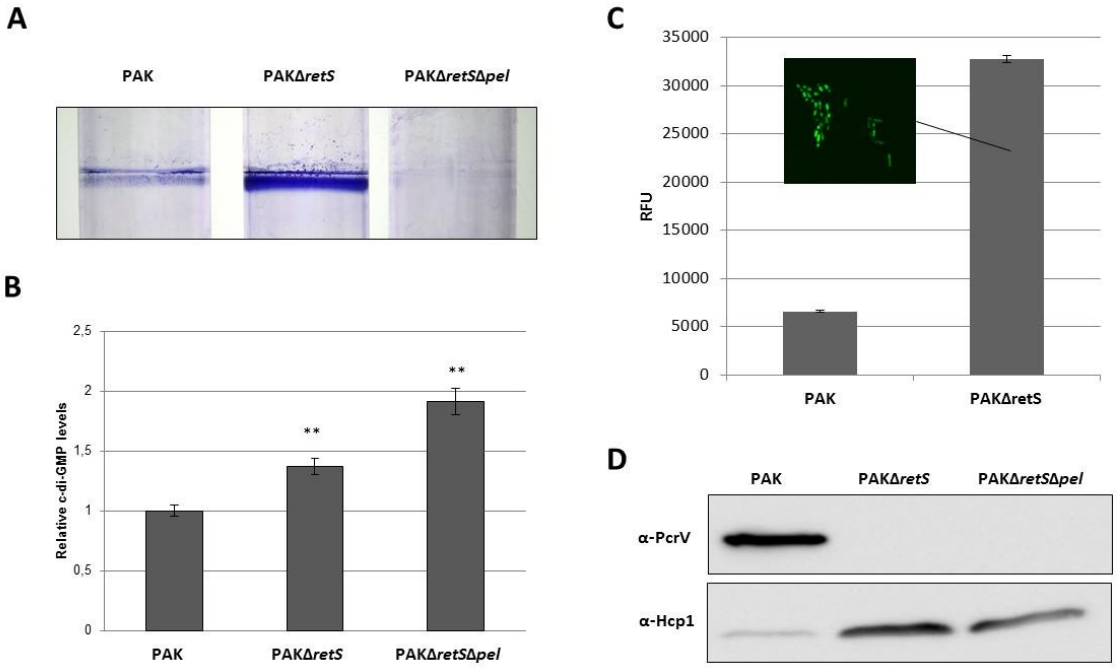

Figure 4.1 A *retS* mutant is a hyperbiofilm former, has elevated levels of c-di-GMP and expresses the T6SS.

(A) Biofilms formed at the air-liquid interface under static conditions are visualized by CV staining. **(B)** Intracellular levels of c-di-GMP measured by LC-MS/MS and compared to the PAK wild-type strain. Statistical Student's *t*-test analysis with $p<0.01$ is based on three technical replicates and significant changes are indicated by asterisks. **(C)** Intracellular levels of c-di-GMP measured with the c-di-GMP transcriptional *gfp* reporter. The reporter was introduced in the *P. aeruginosa* strains specified by electroporation. Relative fluorescence units (RFU) are arbitrary fluorescence intensity units corrected for cell density. A snapshot of fluorescent Δ*retS* cells carrying the reporter was taken using a confocal microscope. **(D)** Western blot performed on whole cell lysates using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS). T3SS was induced by calcium chelation.

To investigate whether the elevated level of c-di-GMP was directly attributable to the *retS* mutation or a consequence of the formation of a hyperbiofilm, the levels of c-di-GMP were determined in a *retS* mutant that is unable to form biofilms due to the inability of producing the Pel exopolysaccharide (Moscoso *et al.*, 2011). In this *retS/pel* mutant, the levels of c-di-GMP remained high (Figure 4.1B), therefore confirming that the deletion of *retS per se* increases the level of c-di-GMP.

4.2 RetS antagonistically controls the T3SS and the T6SS

Besides inducing biofilm formation, deletion of *retS* is also known to induce the expression of the T6SS and to repress the T3SS. This can be visualized by Western blot using antibodies against structural components of both secretion systems: PcrV which sits at the tip of the needle of the T3SS, and Hcp1 which is the main component of the H1-T6SS nanotube.

As illustrated in Figure 4.1D, PcrV is strongly detected in the wild-type strain but not in the *retS* or *retS/pel* mutants. By contrast, the production of Hcp1 is high in the *retS or retS/pel* mutants and low in the wild-type (Moscoso *et al.*, 2011).

In conclusion, the *retS* mutation induces a switch in the expression of the T3SS and T6SS (referred to as T3SS/T6SS switch). This switch is independent of the formation of a hyperbiofilm and occurs in such a way that a *retS* mutant displays an up-regulated T6SS and a down-regulated T3SS.

4.3 Increased levels of c-di-GMP up-regulate the T6SS

If in the *retS* mutant the hyperbiofilm phenotype is associated with high levels of c-di-GMP, it was hypothesized that the same could apply to the T3SS/T6SS switch. To test this hypothesis, the pBBR1-MCS4 and the derivative construct overexpressing *wspR*^{R242A} were conjugated in a *pel* mutant strain. WspR^{R242A} is an active DGC and the effect of an increase in c-di-GMP production on T3SS and T6SS expression was monitored. As presented in Figure 4.2A and B, production of WspR^{R242A} resulted in a more than 2-fold increased level of c-di-GMP and this correlated with a clear T3SS/T6SS switch, i.e. down-regulation of the T3SS concomitant with an up-regulation of the T6SS (Moscoso *et al.*, 2011).

The c-di-GMP-dependent up-regulation of the T6SS was further shown not to be growth phase dependent (Figure 4.2C) and to also occur upon overproduction of YfiN (PA1120), another DGC (Figure 4.2D). This suggested that in these conditions, it is the global increase in c-di-GMP levels that induces the T6SS rather than a specific role of either one of the tested DGCs in the process. Finally, the expression of other structural components of the T6SS involved in the formation of the puncturing device, namely VgrG1a, VgrG1b and VgrG1c, was also up-regulated upon the overexpression of *wspR*^{R242A} (Figure 4.2E). This confirms that the impact of increased levels of c-di-GMP is on the whole T6SS and not particularly on some of the genes or transcription units.

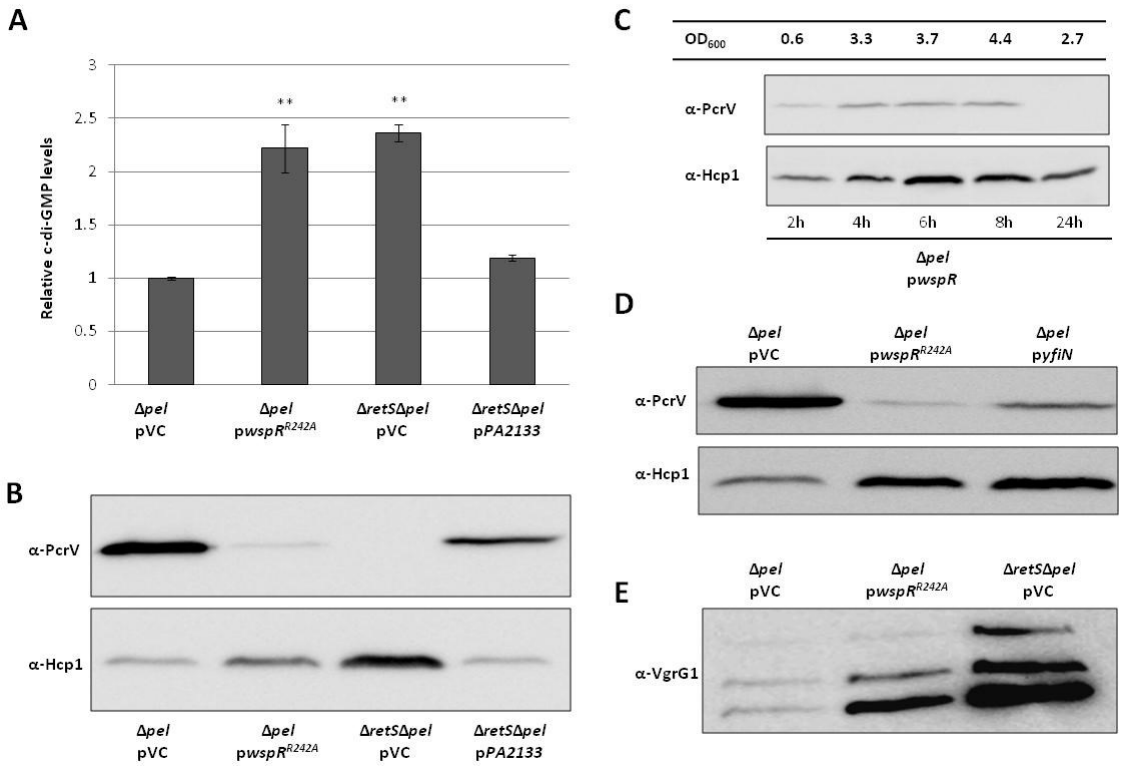

Figure 4.2 Cyclic di-GMP inversely regulates T3SS and T6SS.

The pBBR1-MCS4 vector control (pVC) and its derivatives overexpressing *wspR (*p*wspR*), *wspR*^{R242A} (p*wspR*^{R242A}), PA2133 (pPA2133) or *yfiN* (p*yfiN*) were introduced in PAKΔ*pel* or PAKΔ*retSΔpel* by conjugation. **(A)** Intracellular levels of c-di-GMP measured by LC-MS/MS and compared to PAKΔ*pel* carrying pBBR1-MCS4. Statistical Student's *t*-test analysis with $p<0.01$ is based on three technical replicates and significant changes are indicated by asterisks. **(B)** At low levels of c-di-GMP the T3SS is expressed whereas at high levels of c-di-GMP the T6SS is expressed. T3SS was induced by calcium chelation. **(B)**, **(C)**, **(D)** and **(E)** Western blot performed on whole cell lysates using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS) or VgrG1 (T6SS). In **(E)** the three bands correspond, from top to bottom, to VgrG1b, VgrG1c and VgrG1a (Hachani *et al.*, 2011).

The T3SS, together with the T2SS, is associated with the development of acute infections via the secretion of toxins and hydrolytic enzymes. Since the increase of c-di-GMP is accompanied by a down-regulation of T3SS, the impact of c-di-GMP on T2SS was also checked. In this case, the expression of the secretion system was monitored by Western blot using an antibody against one of its substrates, the elastase LasB. Interestingly and similar to the impact on T3SS, elevated levels of c-di-GMP obtained by overproduction of WspR^{R242A} down-regulated the production of LasB (Figure 4.3).

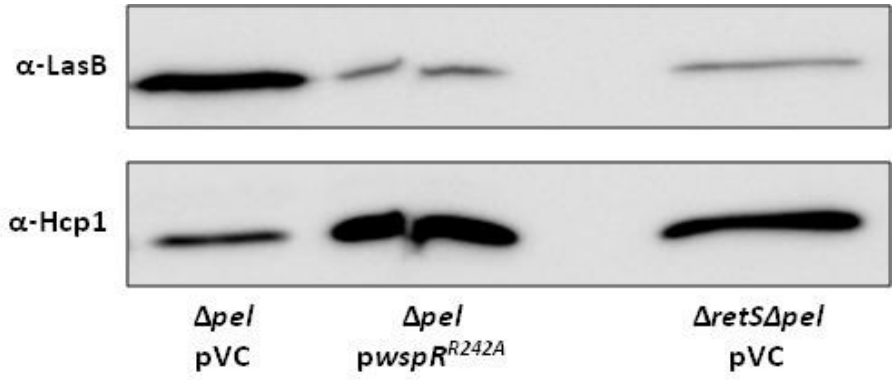

Figure 4.3 Elevated levels of c-di-GMP down-regulate the T2SS effector protein LasB.

The pBBR1-MCS4 vector control (pVC) and its derivative overexpressing *wspR*^{R242A} (p*wspR*^{R242A}) were introduced in PAKΔ*pel* or PAKΔ*retS*Δ*pel* by conjugation. Western blot performed on whole cell lysates using antibodies directed against LasB (T2SS) and Hcp1 (T6SS).

4.4 Decreased levels of c-di-GMP up-regulate the T3SS

To determine if the observable T3SS/T6SS switch can be reverted by lowering the levels of c-di-GMP, the inverse approach was applied. In this case, a construct overexpressing PA2133 which encodes a protein with PDE activity was introduced in the *retS/pel* mutant. As previously described, the production of PcrV and Hcp1 was monitored. Remarkably, the effect of PA2133 mirrored the effect of WspR^{R242A}, hence the T3SS was up-regulated whereas the T6SS was down-regulated (Figure 4.2A and B) (Moscoso *et al.*, 2011).

4.5 The Gac system is required for the c-di-GMP-dependent T3SS/T6SS switch

RetS belongs to a signalling cascade that involves several components like the central GacAS two-component system, the two RsmY and RsmZ sRNAs, and the additional LadS sensor. The fact that the Gac system and the c-di-GMP network exert a similar control on biofilm formation and the T3SS/T6SS switch prompted the question of whether this regulation runs in parallel or is interlinked. To address this question, the plasmid overexpressing *wspR*^{R242A} was introduced in a series of mutants in which the Gac cascade is inactivated, i.e. the *rsmY/rsmZ* double mutant, the *gacS* and *gacA* mutants and the *ladS* mutant.

As illustrated in Figure 4.4A and B, even though overproduction of WspR^{R242A} increased the levels of c-di-GMP in the *rsmY/rsmZ* double mutant, this was not accompanied by an induction of the T6SS or a reduction of the T3SS expression. This suggests that the c-di-GMP levels *per se* are not sufficient to induce the T3SS/T6SS switch and full control requires the production of the sRNAs to sequester RsmA and drive the up-regulation of the T6SS and down-regulation of the T3SS (Moscoso *et al.*, 2011). Furthermore, the switch was not induced in any mutant affected in genes required for the expression of the sRNAs, *gacS* and *gacA*, or positively influencing their expression, *ladS.* Interestingly, when the levels of c-di-GMP were assessed in a *retS* mutant also lacking the *rsmY* and *rsmZ* genes, the strain displayed reduced c-di-GMP levels, indicating that the elevated levels of c-di-GMP in the *retS* mutant is somehow dependent on the production of the two sRNAs (Figure 4.4C). Deletion of the sRNAs individually did not have an impact on the overall levels of intracellular c-di-GMP, suggesting that for this phenotype they may have a redundant function.

In conclusion, the expression of RsmY and RsmZ seems to be central for the c-di-GMP-dependent control of the T3SS/T6SS switch. Furthermore, in all the mutants tested the introduction of the vector control or the plasmid overproducing WspR^{R242} resulted in a wild-type phenotype with up-regulated T3SS and down-regulated T6SS, indicating that LadS, GacS and GacA, acting upstream of RsmY and RsmZ, are also required to observe the c-di-GMP-induced T3SS/T6SS switch.

4.6 Screen for c-di-GMP binding of the components of the Gac system

The data provided so far introduces the notion that the Gac and c-di-GMP cascades are interlinked but does not give yet a clear mechanistic insight on how it happens. One possibility is that c-di-GMP acts as a signal for the expression or activation of components belonging to the Gac signalling cascade. Another possibility is that the production of c-di-GMP is controlled by the Gac system.

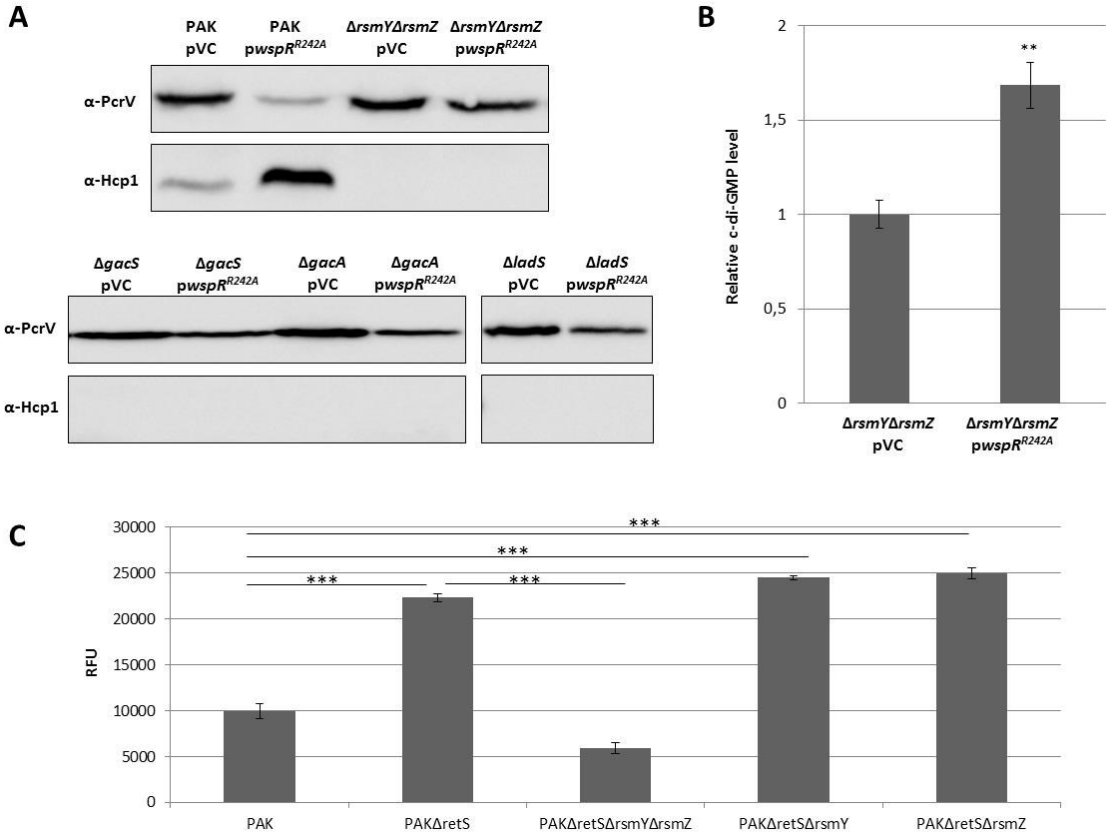

Figure 4.4 The c-di-GMP-dependent T3SS/T6SS switch requires the expression of the two sRNAs RsmY and RsmZ.

The pBBR1-MCS4 vector control (pVC) and its derivative overexpressing *wspR*^{R242A} (p*wspR*^{R242A}) were introduced in PAK or the *rsmY/rsmZ*, *gacS, gacA* and *ladS* mutants by conjugation. **(A)** Western blot performed on whole cell lysates using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS). T3SS was induced by calcium chelation. **(B)** Intracellular levels of c-di-GMP measured by LC-MS/MS and compared to PAKΔ*rsmYΔrsmZ* carrying pBBR1-MCS4. Statistical Student's *t*-test analysis with $p<0.01$ is based on three technical replicates and significant changes are indicated by asterisks. **(C)** Intracellular levels of c-di-GMP measured with the c-di-GMP transcriptional *gfp* reporter. The reporter was introduced in the *P. aeruginosa* strains specified by electroporation. Relative fluorescence units (RFU) are arbitrary fluorescence intensity units corrected for cell density. An ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes. One independent experiment was performed.

To test the first possibility, the ability of RetS, LadS, GacS, GacA and RsmA to allosterically bind c-di-GMP was evaluated. For that reason, the corresponding genes were cloned into pET28a with an N-terminal FLAG tag and expressed in *E. coli* BL21 (DE3) cells (for soluble proteins) or BL21 (DE3) C41 cells (for membrane proteins). In collaboration with the group of Urs Jenal at the Biozentrum (University of Basel), whole cell lysates overexpressing these proteins were tested for c-di-GMP binding using a biochemical approach based on a c-di-GMP capture compound (Nesper *et al.*, 2012). This capture compound was developed by a biotechnology company called Caprotec Bioanalytics and consists of a molecule with three domains: a selectivity domain which contains the c-di-GMP molecule, a reactivity domain that can be activated to cross-link the proteins that bind to the selectivity domain, and a sorting domain which allows the pull-down of the capture compound and any proteins cross-linked to it (Figure 4.5A). In this technique, whole cell lysates overexpressing FLAG-tagged versions of the protein of interest are incubated with the capture compound and proteins that bind c-di-GMP are cross-linked to the capture compound by excitation with UV light. After, the bound proteins are isolated using streptavidin magnetic beads that bind to the biotin in the sorting domain. Using antibodies directed against the FLAG-tag of the protein it is then possible to analyse by Western blot whether the proteins of interest are "captured" by the compound. In the event of binding, specific binding to c-di-GMP can be determined by prior incubation of the lysate with free c-di-GMP so that the protein is first saturated with c-di-GMP and later unable to bind to the capture compound.

To carry out the assay, whole cell lysates containing the positive control DgrA, a soluble PilZ domain protein from *C. crescentus* known for binding c-di-GMP was used. Whereas DgrA could be specifically captured, the same was not true for the *P. aeruginosa* RsmA soluble protein (Figure 4.5B). As for GacA, it was observed that some of the protein was captured, but it was not possible to discriminate if this binding was specific as pre-incubation with free c-di-GMP did not prevent the binding of GacA to the capture compound.

In relation to the membrane proteins, specific binding to c-di-GMP could not be conclusively detected for any of the proteins tested (Figure 4.5C), including for the known DGC protein YfiN. Similarly to GacA, YfiN and GacS were able to bind the capture compound but this binding was not prevented by pre-incubation with free c-di-GMP. For LadS and RetS, no interaction with the capture compound was detected.

To further investigate the ability of GacA and GacS to bind c-di-GMP, an additional method was used. As explained in the previous chapter, the DRaCALA assay is another assay that allows the identification of c-di-GMP binding proteins and was performed in collaboration with Vincent Lee. As

shown in Figure 4.6, c-di-GMP binding was clear for DgrA but negligible for GacS, GacA, RetS and RsmA. However, for whole cell lysates overexpressing LadS a weak but reproducible c-di-GMP binding ability was detected, challenging the result obtained by the capture compound assay.

In summary, the two methods employed here to determine whether RetS, LadS, GacS, GacA and RsmA components of the Gac system are able to bind c-di-GMP yielded inconclusive data and it remains to be determined if LadS, GacS or GacA have any binding affinity to c-di-GMP. In addition, the sensitivity of these methods is unclear and low affinity binders may be hard to identify.

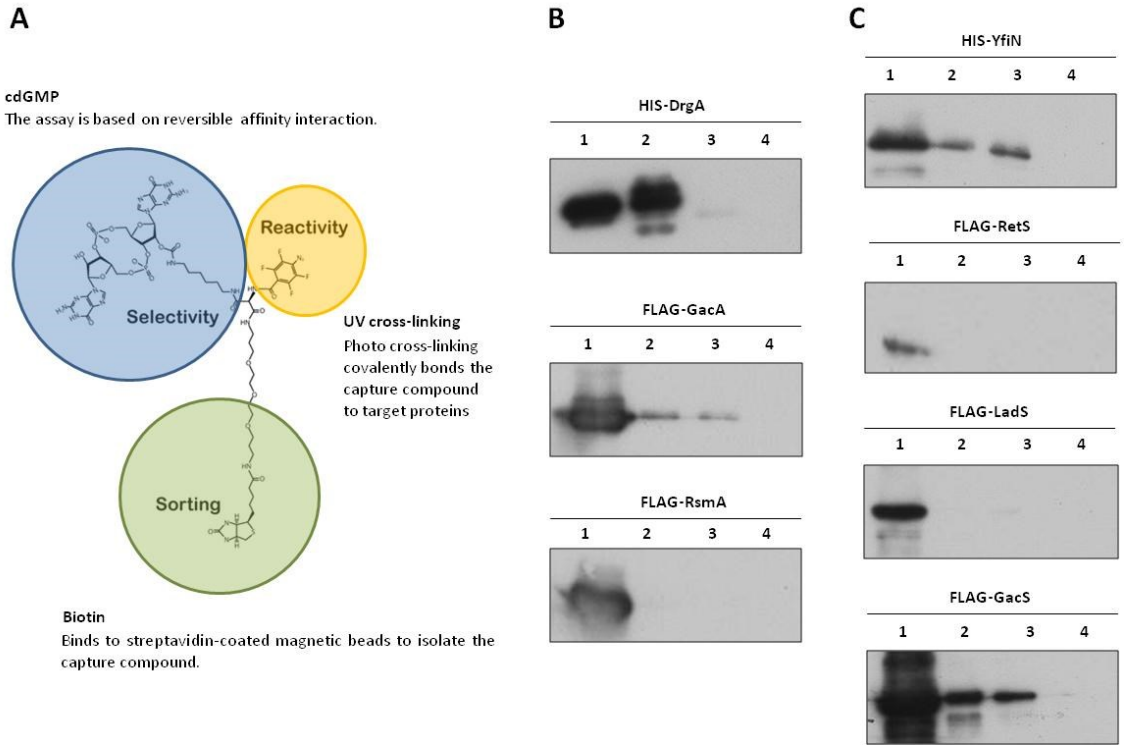

Figure 4.5 Assessment of the c-di-GMP binding ability of RetS, LadS, GacS, GacA and RsmA using the c-di-GMP capture compound.

(A) Chemical structure and domain specification of the c-di-GMP capture compound developed by Caprotec Bioanalytics GmbH, Berlin. **(B)** Western Blot analysis of whole cell lysates overproducing the soluble proteins DgrA, GacA and RmsA or **(C)** the membrane proteins YfiN, RetS, LadS and GacS. All proteins were HIS- or FLAG-tagged and detected using anti-HIS or anti-FLAG antibodies. Lanes 1 were loaded with the cell extracts. Lanes 2 were loaded with cell extracts incubated with 10 µM of capture compound. Lanes 3 were loaded with cell extracts pre-incubated with 100x excess of c-di-GMP (specificity control). Lanes 4 were loaded with cell extracts incubated with the streptavidin beads (beads control).

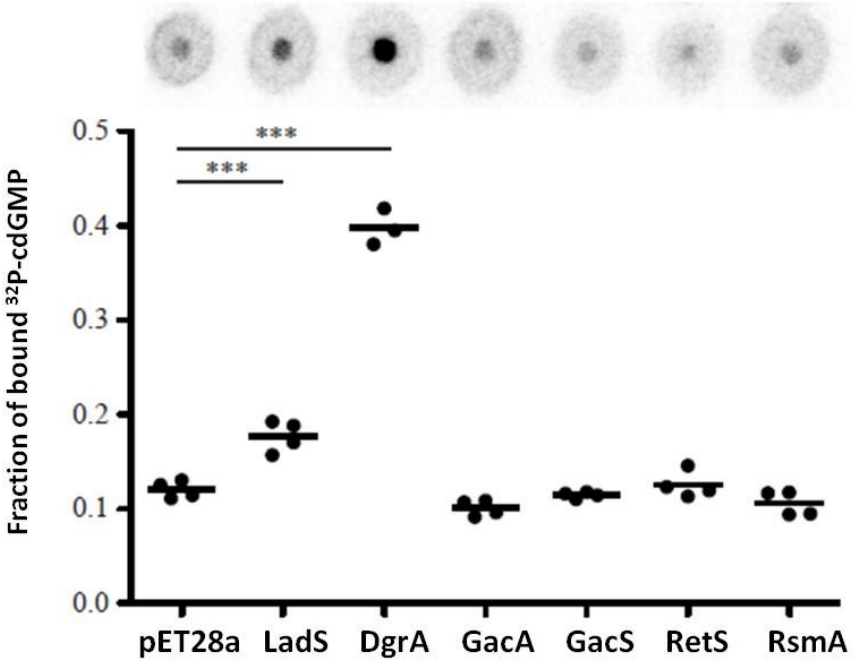

Figure 4.6 Assessment of the c-di-GMP binding ability of RetS, LadS, GacS, GacA and RsmA by DRaCALA.

Graph of ^{32}P-c-di-GMP binding by *E. coli* whole cell lysates overproducing the indicated proteins. The average indicated by a horizontal bar. Asterisks indicate significant difference by Dunnet's test (*** represents $p<0.01$). Figure provided by Vincent Lee (unpublished).

4.7 Screen for putative DGCs and PDEs involved in the T3SS/T6SS switch

To test if any c-di-GMP related protein is specifically associated with the regulation of the T3SS/T6SS switch, a screen using a PA14 transposon collection in genes encoding proteins with a GGDEF, EAL, HD-GYP or PilZ domains was performed. The PA14 strain was chosen in detriment of the PAO1 strain because in PAO1 the T3SS/T6SS switch is not as clearly identifiable as in PA14. Hence, the PA14 collection was obtained from the laboratory of Frederick Ausubel (Liberati *et al.*, 2006) and consisted of transposon insertion mutants in all the genes encoding proteins with the referred domains, including a mutant in the *pvrR* gene which encodes a protein with an EAL domain found only in PA14; and excluding PA2771 and PA2818 which are not found in PA14; PA0012 and PA2870 which are not available in the library; and *pelD* and *toxR* which were not available in the Filloux's laboratory collection.

The rationale of this screen was based on the overproduction of LadS, an antagonist of RetS which is able to induce the T3SS/T6SS switch, up-regulating T6SS and down-regulating T3SS. The collection

was thus tested for mutants unable to switch upon introduction of the pBBR1-MCS4 derivative carrying the *ladS* gene. The transposon mutants in PA2072, PA4781 and PA2989 were not considered in this evaluation because they were not able to incorporate the plasmid but among the remaining 43 transposon mutants, the *wspR* mutant was the only one unable to up-regulate T6SS and down-regulate T3SS upon overexpression of *ladS* (Figure 4.7A). However, subsequent attempts to confirm this phenotype by reconstructing a clean *wspR* deletion mutant did not confirm the phenotype and the T3SS/T6SS switch was perfectly effective (Figure 4.8) (Moscoso *et al.*, 2012).

In addition to this, another four mutants were partially affected for the T3SS/T6SS switch as LadS was able to induce the up-regulation of the T6SS but no down-regulation of the T3SS was observable. These were mutants in PA0290, PA3258, *morA* and PA2200 and suggest that these proteins are specifically involved in the regulation of the T3SS, but this line of investigation was not carried further.

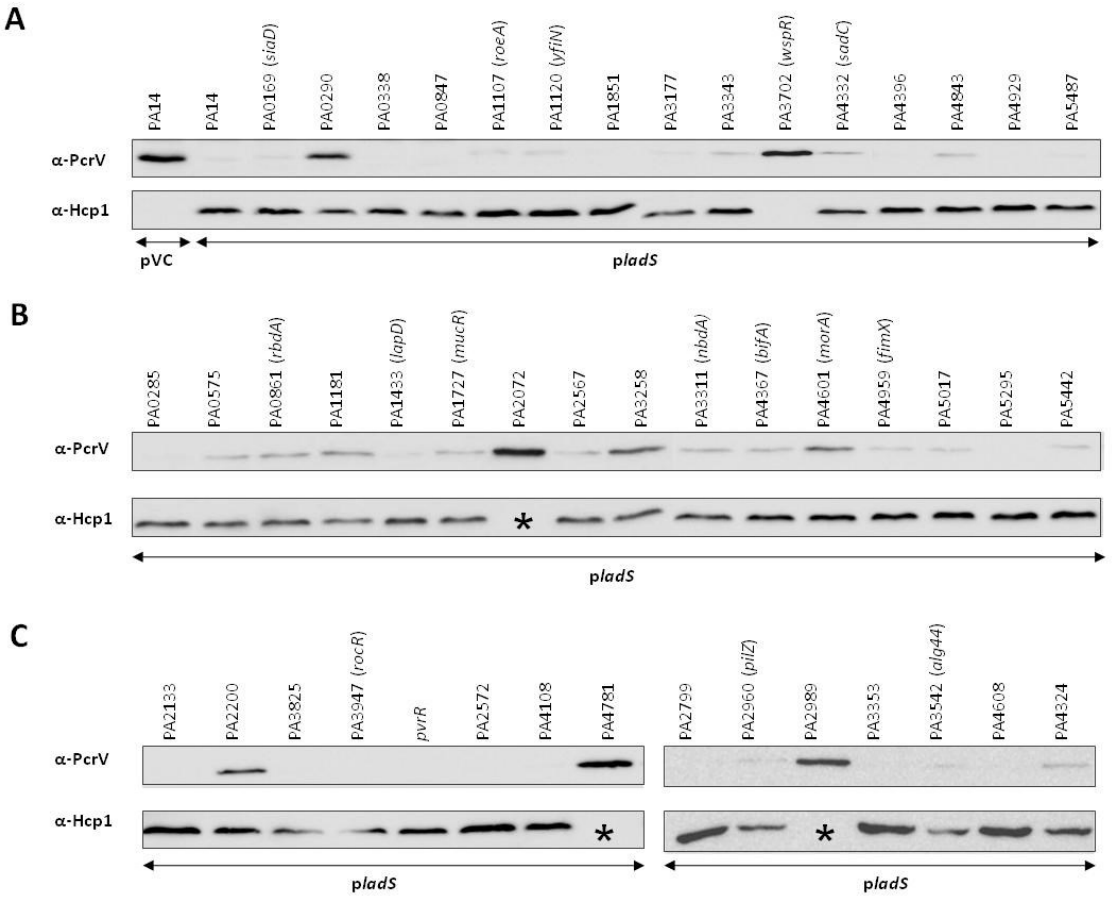

Figure 4.7 Screen of a PA14 transposon collection for loss of T3SS/T6SS switch. (Figure legend continues on the next page.)

The pBBR1-MCS4 vector control (pVC) and its derivative overexpressing *ladS* (p*ladS*) were introduced in all the strains by conjugation and Western blot was performed on whole cell lysates using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS). Asterisks indicate strains that did not incorporated the p*ladS* plasmid. **(A)** Overexpression of *ladS* induces the T3SS/T6SS in PA14 and mutants in genes encoding proteins with a GGDEF domain are shown. **(B)** Mutants in genes encoding proteins with GGDEF and EAL domains are shown. **(C)** Mutants in genes encoding proteins with EAL or HD-GYP domains (left) and in genes encoding proteins with PilZ domains (right) are shown.

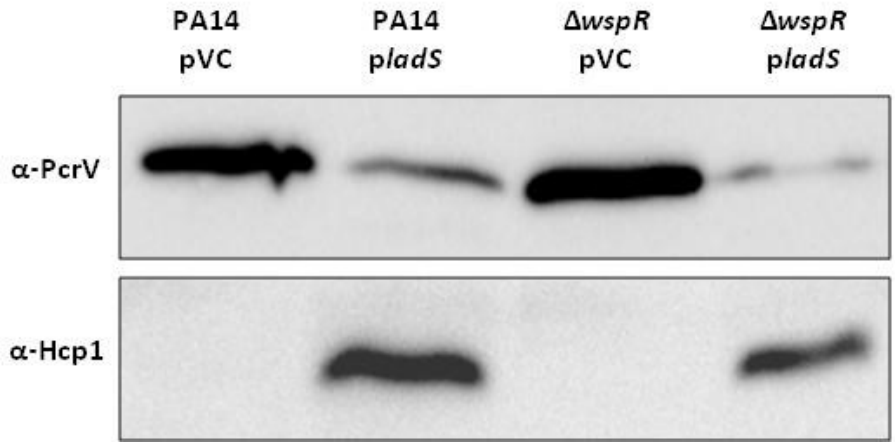

Figure 4.8 The T3SS/T6SS switch occurs in a *ΔwspR* mutant upon overproduction of LadS.

Western blot analysis of *P. aeruginosa* PA14 and PA14Δ*wspR* mutant carrying pBBR1-MCS-4 (pVC) or its derivative overexpressing *ladS* (p*ladS*). The antibodies used are directed against PcrV (T3SS) and Hcp1 (T6SS).

4.8 Discussion

The relation between the Gac system and the control of the *P. aeruginosa* mode of infection has been established in the last decade through numerous observations. In the course of an acute infection, the T3SS is activated upon host-cell contact and drives the injection of toxins directly in the cytosol of the host cells. In laboratory conditions, the T3SS can be induced in calcium depleted medium. In various animal models of acute infection (Hauser *et al.*, 2002; Roy-Burman *et al.*, 2001), including the cost-effective and easy to use *Galleria mellonella* caterpillar (Miyata *et al.*, 2003), the T3SS has been shown to be required for full virulence. Moreover, isolates from chronically infected patients frequently do not have an active T3SS indicating that the secretion of T3SS effectors is not a requirement for the establishment of long-term chronic infections (Jain *et al.*, 2004).

Interestingly, transcriptome analysis of a *retS* mutant has shown that RetS is required not only for the expression of the T3SS but also for the expression of other virulence factors such as T2SS and T4P (Goodman *et al.*, 2004). In addition, *in vivo* studies with murine models for acute infections (Goodman *et al.*, 2004; Zolfaghar *et al.*, 2006) and *G. mellonella* (unpublished data, Figure 4.9) showed that the *retS* mutant is attenuated in virulence. In contrast, the *retS* mutant displays increased ability to adhere to surfaces, biotic or abiotic, and transcriptome analysis revealed an up-regulation of the *pel* and *psl* exopolysaccharide genes together with an up-regulation of the genes in the H1-T6SS. Both the production of exopolysaccharides, an intrinsic characteristic of biofilms, and the expression of T6SS are traits that have been associated with *P. aeruginosa* chronic infections. In the lungs of cystic fibrosis patients, bacteria establish as a biofilm (Singh *et al.*, 2000) and high titers of antibodies against the T6SS are detected in their serum (Mougous *et al.*, 2006). Furthermore, in a murine chronic lung infection model, mutants in the HI-T6SS were identified as being attenuated in virulence (Potvin *et al.*, 2003).

RetS is an orphan sensor that belongs to the Gac signalling cascade. This cascade is known to coordinate the expression of more than 500 genes via the translational repressor RsmA (Figure 1.6). This is a sRNA-binding protein that inhibits the translation of 2/3 of its regulon by directly binding to mRNA, including the *psl* and T6SS mRNA (Brencic & Lory, 2009; Irie *et al.*, 2010). Indirectly, RsmA positively impacts the expression of 1/3 of its regulon, including T3SS, T2SS and T4P genes. The function of RsmA is counteracted by the expression of two sRNAs that compete with the mRNA targets for RsmA binding. The expression of these sRNAs, RsmY and RsmZ, is induced by the central two-component system GacAS and positively affected by the additional orphan sensor LadS.

Given that the Gac system plays a central role in the modulation of the two *P. aeruginosa* modes of infection, this work addressed the question of whether c-di-GMP is related to the Gac regulatory pathway. In other bacteria that have a conserved GacAS two-component system, such as *E. coli* and *S. enterica*, a connection between the Gac and c-di-GMP networks has been established in the regulation of the PGA exopolysaccharide production and the biogenesis of cellulose, respectively. In *P. aeruginosa* a link between c-di-GMP and the Gac system has never been explored experimentally although two reports provide hints on such a link. In the paper by Goodman *et al.* (2004), the GGDEF domain protein PA4332 (*sadC*) was identified in a transposon screen for suppressors of the *retS* mutant; and in the paper by Brencic & Lory (2009), the GGDEF domain proteins PA0338 and PA4929 were identified as being part of the RsmA regulon, the former being up-regulated in a *rsmA* mutant whereas the latter is down-regulated.

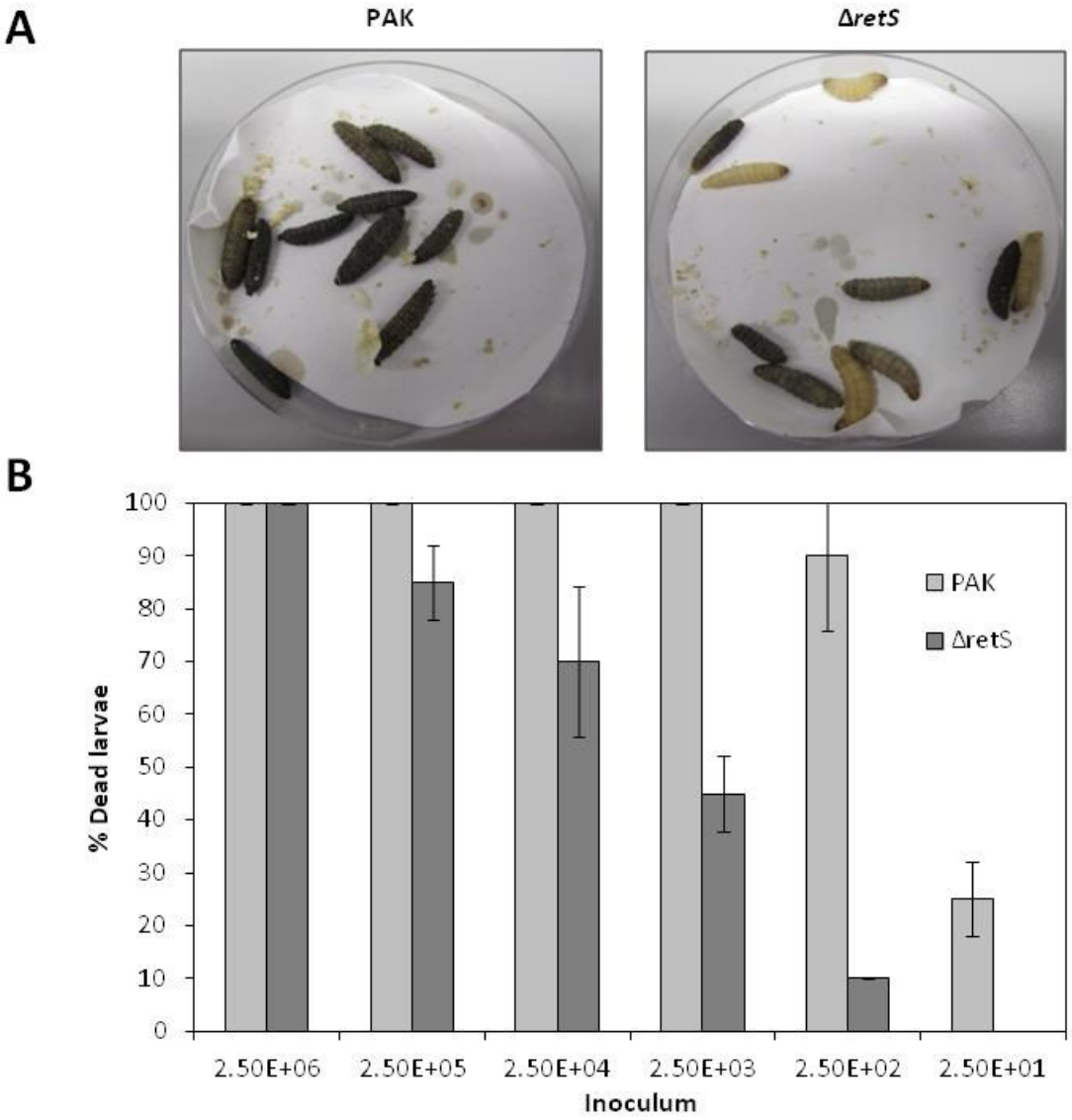

Figure 4.9 *G. mellonella* killing is attenuated in the *retS* mutant.

This experiment was performed together with Isabel Cortés. **(A)** Photograph of *G. mellonella* inoculated with $2.5x10^4$ bacteria and incubated for 1 day. Dead larvae are black. **(B)** The PAK wild-type strain and the *retS* mutant were injected into *G. mellonella* and the percentage of dead larvae after 1 day of incubation is shown.

The signalling molecule c-di-GMP is known to regulate the switch between motility and sessility, so the first thing to test was if the *retS* hyperbiofilm mutant was associated with high levels of c-di-GMP. Although the overall intracellular concentrations of c-di-GMP varies between different species, the levels of c-di-GMP are typically very low, in the picomolar range (Simm *et al.*, 2009), and its quantification is not trivial. Nevertheless, simple methods that allow the detection and relative quantification of c-di-GMP have become available recently (Table 4.1). By using LC-MS/MS and the c-di-GMP reporter, it was possible to observe that the hyperbiofilm phenotype of the *retS* mutant was associated with elevated levels of c-di-GMP. Importantly, this was independent of the formation of biofilms as the *retS/pel* mutant displayed even higher levels of c-di-GMP than the *retS* mutant (Figure 4.1).

The finding that c-di-GMP is involved in the hyperbiofilm phenotype of the *retS* mutant is consistent with the pre-established concept that high levels of c-di-GMP promote biofilm formation. Recently, in *X. campestris*, the *rsmA* hyperbiofilm mutant was also associated with increased levels of c-di-GMP and three DGCs were identified as RsmA targets, providing an explanation on how the c-di-GMP levels are elevated in the mutant (Lu *et al.*, 2012).

In *P. aeruginosa* the finding that the *retS* mutant has high levels of c-di-GMP left open the possibility that c-di-GMP could be involved in the inverse regulation of the T3SS and T6SS, two secretion systems that play a major role in determining the virulence status of *P. aeruginosa*. To investigate this question, the levels of c-di-GMP were artificially modulated by means of overexpression of a DGC, $WspR^{R242A}$, and a PDE, PA2133 (see Chapter 3). It was observed that increased levels of c-di-GMP caused the wild-type strain to switch from an up-regulated T3SS to an up-regulated T6SS, and low levels of c-di-GMP caused the *retS* mutant to switch from an up-regulated T6SS to an up-regulated T3SS (Figure 4.2). In other words, low levels of c-di-GMP promoted the expression of T3SS while high levels of c-di-GMP were associated with the expression of the T6SS. Furthermore, the T2SS which is co-regulated with the T3SS in the *retS* mutant, was also shown to be down-regulated by high levels of c-di-GMP (Figure 4.3).

Table 4.1 Chemical and biochemical methods for detection of c-di-GMP and identification of c-di-GMP binding proteins.

Method	Description	References
Detection/quantification of c-di-GMP		
2D-TLC	Two-dimensional thin layer chromatography of nucleotide extracts	Bochner & Ames, 1982; Merritt *et al.*, 2007
Liquid chromatography coupled with mass spectrometry	Following nucleotide extraction, the amount of c-di-GMP is determined by mass spectrometry after separation by reversed-phase high-performance liquid chromatography.	Simm *et al.*, 2009; Spangler *et al.*, 2010
FRET	Genetically engineered c-di-GMP binding protein fused to two fluorescent proteins. When c-di-GMP binds, fluorescence decreases.	Christen *et al.*, 2010
Thiazole orange	Fluorescent detection of c-di-GMP quadruplexes.	Nakayama *et al.*, 2011
c-di-GMP-proflavine-hemin complex	Colorimetric assay based on the peroxidase activity of the complex.	Nakayama *et al.*, 2012
c-di-GMP reporter (P_{cdrA}-*gfp*)	Fluorescence-based transcriptional reporter that uses the c-di-GMP-responsive promoter of the adhesin encoding gene *cdrA*. The presence of c-di-GMP activates transcription.	Rybtke *et al.*, 2012
Identification of c-di-GMP binding proteins		
c-di-GMP analog bound to sepharose beads	The functional c-di-GMP analog 2'-aminohexylcarbamoyl-c-di-GMP is covalently coupled to sepharose beads and proteins are isolated by affinity chromatography.	Duvel *et al.*, 2012
DRaCALA	Differential radial capillary action of ligand assay. Radioactive labelled c-di-GMP is synthesized and diffusion of the ligand in a membrane is measured after contact with the protein. Diffusion is slower when c-di-GMP* binds the protein.	Roelofs *et al.*, 2011
c-di-GMP-capture compound	Chemical molecule composed of c-di-GMP, UV cross-linking domain and biotin. Proteins that bind c-di-GMP are covalently bound to the capture compound and isolated using streptavidin magnetic beads.	Nesper *et al.*, 2012

To elucidate the link between the c-di-GMP and Gac cascades in the regulation of biofilms and the secretion systems, the c-di-GMP levels were manipulated by overproducing $WspR^{R242A}$ in strains lacking genes of the Gac system that are required for the expression of the T6SS. In all the mutants tested, i.e. *ladS, gacS, gacA and rsmY/rsmZ* mutants, $WspR^{R242A}$ was unable to induce the T3SS/T6SS switch, indicating that the expression of the RsmA antagonists, RsmY and RsmZ, is required for the c-di-GMP-dependent T3SS/T6SS switch (Figure 4.4). Furthermore, the levels of c-di-GMP in a *retS/rsmY/rsmZ* mutant were reduced in comparison to the *retS* mutant, suggesting that the increase on c-di-GMP levels is a consequence of the activity of the Gac pathway. Although it is not known how c-di-GMP impacts the T3SS or the T6SS, the fact that the up-regulation of the T6SS via c-di-GMP is dependent on the sRNAs is indicative of an indirect role of c-di-GMP in the posttranscriptional regulation of the T6SS.

This link between c-di-GMP and the regulation of the T2SS, T3SS and T6SS, together with the observation that the *retS* mutant displays high levels of c-di-GMP constitute a novel finding. At the same time that this research was carried out, a few publications correlating the expression of the T3SS and c-di-GMP started to appear and are in accordance with these findings. In the phytopathogen *Dickeya dadantii* (ex *Erwinia chrysantemi*), one PDE (EcpC) and one protein with GGDEF and EAL domains with putative PDE activity (EcpB) were positively correlated to the expression of T3SS and shown to be required for virulence *in vivo* (Yi *et al.*, 2010). In *S. enterica*, overexpression of a DGC, AdrA, was shown to lead to reduced virulence and secretion of the T3SS effectors (Lamprokostopoulou *et al.*, 2010). More recently, a mutant in STM2215, encoding a PDE, was also shown to be attenuated in virulence and to have reduced expression of T3SS effectors (Zheng *et al.*, 2013).

To understand the hierarchy between the two signalling networks i.e. if c-di-GMP influences the Gac system or if c-di-GMP variation is a consequence of the activity of the Gac system, two strategies were employed. Firstly, the ability of the Gac system components to bind c-di-GMP was assessed. Secondly, a screen to identify putative c-di-GMP related proteins specifically involved in the T3SS/T6SS switch was performed.

Like for the detection of c-di-GMP, the identification of c-di-GMP binding proteins has been hampered by the lack of simple procedures and high throughput techniques to verify c-di-GMP binding ability. Until recently, the only proteins known to bind c-di-GMP were proteins with a PilZ domain and studies were quite limited to the conventional bioinformatic predictions for the presence of the domain. However, the disproportion between the number of proteins with a PilZ domain and the number of putative DGCs and PDEs has always suggested that other c-di-GMP

receptors ought to exist and other types of receptors have slowly started to be discovered (see Chapter 1, section 1.3.2). Recently, a few groups developed assays suitable for high throughput analysis of c-di-GMP binding proteins (Table 4.1). One of them is the DRaCALA assay previously described and another one is from the group of Urs Jenal in Switzerland who developed a technique based in the usage of a c-di-GMP capture compound. To test whether LadS, RetS, GacS, GacA or RsmA were able to bind c-di-GMP both the capture compound and the DRaCALA assays were used.

For RetS and RsmA, none of the assays detected any c-di-GMP binding affinity but for GacS, GacA and LadS the results were inconclusive (Figure 4.5). This divergency between the two methods was not surprising as previous screens performed with the capture compound methodology and another technique developed by the group of Susanne Haussler (Table 4.1) (Duvel *et al.*, 2012) also yielded a number of divergent results. Using the c-di-GMP capture compound, GacS and GacA were pulled-down with the capture compound, but it was not possible to show that the binding was specific to c-di-GMP. This could mean that there is no c-di-GMP binding affinity or, instead, it can reflect a weak affinity for c-di-GMP under the conditions tested. One explanation for this last hypothesis is that the tested proteins were expressed in *E. coli* so it is possible that additional factors required to increase GacS or GacA c-di-GMP binding affinity were missing. For instance, GacS is known to phosphorylate GacA, and it is possible that the phosphorylation of the proteins alters their c-di-GMP binding affinity. Using the DRaCALA assay, a weak c-di-GMP binding affinity was determined for LadS but until further validation with purified protein or an alternative assay, it remains questionable if this binding is real or physiologically relevant (Figure 4.6). Hence, with the results presented here, it cannot be concluded that any of the components of the Gac system tested are able to sense c-di-GMP.

Noteworthy, even if it is unlikely that c-di-GMP binds any of the sensors and response regulators of the Gac system, it is still conceivable that c-di-GMP binds to the two sRNAs or that c-di-GMP acts in the regulation of the Gac components at the transcriptional or posttranscriptional level.

Although the RetS and LadS regulons do not entirely overlap, a subset of genes positively regulated by LadS corresponds to genes that are regulated negatively by RetS (Figure 1.7). This includes genes involved in biofilm formation, T3SS and T6SS, and a strain overproducing LadS displays an up-regulated T6SS like the *retS* mutant does. Exploiting this fact, a screen of a collection of PA14 transposon mutants was performed in search of mutants that did not switch upon overexpression of *ladS* but the screen did not identify any specific GGDEF, EAL, HD-GYP or PilZ domain proteins involved in the T3SS/T6SS switch (Figure 4.7 and 4.8) (Moscoso *et al.*, 2011; Moscoso *et al.*, 2012).

The regulation of the expression and activity of the T3SS in *P. aeruginosa* is known to be dependent on the transcriptional activator ExsA and on the production of cAMP. Briefly, ExsA (PA1713) binds the promoters of T3SS genes, including its own, and via ExsC, ExsD and ExsE, it couples the activity of the machinery with its transcription. When the T3SS is not active, ExsE (PA1711) accumulates in the cytoplasm and binds ExsC (PA1710), preventing the release of ExsA from the anti-activator ExsD (PA1714). When T3SS is active, ExsE is secreted and ExsC becomes available to bind the anti-activator ExsD, allowing ExsA to bind its target promoters (Hauser, 2009). In addition to this, the nucleotide cAMP has been shown to be required for T3SS activation, probably by binding to the transcriptional regulator Vfr, but the precise mechanism linking cAMP and T3SS activation is not clearly understood (Wolfgang *et al.*, 2003; Yahr & Wolfgang, 2006). Remarkably, mutants lacking cAMP have reduced expression of T2SS, T3SS and T4P, just like the *retS* mutant which has high levels of c-di-GMP. This suggests that there may be a balance between the intracellular concentrations of cAMP and c-di-GMP in such a way that high levels of c-di-GMP are correlated to low levels of cAMP and that these are involved, respectively, in inducing biofilm formation and repressing T3SS. Conversely, low levels of c-di-GMP may be correlated to high levels of cAMP, preventing biofilm formation and inducing the expression of the T3SS.

On the other hand, the regulation of the T6SS varies greatly between different species and even between different clusters in a same species. In *P. aeruginosa*, the T6SS is regulated at different levels, i.e. transcriptional, posttranscriptional and posttranslational (Miyata *et al.*, 2013; Silverman *et al.*, 2012). Transcriptionally, two quorum sensing related transcriptional regulators, LasR and MvfR (PA1013), have been shown to repress the H1-T6SS and induce H2- and H3-T6SS (Lesic *et al.*, 2009). Posttranscriptionally, H1-T6SS is known to be regulated directly by the translational repressor RsmA (Brencic & Lory, 2009) and in light of the data herein presented, indirectly by c-di-GMP. Posttranslationally, the H1-T6SS assembly and export is dependent on the phosphorylation of a T residue in Fha1 which is controlled by PpkA (kinase) and PppA (phosphatase) (Mougous *et al.*, 2007). The precise mechanism by which Fha1 allosteric modification induces the T6SS is still unknown but Fha1 has been observed to form stable complexes with the ATPase ClpV1 and to be recruited to the T6SS machinery upon PpkA phosphorylation (Hsu *et al.*, 2009).

In conclusion, the results show that the regulation by c-di-GMP goes beyond the switch between motitlity and sessility as it also acts in the regulation of secretion machineries that are major determinants of the *P. aeruginosa* pathogenicity, namely the T2SS, T3SS and T6SS. Furthermore, it establishes a link to the Gac system, demonstrating that the c-di-GMP-dependent T3SS/T6SS switch does not bypass the regulation exerted by RsmA. Importantly, there are multiple possibilities by

which c-di-GMP can regulate the T3SS/T6SS switch and this deserves further investigation. The mechanisms of action involving c-di-GMP signalling have proven to be very versatile and range from transcriptional regulation (e.g. FleQ in *P. aeruginosa* (Hickman & Harwood, 2008), posttranscriptional regulation (e.g. riboswitches in *B. subtilis* (Sudarsan *et al.*, 2008)), regulation of protein activity (e.g. flagellar brake in *E. coli* (Boehm *et al.*, 2010)), and cross-envelope inside-out signalling (e.g. LapD in *P. fluorescens* (Newell *et al.*, 2011)) (Moscoso *et al.*, 2011). Understanding how the molecular mechanism by which c-di-GMP is operating in the regulation of the different secretion systems may not only constitute a great leap on the understanding of bacterial signalling, but also help designing new drugs to efficiently combat infections by *P. aeruginosa* and many other pathogens.

5) Link between cyclic di-GMP and the Gac system

Chapter 5 – Link between cyclic di-GMP and the Gac system

In the previous chapters it was shown that high levels of c-di-GMP promote hyperbiofilm formation and the expression of the T6SS, whereas low levels of c-di-GMP promote a motile lifestyle and the expression of the T3SS and T2SS. In addition, the intricate c-di-GMP signalling network within the cell that operates at high specificity in the regulation of a variety of cellular processes was presented. Subsequently, evidence on how the c-di-GMP network is linked to the Gac signalling cascade and how these two pathways can be important in determining the *P. aeruginosa* course of infection was provided.

In this chapter, the previous observation that the *retS* hyperbiofilm mutant has elevated c-di-GMP levels sets the starting point of the investigation. Here, I looked for what protein is accountable for the elevated levels of c-di-GMP in the *retS* mutant and then for the downstream components that are involved in the specific c-di-GMP-dependent lifestyle switch orchestrated by the Gac system.

5.1 Screen for up-regulated DGCs in the *retS* mutant

The fact that the *retS* hyperbiofilm mutant displays high levels of c-di-GMP (Figure 4.1A, B and C) was not surprising as biofilm formation has been associated with elevated levels of the second messenger for quite some time now (Figure 1.10). However, because the c-di-GMP network operates at high specificity, this observation prompted the question of which protein, or proteins, are responsible for increasing the overall intracellular concentration of c-di-GMP in the *retS* mutant background. In theory, the elevated levels could be a consequence of (i) an up-regulation or activation of proteins with DGC activity, and/or (ii) a down-regulation or inactivation of proteins with PDE activity. In order to explore the first hypothesis, the mRNA levels of all the identified genes encoding putative DGCs, i.e. proteins with a GGDEF or a GGDEF/EAL domain, were analysed by qRT-PCR (real time reverse transcription PCR).

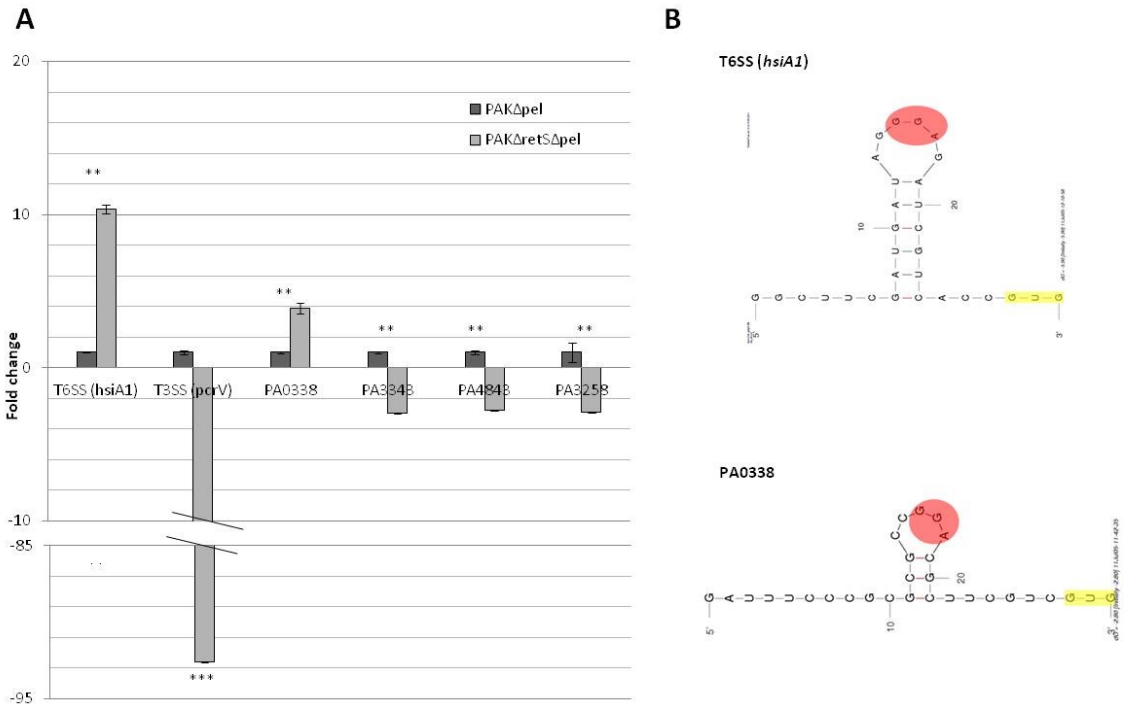

Figure 5.1 PA0338 is up-regulated in the *retS* mutant.

(A) Genes up- or down-regulated in the qRT-PCR screen of all the 34 proteins with a GGDEF domain found in *P. aeruginosa* PAO1 genome. Expression levels were normalized to *gyrA.* Data represents two independent experiments performed with three technical replicates each. Statistical Student's *t*-test analysis with $p<0.01$ is based on three replicates and significant changes are indicated by asterisks. **(B)** RNA secondary structures of the 5' untranslated region of *hsiA1* and PA0338 predicted by Mfold. GGA motifs are highlighted in red and the predicted start codon is in yellow.

A total of 34 candidates (see list of primers/genes on Table 2.2) were assessed and the mRNA levels of a particular gene in the *retS* mutant were compared to the mRNA levels of the same gene in the PAK wild-type strain. With a cut off value of 1.5-fold change, only four out of the 34 genes tested were up- or down-regulated in the *retS* mutant (Figure 5.1A). These were PA0338, encoding a protein with a GGDEF domain which was ~3.8-fold up-regulated; and another three genes that were in average 2.8-fold down-regulated: PA3343 and PA4843 which encode proteins with a GGDEF domain, and PA3258 which encodes a protein with a GGDEF/EAL domain.

In agreement with the up-regulation of PA0338 which suggests that PA0338 is a RsmA target, a putative RsmA binding site is recognizable in the 5' untranslated region of the PA0338 mRNA (Figure 5.1B). This is a GGA motif that sits in an exposed region of a putative stem-loop and overlaps with the predicted ribosome binding site, 12 nucleotides upstream of the start codon.

5.2 PA0338 is not responsible for the hyperbiofilm phenotype

Since PA0338 was up-regulated in the *retS* mutant and encodes a protein with a C-terminal GGDEF domain harbouring conserved I- and A-sites, it was hypothesized that the elevated levels of c-di-GMP in the *retS* mutant were due to the production and consequent DGC activity of PA0338. To investigate this hypothesis, the deletion of PA0338 was engineered in the *retS* mutant and the biofilm, swimming and T3SS/T6SS switch phenotypes were tested.

As illustrated in Figure 5.2A, B and C, deletion of PA0338 did not have an effect on the *retS* phenotypes. Similarly to the *retS* mutant, the *retS/*PA0338 double mutant still exhibited enhanced Congo Red binding (indicative of hyperbiofilm formation), reduced swimming motility and expression of the T6SS in detriment of the T3SS.

To check the levels of c-di-GMP in the *retS/*PA0338 double mutant, the c-di-GMP reporter described in the previous chapter was introduced by electroporation in the strains of interest and the produced fluorescence was measured. Consistent with the previous phenotypes, the *retS/*PA0338 double mutant displayed high levels of c-di-GMP (Figure 5.2D), refuting the hypothesis that PA0338 is a main player in the synthesis of c-di-GMP in the *retS* mutant background.

5.3 PA3343 is encoded in the genetic region of *hptB*

Among the genes that were down-regulated in the qRT-PCR screen, PA3343 stood out for being located close to *hptB* (PA3345). HptB is a protein with an Hpt domain that has been shown to be involved in a phosphorelay between RetS, PA1611 and HsbR, eventually contributing for the regulation of *rsmY* expression (Figure 1.6 and 1.8).

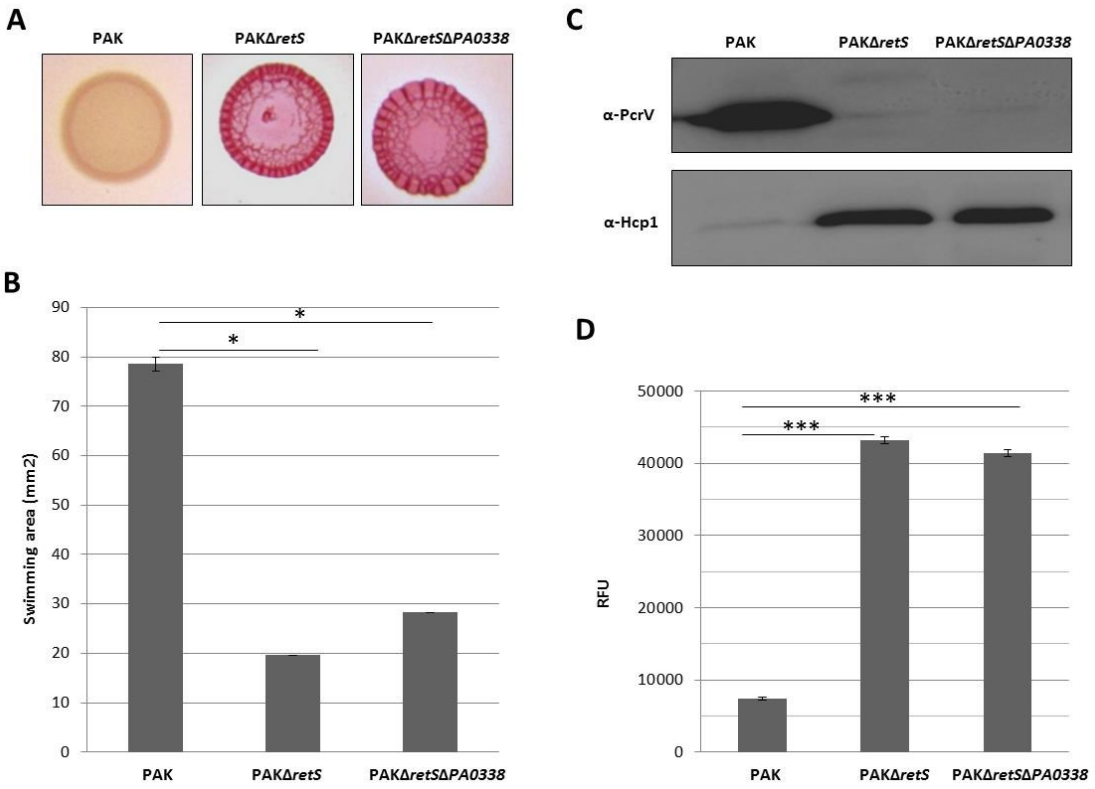

Figure 5.2 Phenotypes of the *retS*/PA0338 double mutant.

(A) Congo Red binding. The photograph was taken after 2 days of incubation. **(B)** Swimming motility in 0.3% LB agar plates from one independent experiment. **(C)** T3SS/T6SS switch. Western blot performed on whole cell lysates using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS). **(D)** Intracellular levels of c-di-GMP measured with the c-di-GMP transcriptional *gfp* reporter. The reporter plasmid was introduced in the *P. aeruginosa* strains specified by electroporation. Relative fluorescence units (RFU) are arbitrary fluorescence intensity units corrected for cell density. At least three independent experiments were performed. In (B) and (D) an ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes.

Given the proximity of PA3343 to *hptB*, RT-PCR (reverse transcription PCR) was performed to check if PA3343 was co-transcribed with *hptB* in the same polycistronic mRNA. To do this, a set of eight primer pairs were designed to amplify the intergenic regions between all the genes from PA3348 to PA3340 (Figure 5.3A). The products amplified by RT-PCR are shown in Figure 5.4B. By comparing the PCR products obtained from gDNA and cDNA, it is likely that *hptB* is encoded within an operon that extends from PA3347 to PA3341 and includes PA3343, the gene encoding a protein with known DGC activity (Kulasakara *et al.*, 2006).

Despite this very interesting observation, the fact that the qRT-PCR data revealed a down-regulation of PA3343 in the *retS* mutant rather than an up-regulation can hardly explain how a deletion of *retS* leads to increased levels of c-di-GMP. Therefore, for the purpose of this study this was not investigated further.

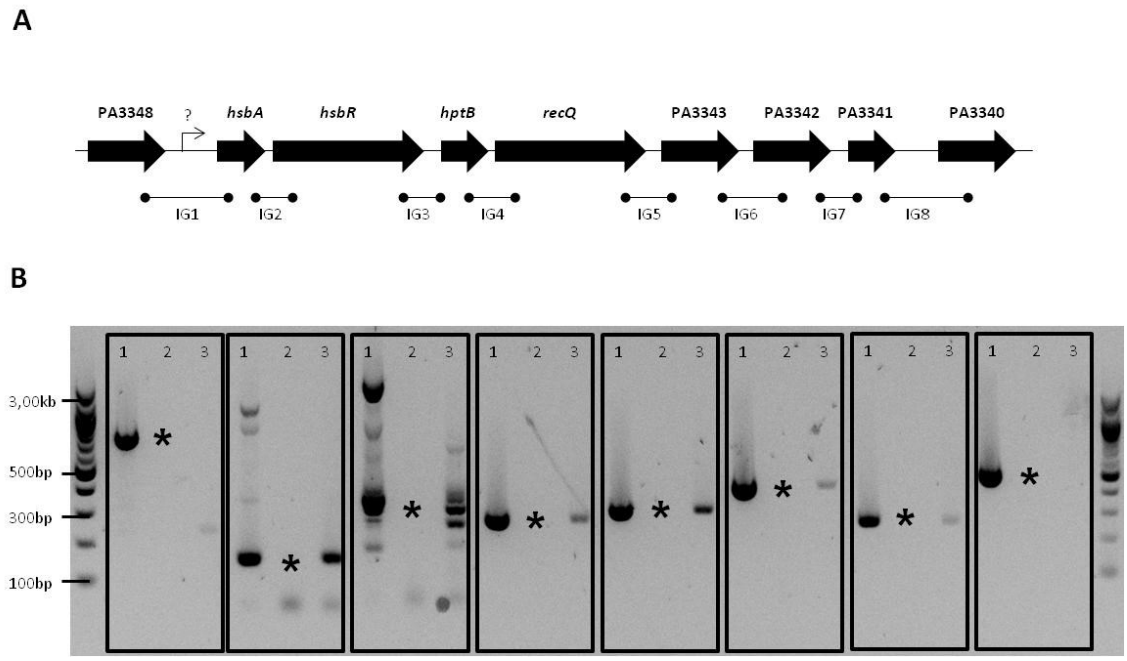

Figure 5.3 The genetic context of PA3343.

(A) Gene map of the region harbouring PA3343. Thick arrows denote open reading frames and the different intergenic regions (IG) are numbered 1-8 and indicated by lines with closed circles. **(B)** RT-PCR analysis of IG1-8 resolved on a 1.2% agarose gel. Lane 1 corresponds to PCR products from gDNA (positive control). Lane 2 corresponds to PCR products from the RNA extract (negative control). Lane 3 corresponds to PCR products from cDNA. Asterisks indicate the PCR product of the expected size.

5.4 SadC is responsible for the hyperbiofilm phenotype of the *retS* mutant

Since PA0338 was not responsible for the elevated levels of c-di-GMP in the *retS* mutant, another candidate was considered. According to the paper by Goodman *et al.* (2004) which characterized RetS, two *sadC* transposon insertion mutants were identified as suppressors of the *retS* hyperbiofilm mutant. SadC (PA4332) is a membrane protein and has a cytoplasmic C-terminal GGDEF domain with known DGC activity. Although *sadC* (PA4332) was not found to be up-regulated in the *retS* mutant, the impact of deleting *sadC* in the *retS* mutant was analysed in a similar way to PA0338.

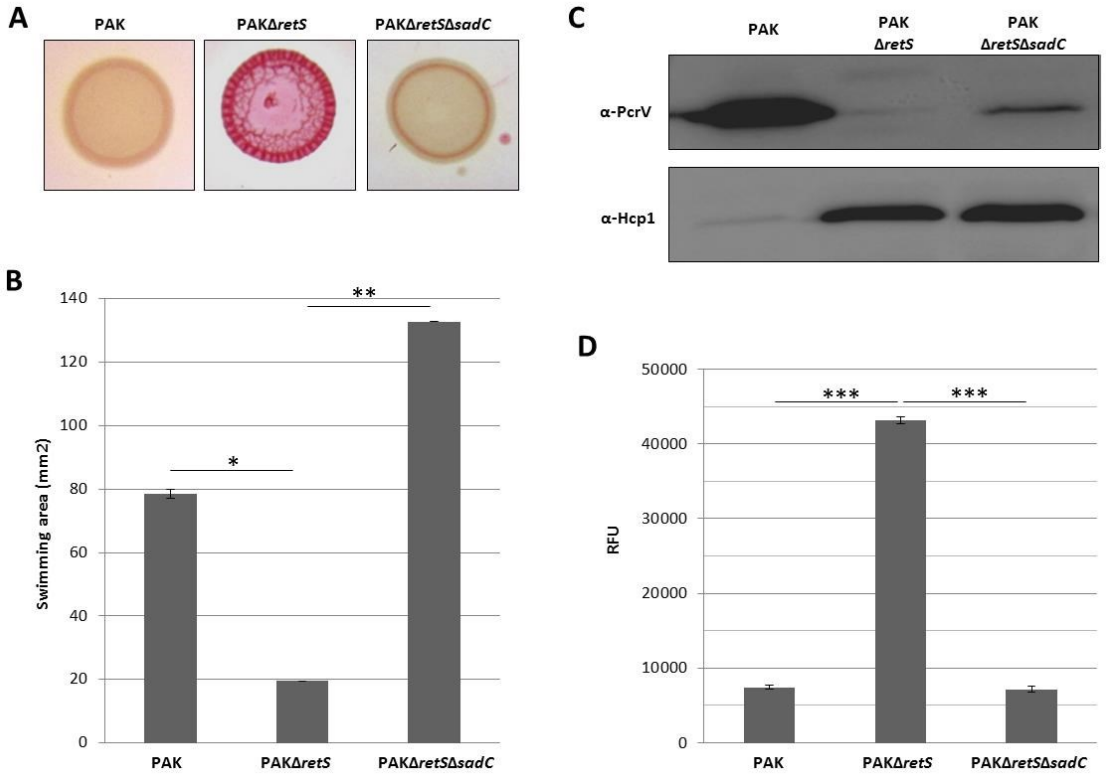

Figure 5.4 Phenotypes of the *retS/sadC* double mutant.

(A) Congo Red binding. The photograph was taken after 2 days of incubation. **(B)** Swimming motility in 0.3% LB agar plates from one independent experiment. **(C)** T3SS/T6SS switch. Western blot performed on whole cell lysates using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS). **(D)** Intracellular levels of c-di-GMP measured with the c-di-GMP transcriptional *gfp* reporter. The reporter plasmid was introduced in the *P. aeruginosa* strains specified by electroporation. Relative fluorescence units (RFU) are arbitrary fluorescence intensity units corrected for cell density. At least three independent experiments were performed. In (B) and (D) an ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes.

As shown in Figure 5.4A, the *retS/sadC* double mutant did not bind Congo Red like the *retS* mutant did, indicating that without *sadC* the hyperbiofilm phenotype induced by deletion of *retS* is lost.

In accordance to the loss of the hyperbiofilm phenotype, the swimming ability of the *retS/sadC* double mutant was also recovered and even surpassed that of the PAK wild-type strain (Figure 5.4B). In addition, the wild-type T3SS/T6SS switch phenotype was partially restored so that the T3SS was up-regulated (Figure 5.4C).

To check if the observed phenotypes were due to low levels of c-di-GMP, the c-di-GMP reporter was used. Indeed, the elevated c-di-GMP levels of the *retS* mutant were drastically reduced in the *retS/sadC* double mutant (Figure 5.4D). Taken together, these results strongly support the idea of SadC being responsible for the elevated levels of c-di-GMP in the *retS* mutant.

5.5 SadC is central to the Gac signalling system

Given that SadC was responsible for the elevated levels of c-di-GMP in the *retS* mutant, the requirement of SadC in the broader context of the Gac system was inspected. Besides deletion of *retS*, the deletion of another two components of the Gac signalling cascade, *hptB* and *rsmA*, also leads to hyperbiofilm formation. Inversely, overexpression of a RetS antagonist, LadS, or an HptB antagonist, HsbR, causes a similar hyperbiofilm outcome.

As illustrated in Figure 5.5A and B, the *hptB* hyperbiofilm mutant displayed an intermediate Congo Red binding phenotype in relation to the wild-type and the *retS* mutant, and this correlated to an intermediate level of c-di-GMP. Strikingly, like deletion of *sadC* in the *retS* mutant, the *hptB/sadC* double mutant was unable to bind Congo Red and showed reduced c-di-GMP levels. Concomitantly, overexpression of the antagonists LadS and HsbR in a *sadC* deletion mutant was unable to induce Congo Red binding (Figure 5.5C and D).

In relation to RsmA, a downstream target of both the HptB and RetS pathways, an *rsmA/sadC* double mutant was engineered and the Congo Red binding phenotype tested. As shown in Figure 5.6A, deletion of *sadC* in the *rsmA* mutant abrogated Congo Red binding, indicating that the production of SadC is also required for the hyperbiofilm phenotype of the *rsmA* mutant and further supporting a theory in which SadC is central to the Gac system and to signaling cascades converging onto RsmA.

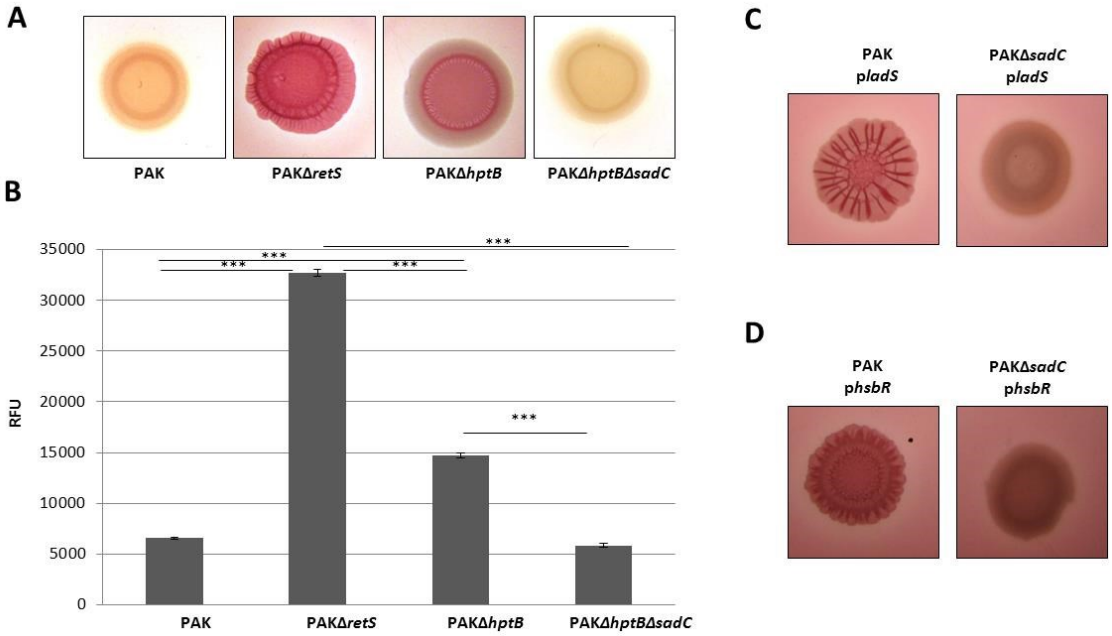

Figure 5.5 SadC is required for the c-di-GMP-dependent biofilm phenotype regulated by the Gac system.

(A) Congo Red binding. The photograph was taken after 2 days of incubation. **(B)** Intracellular levels of c-di-GMP measured with the c-di-GMP transcriptional *gfp* reporter. The reporter was introduced in the *P. aeruginosa* strains specified by electroporation. Relative fluorescence units (RFU) are arbitrary fluorescence intensity units corrected for cell density. An ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes. Three independent experiments were performed. PAK and PAKΔ*sadC* were conjugated with pBBR1-MCS4-*ladS* (p*ladS*) **(C)** or pBBR1-MCS4-*hsbR* (p*hsbR*) **(D)**. Congo Red binding is from day 2 of incubation.

To investigate the relation between SadC and the Gac system, the ability of SadC to induce biofilm formation in an *rsmY/rsmZ* mutant was checked. This mutant is unable to form biofilms because it lacks the two sRNAs that antagonize the repression exerted by RsmA. In order to induce biofilm formation via the activity of SadC, the *sadC* gene was cloned under the regulation of an arabinose inducible promoter into miniCTX1. This plasmid allows the integration of genes into the *att* site of the *P. aeruginosa* chromosome. Site-directed mutagenesis on *sadC* was carried out to release the DGC activity from the c-di-GMP feedback inhibition and rendered the I-site nonfunctional through the substitution to A of the R392 residue. As presented in Figure 5.6B, in the wild-type strain, overexpression of $sadC^{R392A}$ induced Congo Red binding as it was expected. However, in the *rsmY/rsmZ* double mutant the same was not true, demonstrating that SadC depends on the expression of the two sRNAs to induce biofilm formation.

All together, these results confirm that SadC plays a central role in the Gac signalling pathway and consolidates the notion that the Gac and c-di-GMP networks are interlinked. Moreover, the fact that overexpression of *sadC*^{R392A} *per se* was not sufficient to induce biofilm formation in the absence of RsmY and RsmZ expression, indicates that SadC acts downstream of the two sRNAs.

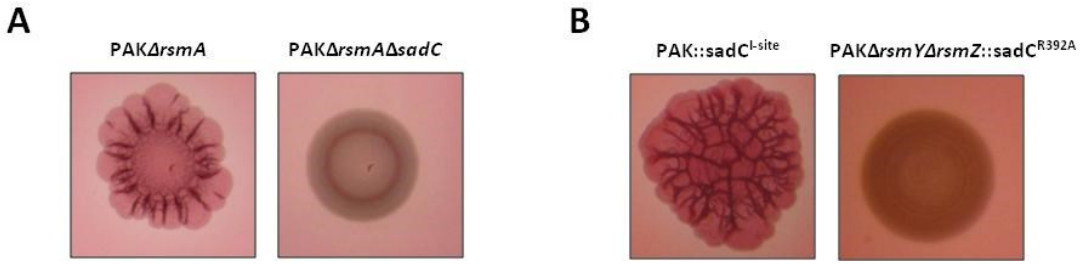

Figure 5.6 Interdependence of the Gac and c-di-GMP signalling cascades.

Congo Red binding phenotypes visualized on day 2 of incubation. **(A)** The Δ*rsmA* mutant phenotype is lost upon deletion of *sadC.* **(B)** Overexpression of Sad$^{I\text{-}site}$ in the wild-type strain but not in the PAKΔ*rsmY*Δ*rsmZ* induces Congo Red binding. Sad$^{I\text{-}site}$ was cloned in miniCTX1-*araC*-P_{BAD} and expressed using 0.4% arabinose after integration in the chromosome following conjugation.

5.6 SadB is also required for the hyperbiofilm phenotype of the *retS* and *hptB* mutants

Based on research performed by the group of George O'Toole, SadC was characterized for its role in reciprocally regulating biofilm formation and swarming motility (Merritt *et al.*, 2007). This observation derived mainly from the fact that a *sadC* deletion mutant had a hyperswarming phenotype and was defective in the transition to irreversible attachment during biofilm formation. Hinted by the fact that a previously described *sadB* (PA5346) transposon mutant displayed a similar phenotype (Caiazza & O'Toole, 2004), and through a series of epistasis experiments, the authors were able to place *sadB* and *sadC* in the same genetic pathway and demonstrated that SadB acts downstream of SadC.

In light of this, the effect of deleting *sadB* in the *retS* and *hptB* mutants was also investigated. If it is true that SadB is downstream of SadC, it is expected that deletion of *sadB* in the *retS or hptB* mutants causes a similar effect on biofilm formation as deletion of *sadC* does. As shown in Figure 5.7A and B, deletion of *sadB* impairs the hyperbiofilm phenotype of both mutants, although this was not as pronounced as the impairment caused by deletion of *sadC*. Nonetheless, the results support

the concept of SadB being in the same genetic pathway as SadC and further indicate that SadB may not be the sole player in the signalling cascade that operates downstream of SadC.

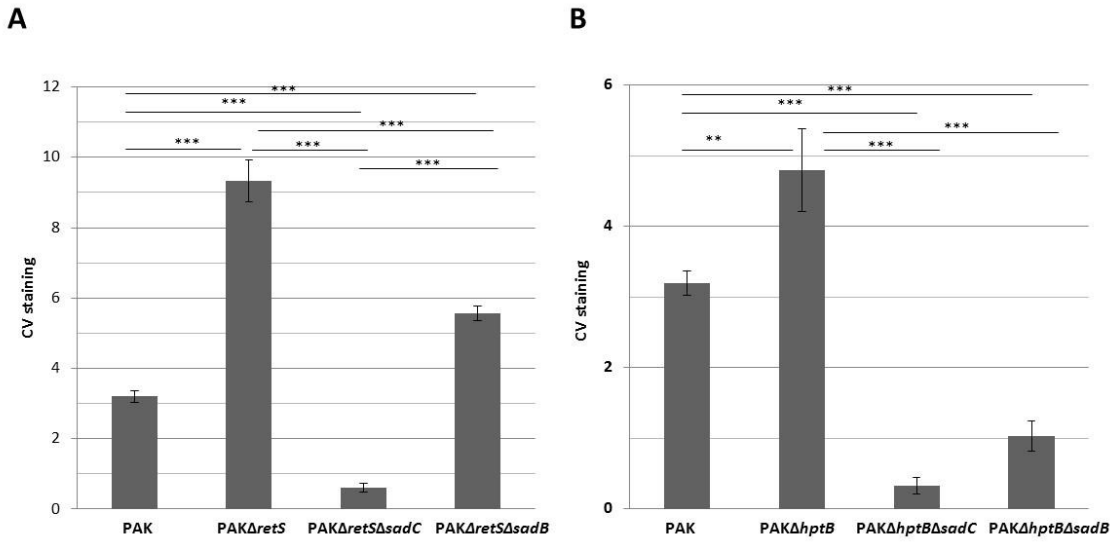

Figure 5.7 SadB is also required for the c-di-GMP-dependent biofilm phenotype regulated by the Gac system.

Biofilms formed in a MTP under shaking conditions for 24 h in M9 medium with 0.4% glucose were quantified by CV staining from three technical replicates. Deletion of *sadB* in the *retS* **(A)** or the *hptB* **(B)** mutant backgrounds negatively impacts the hyperbiofilm phenotype. An ANOVA analysis was performed with a *p* value of 0.05. Asterisks indicate statistically significant changes. Two independent experiments were performed.

5.7 The C-terminus of SadB has structural homology with the HD-GYP domain

The biochemical function of SadB is unknown. SadB is a cytoplasmic protein with 469 amino acids. It is predicted to have two domains: an N-terminal YbaK domain which is a domain of unknown function found in prokaryotic cells and frequently associated with prolyl-tRNA synthetases; and a C-terminal HD domain which typically harbours phosphohydrolase activity. For instance, in the YfbR protein from *E. coli* the HD domain works as a nucleotidase by dephosphorylating nucleoside 5′-monophosphates (Zimmerman *et al.*, 2008).

Due to the fact that the first crystal structure of the c-di-GMP related HD-GYP domain, a variant of the HD superfamily, was only recently solved (Lovering *et al.*, 2011), the structural homology of the HD domain of SadB with the HD-GYP domain of Bd1817 from *Bdellovibrio bacteriovorus* was examined. To do this, the crystal structure of Bd1817 (PBD code 3TM8) was searched against the *P. aeruginosa* PAO1 genome by doing a BackPhyre analysis.

Table 5.1 Proteins with a putative HD-GYP domain identified by Phyre2.

Hit	Alignment coverage (amino acids)	Confidence (%)
Previously identified		
PA4108	**5-306**	**100**
PA4781	**82-306**	**100**
PA2572	**31-307**	**100**
Previously unidentified		
PA0758	148-306	99.4
PA0356	148-306	99.1
PA5346 (*sadB*)	**148-306**	**98.7**
PA0267	148-287	98.3
PA2804	118-238	97.6
PA3043	150-266	97.3
PA5241	150-270	97.1
PA4955	148-269	96.2
PA1124	150-266	96

As expected, the first three hits of the BackPhyre analysis, predicted with a confidence of 100%, were the three HD-GYP domain proteins known for *P. aeruginosa*: PA2572, PA4106 and PA4781 (Table 5.1). Strikingly, another nine proteins with structural homology to the HD-GYP domain were also identified with high levels of confidence (above 95%). Interestingly, the region encompassing the HD domain of SadB was one of them, despite the fact that the amino acid sequence of SadB displays a degenerated HD motif and an unrecognizable GYP motif (Figure 5.8).

Figure 5.8. Phyre2 alignment of the structure and sequence of Bd1817, an HD-GYP domain protein, and SadB.

Alignment covers amino acids 148 to 307 of Bd1817 and amino acids 250 to 450 of SadB. Asterisks indicate HD and GYP motifs. Grey boxes indicate conserved residues and α-helices are indicated by the green helices.

5.8 Screen for proteins that interact with SadC

The idea that SadC signals via SadB left open the possibility that SadB could be interacting with SadC. To test this hypothesis, a bacterial two-hybrid approach was used. This method is based on the reconstitution of the AC catalytic domain of CyaA when two proteins that carry the T18 and T25 subdomains interact (Battesti & Bouveret, 2012; Karimova *et al.*, 1998). This can then be visualized as red colonies in MacConkey agar plates supplemented with maltose, IPTG and the appropriate antibiotics (Figure 5.9A).

Firstly, the genes of interest were cloned into specially designed vectors that allow the production of chimeric proteins harbouring the T18 or T25 subdomains. Because SadC has N-terminal transmembrane domains, two versions were engineered: one expressing the full length protein ($SadC^{fl}$) with a C-terminal T18 or T25 subunit, and another one expressing the cytoplasmic portion of the protein ($SadC^{ct}$) with N-terminal T18 or T25 subunits. For SadB, the engineered plasmid encoded an N-terminal T18 or T25 subunit. When *E. coli* DHM1 cells were co-transformed with the relevant plasmids and plated in MacConkey agar, positive interactions were observed for self-interactions of $SadC^{fl}$, $SadC^{ct}$ and SadB (Figure 5.9B). However, interactions between $SadC^{fl}$ and SadB or $SadC^{ct}$ and SadB were not detected.

To search for other potential interaction partners of SadC, a genome-wide screen was performed in collaboration with Christophe Bordi at the CNRS in Marseille. Recently, a bacterial two-hybrid

genome fragment library with an estimated 3-fold coverage of the whole *P. aeruginosa* PAO1 genome was constructed (Houot *et al.*, 2012) and SadC^{fl} was used as a bait to probe against the library. In total, an estimated number of 30 000 clones was obtained and among these, 20 red colonies were identified in the course of one-week of incubation on MacConkey agar plates. After re-streaking to confirm positive interaction and sequencing, a total of 12 hits were recognized (Table 5.2). One of them, PA4379, was identified four times as a strong interaction that was apparent on the second day of incubation; another one, PilS (PA4546), was identified two times as a weaker interaction. Other identified hits were FimV (PA3115), PA3926 and PA4459 which appeared after two days of incubation only, and PA1378, PA1891, PA3403, CysE (PA3816), InaA (PA4378), PA4929 and AtpF (PA5558) which appeared later. Noteworthy, both FimV and PilS are proteins that have previously been related to twitching motility. Also, InaA is predicted to be encoded in the same operon as PA4379, and PA4929 is a protein with a C-terminal GGDEF domain and an N-terminal 7TMR_DISM domain (like RetS and LadS).

Table5.2 Prey proteins in the bacterial two-hybrid screen using SadC as bait.

Hit	Comments	Gene product
Strong interactions (after 2 days of incubation)		
FimV (PA3115)	Hits the N-terminus	Membrane protein
PA3926	Hits the C-terminus	Transporter
PA4379	Hit 4x	Methyltransferase
PA4459		Lipopolysaccharide assembly
Weaker interactions (after 4-6 days of incubation)		
PA1378		Hypothetical
PA1891		Membrane protein
PA3403		Hypothetical
cysE (PA3816)	Hits the C-terminus	O-acetylserine synthase
InaA (PA4378)	Same operon as PA4379	Lipopolysaccharide kinase
PilS (PA4546)	Hit 2x	Two-component membrane sensor
PA4929	Hits the C-terminus	Diguanylate cyclase
AtpF (PA5558)		ATP synthase B chain

In relation to PA4379, the gene encodes a protein of 227 amino acids with a putative methyltransferase domain. The detected interaction with SadC in the screen was further confirmed by cloning the full length PA4379 into pKT25 and pUT18C, the vectors allowing the expression of PA4379 chimeric proteins carrying the T25 or T18 subunit at the N-terminus. As shown in Figure 5.9C, PA4379 was able to interact with SadC^{fl}. Moreover, the interaction with PA4379 still occured with the cytoplasmic truncated SadC^{ct} version, indicating that the transmembrane portion of SadC is not essential for the interaction between SadC and PA4379. Like for SadC, an interaction between PA4379 and SadB was not observable.

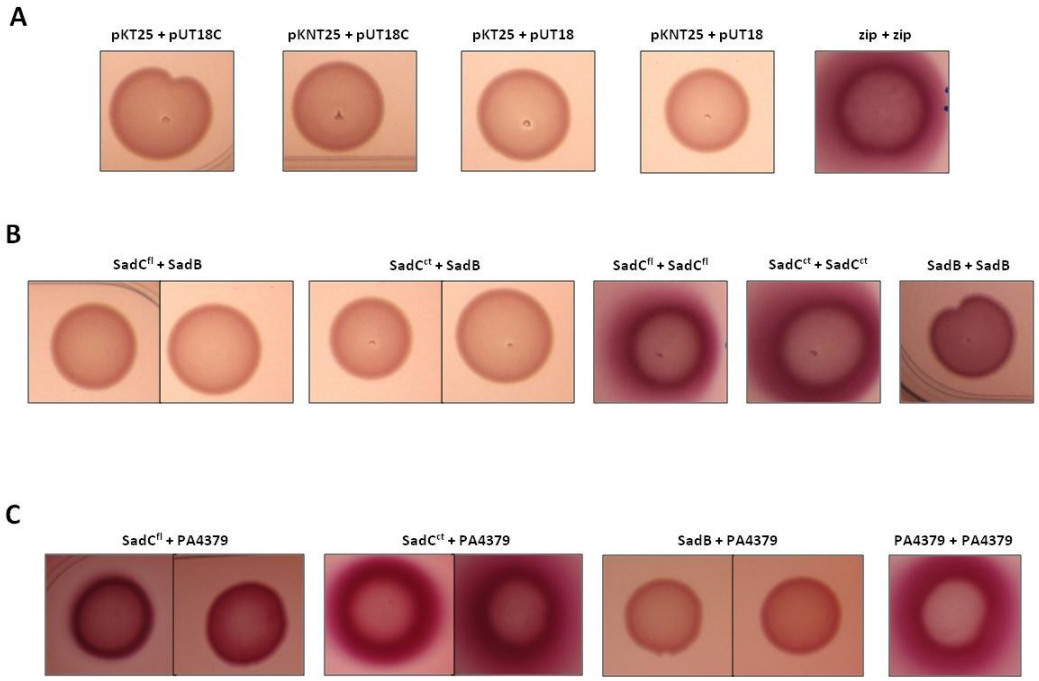

Figure 5.9 SadC interacts with PA4379.

Bacterial two-hybrid analysis of possible interactions between SadC and SadB or SadC and PA4379 visualized on MacConkey agar plates after 2 days at 30°C. The different pKT25/pKNT25 and pUT18C/pUT18 recombinant plasmids harboring the proteins of interest were co-transformed into *E. coli* DHM1. SadC^{fl} indicates expression of the full length *sadC* and was cloned in pKNT25 or pUT18. All the other proteins of interest, including the cytoplasmic truncation of *sadC* (SadC^{ct}), were expressed from pKT25 or pUT18C. **(A)** Co-expression of the empty vectors encoding the T25 and T18 subunits does not give an interaction but the positive control expressing recombinant *zip* leads to the appearance of red colonies. **(B)** SadC^{fl} or SadC^{ct} do not seem to interact with SadB but the three are able to interact with the self. **(C)** PA4379 is able to interact with itself and with SadC but does not interact with SadB. The transmembrane N-terminal region of SadC is not essential for the interaction with PA4379.

5.9 Discussion

Although the complexity of the Gac system varies between different species, the central GacAS two-component system and its downstream targets, namely the sRNAs and the translational repressor, are conserved in the *Gammaproteobacteria* class (Lapouge *et al.*, 2008). In some species, a link between the Gac system and the c-di-GMP network in the regulation of specific features became apparent in the past few years. In *E. coli*, the GacAS two-component system is known as BarA/UvrY and controls the expression of two sRNAs, CsrB and CsrC. These, in turn, modulate the activity of the CsrA translational repressor which targets, among many other genes, two genes encoding proteins with a GGDEF domain, YdeH and YcdT (Jonas *et al.*, 2008). In *S. enterica*, the GacAS two-component system is known as BarA/SirA and modulates the CsrA translational repressor via CsrB and CsrC. In this case, CsrA is known to regulate eight genes encoding GGDEF, GGDEF/EAL or EAL domain proteins, five of which by direct binding to the mRNA (Jonas *et al.*, 2010). In *X. campestris*, RsmA has been shown to control posttranscriptionally at least three GGDEF domain proteins (XC_1803, XC_2715 and XC_2866) among the 22 genes encoding GGDEF or EAL domain proteins. The three contribute additively for the elevated levels of c-di-GMP in the *rsmA* mutant (Lu *et al.*, 2012).

In *P. aeruginosa*, the first direct evidence that the Gac system and the c-di-GMP signalling were interlinked came from the observation that the *retS* mutant displays high levels of c-di-GMP and that the c-di-GMP-induced T3SS/T6SS switch is dependent on the two sRNAs, RsmY and RsmZ (see Chapter 4) (Moscoso *et al.*, 2011). Here, the link between the Gac system and c-di-GMP was studied further and evidence is provided showing that if, on the one hand, PA0338 seems to be a direct target of RsmA, on the other hand, SadC is the DGC responsible for the elevated levels of c-di-GMP in the *retS, hptB* and *rsmA* mutants. Based on the published literature, these two proteins were considered of interest because PA0338 had been seen to be up-regulated in the *rsmA* mutant (Brencic & Lory, 2009) and a *sadC* transposon mutant had been identified as a suppressor of the *retS* mutant hyperbiofilm phenotype (Goodman *et al.*, 2004).

To confirm the up-regulation of PA0338, a qRT-PCR screen was performed to check the expression of all the proteins with a GGDEF domain in the *retS* mutant. Indeed, PA0338 was ~3.8-fold up-regulated and this was in good agreement with the result obtained by Brencic & Lory (2009) which also observed a 3-fold change in the *rsmA* mutant. RsmA is known to exert its repression by binding to consensus GGA motifs at or near the ribosome binding site and residing in a single-stranded region of a stem-loop structure of the target mRNAs (Lapouge *et al.*, 2008). Noteworthy, taking into account this information and according to the secondary mRNA structure predicted by Mfold, a GGA

motif in PA0338 mRNA was found and provided further ground to conclude that PA0338 is post-transcriptionally regulated by RsmA (Figure 5.1).

When PA0338 was deleted in the *retS* mutant background, the *retS/PA0338* double mutant still displayed elevated levels of c-di-GMP. Hence, although being up-regulated, PA0338 does not seem to account for the production of c-di-GMP in the *retS* mutant (Figure 5.2). This may be because PA0338 (i) does not have DGC activity (despite having conserved I- and A-sites), (ii) is not catalytic active under the conditions tested, or (iii) has weak DGC activity and its contribution to the overall level of the second messenger is minimal. According to Tina Jaeger from the laboratory of Urs Jenal, overexpression of PA0338 causes a slight increase in the ability of PAO1 to form biofilms, indicating that PA0338 has some DGC acitivity. Interestingly, PA0338 is predicted to be encoded in an operon with two other upstream genes. PA0336 is homologous to the *E. coli* protein RppH (formerly NudH/YgdP) that has been shown to be involved in mRNA decay by 5' pyrophosphate removal (Deana *et al.*, 2008) and PA0337 is homologous to the *E. coli* protein PstP which in *P. putida* has been shown to be a component of a phosphotranferase system important in the regulation of carbon and nitrogen metabolism (Velazquez *et al.*, 2007). In *P. aeruginosa* it has also been reported that PstP is involved in pyocyanin production via the two quorum sensing systems *las* and *rhl* (Xu et al., 2005) and to be required, similarly to GacS and GacA, for virulence in various infection models (Tan *et al.*, 1999).

In addition to the up-regulated PA0338, the qRT-PCR screen performed showed a down-regulation of PA3343, PA4843 and PA3258 in the *retS* mutant. PA3343 is known to have DGC activity and PA3258 is homologous to RapA from *P. fluorescens* which has PDE activity (Monds *et al.*, 2007). As for PA4843, the protein harbours two N-terminal REC domains and a C-terminal GGDEF domain with conserved I- and A-sites, but its catalytic function has not been demonstrated.

Due to the proximity to *hptB,* PA3343 was hypothesized to be encoded in the same operon as *hptB*. Previously, it had been demonstrated by Hsu *et al.* (2008) that *hptB* was forming an operon with PA3347 and PA3346. Although the data is preliminary, the RT-PCR analysis suggested that PA3343 belongs to a seven gene operon starting with PA3347 and finishing with PA3341 (Figure 5.3). Besides the putative DGC, HptB, HsbR (phosphatase) and HsbA (anti-anti-sigma factor), the operon includes a putative helicase (PA3344), a putative transcriptional regulator (PA3341) and a protein of unknown function (PA3342). Curiously, next to PA3347 is PA3348, a putative chemotaxis methyltransferase that was identified in the screen by Nesper *et al.* (2012) and Duvel *et al.* (2012) as being able to bind c-di-GMP. Based on these observations, one can speculate that these proteins may converge in the

regulation of a common cellular function that is dependent on both the Gac and c-di-GMP signalling via HptB and PA3343.

Contrary to PA0338, *sadC* was not up-regulated in the *retS* mutant (Figure 5.1) or the *rsmA* mutant (Brencic & Lory, 2009). However, when *sadC* was deleted from both backgrounds the levels of c-di-GMP were reduced and the phenotypes reverted (Figure 5.4 and 5.6). Thus, SadC seems to be the DGC responsible for the elevated levels of c-di-GMP in the *retS* or *rsmA* mutants, but it is unclear how the Gac system influences SadC to promote the production of c-di-GMP. If *sadC* was a direct target of RsmA, it would be expected to observe an up-regulation in the qRT-PCR screen or it would be possible to recognise a RsmA binding motif in the 5' untranslated region of the corresponding mRNA, but neither one of these were identified. In theory, the regulation of *sadC* by the Gac system at the transcriptional level, directly or indirectly, can be disregarded. At the posttranscriptional level, the results do not support a direct regulation by RsmA but it is still possible that RsmA regulates *sadC* expression indirectly. For instance, RsmA could bind to an unknown allosteric regulator of SadC. Although the molecular link between SadC and the Gac system remains elusive, the link between the Gac system and SadC is strengthened by the fact that the two sRNAs, RsmY and RsmZ, were required for the SadC-induced biofilm phenotype. Additionally, the hyperbiofilm phenotype of the *hptB* mutants was also dependent on SadC (Figure 5.5), positioning SadC as a central player of the Gac signalling pathway that acts downstream of the two sRNAs.

Given that deletion of *sadC* in the *retS* mutant completely reverted the c-di-GMP, Congo Red or swimming phenotypes to levels that are similar or even greater than the wild-type strain, it is unlikely that other DGCs cooperate to increase the c-di-GMP level in the *retS* mutant. However, when analysing the T3SS/T6SS switch in the *retS/sadC* double mutant, only a partial switch was observed as T6SS did not appeared down-regulated (Figure 5.4). This raises a question about the stringency at which c-di-GMP is able to regulate T6SS. While the expression of the T3SS seems to rapidly respond to the levels of c-di-GMP in both up and down directions, the down-regulation of T6SS at low levels of c-di-GMP may be more complex and dependent on additional factors.

SadC was previously characterized for its role in swarming motility and placed in the same genetic pathway as BifA, a PDE, and SadB, a protein of unknown function. In addition, RoeA, a DGC, has been shown to have an additive effect to SadC in *P. aeruginosa* PA14 strain (Merritt *et al.*, 2010). Since SadB has been demonstrated to act downstream of SadC, the impact of its deletion in the *retS* and *hptB* mutants was also investigated and the results confirmed the requirement of SadB downstream of SadC (Figure 5.7).

How SadB acts in the SadC signalling is not clear. Previously, Caiazza & O'Toole (2004) have shown that both the N-terminal YbaK domain of SadB and the C-terminal HD domain of SadB are required for function and no PDE activity has been demonstrated (Caiazza *et al.*, 2007). Hypothetically, it was considered that SadB could act upstream of the c-di-GMP synthesis, somehow assisting SadC in its DGC activity, or that SadB could function as a c-di-GMP receptor, somehow transmitting the c-di-GMP signal to downstream targets. In light of the results presented here, the latter hypothesis is favoured. Firstly, because no direct interaction between SadC and SadB could be detected using the bacterial two-hybrid approach (Figure 5.9). Secondly, because the C-terminus HD domain of SadB was shown to actually have high structural homology to an HD-GYP domain (Table 5.1), suggesting that the protein may be able to bind c-di-GMP and act as a c-di-GMP receptor.

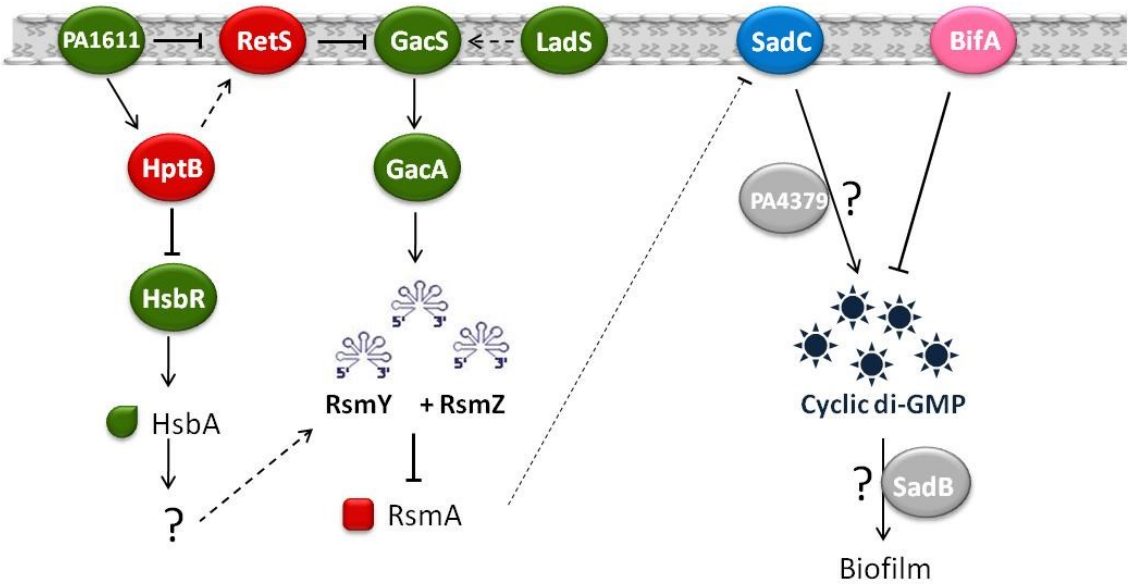

Figure 5.10 Model for the role of SadC in modulating biofilms downstream of the Gac system.

The Gac system is a complex signalling cascade that inversely regulates attributes related to the switch between acute and chronic infections. One of those traits is the development of biofilms. The proteins belonging to this cascade that negatively impact the formation of biofilms are indicated in *red.* All the proteins indicated in *green* have a positive impact on biofilm formation. The hyperbiofilm phenotype displayed by the deletion of *retS*, *hptB* and *rsmA* is dependent on the DGC activity of SadC. Although it cannot be conclusively ruled out that SadC is a direct target of RsmA, the activity of SadC is likely to be regulated indirectly by RsmA. SadB was hypothesized to be able to bind c-di-GMP via its newly recognized HD-GYP domain and to transmit the signal relayed by SadC. This signal may be further regulated by the putative methyltransferase activity of PA4379 and the confirmed PDE acitivity of BifA. SadB is known to act upstream of the Pil-Chp cluster which is related to T4P biogenesis, and SadC has also been identified as a putative interaction partner of the T4P-related genes FimV and PilS. Possibly, the Gac/SadC-dependent high c-di-GMP level and the subsequent biofilm modulation is closely related with the production of T4P.

In addition to this, SadB is known to act upstream of the Pil-Chp chemotactic cluster (Caiazza *et al.*, 2007). This cluster is required for the T4P biogenesis and interestingly, other T4P related genes, namely the peptidoglycan-binding FimV and the sensor PilS, were identified in this study as possible interaction partners of SadC (Table 5.2). Together this provides a strong indication that the Gac system and SadC may be interlinked in the regulation of the T4P which contributes for biofilm formation.

In *P. fluorescens*, a link between SadB and the Gac system has recently been established for the negative regulation of flagella-driven motility during exponential phase (Martinez-Granero *et al.*, 2012). In this bacterium, the Gac system comprises three translational repressors, RsmA, RsmE and RsmI, and three sRNAs, RsmX, RsmY and RsmZ, but RsmI and RsmY do not participate in the motility regulation. In relation to SadB and the Gac system, the two signalling cascades were shown to intersect in the cooperative regulation of the σ^{22} sigma factor, also known as AlgT or AlgU. On the one hand, SadB is required for the transcription of σ^{22}, and on the other hand, RsmA and RsmE act as translational repressors of σ^{22}. Once produced, σ^{22} is necessary for the expression of the transcriptional regulator *amrZ* (also referred to as *algZ*) which functions to down-regulate *fleQ*, the master regulator of the flagellar components. In *P. aeruginosa,* it is not known if σ^{22} is a member of the RsmA regulon, but AmrZ belongs to the σ^{22} regulon and has been shown to be involved in both the down-regulation of flagella and Psl exopolysaccharide, and the up-regulation of twitching motility and alginate production (Baynham *et al.*, 2006; Jones *et al.*, 2013; Tart *et al.*, 2005; Wozniak *et al.*, 2003).

Another identified interaction partner of SadC was PA4379. PA4379 is a putative methyltransferase and may belong to an operon with PA4377, a small protein of unknown function, and PA4378, a putative lipopolysaccharide kinase. The biological role of PA4379 is unknown but one can speculate that PA4379, as a methyltransferase, may be involved in the allosteric modification of SadC.

In summary, a model is proposed in which the biofilm lifestyle directed by the Gac system is dependent on the production of c-di-GMP by SadC. Subsequently, the synthesized c-di-GMP is likely to be perceived by SadB which relays the signal and modulates biofilm formation (Figure 5.10).

6) Conclusion

Chapter 6 – Conclusion

Bacteria are able to adapt to the environment and progress during the biofilm developmental cycle due to multiple signal transduction pathways that operate to allow the integration of extracellular signals into intracellular messages and the concerted action of different biological functions. These signal transduction pathways can have different degrees of complexity, varying from the simplest mode which consists of the direct binding of a signal to an effector protein, to the more complex mode which encompasses cascades of reactions like the ones directing chemotaxis.

Two important signalling pathways in *P. aeruginosa* are the Gac system, pivotal in the inverse regulation between acute and chronic infections; and the c-di-GMP network, central to the inverse regulation between a motile and a sessile lifestyle. Here, a link between these two pathways has been established.

In Chapter 3, the complexity of the c-di-GMP network in *P. aeruginosa* was demonstrated. A total of 51 proteins have identifiable GGDEF, EAL, HD-GYP or PilZ domains which are domains that can be involved in synthesizing (GGDEF domain), degrading (EAL or HD-GYP domains) or binding to c-di-GMP (PilZ domain). In relation to the later, many more proteins are thought to exist but remain unknown. As for the proteins with a GGDEF, EAL or HD-GYP domains, not all of them seem to have catalytic activity and some of them, like MucR, may have both the DGC and PDE activities. Nonetheless, the numerous c-di-GMP related proteins seem to work in a concerted manner to regulate a variety of biological functions in a highly specific manner. From the proteins that have been characterized so far, attributes like biofilm formation and dispersal, flagella and T4P motility, or the production of exopolysaccharides have been shown to be c-di-GMP regulated (Figure 6.1). Overall, the paradigm of c-di-GMP signalling has been established based on the overexpression of DGCs or PDEs and dictates that high levels of c-di-GMP promote the sessile lifestyle of the bacteria whereas low levels of c-di-GMP promote motility (Figure 3.1). However, when the production of individual DGCs or PDEs is impaired, the impact on biofilm formation or dispersal is not uniform (Figure 3.4), reflecting the high level of specificity at which the c-di-GMP network functions.

Despite the fact the substantial progress has been made in characterizing the GGDEF, EAL and HD-GYP domain proteins found in *P. aeruginosa* during the last years, the physiolocally role of more than half remains elusive and should be the focus of future work. For instance, it was established in this work that PA2072 is likely to function as a PDE, but further demonstration of this catalytic activity with an *in vitro* assay would be necessary to make a solid conclusion. Moreover, since

PA2072 exhibits both a GGDEF and EAL domain, an *in vitro* biochemical assay would also allow the investigation of the catalytic function of the GGDEF domain. Additionally, the fact that over the last year at least three different proteins (NbdA, RbdA and DipA) were implicated in biofilm dispersal by three independent studies, raises the question of whether they are expressed under the same conditions (at least RbdA and DipA seem to, Table 3.1) and if so, if their function is redundant or additive.

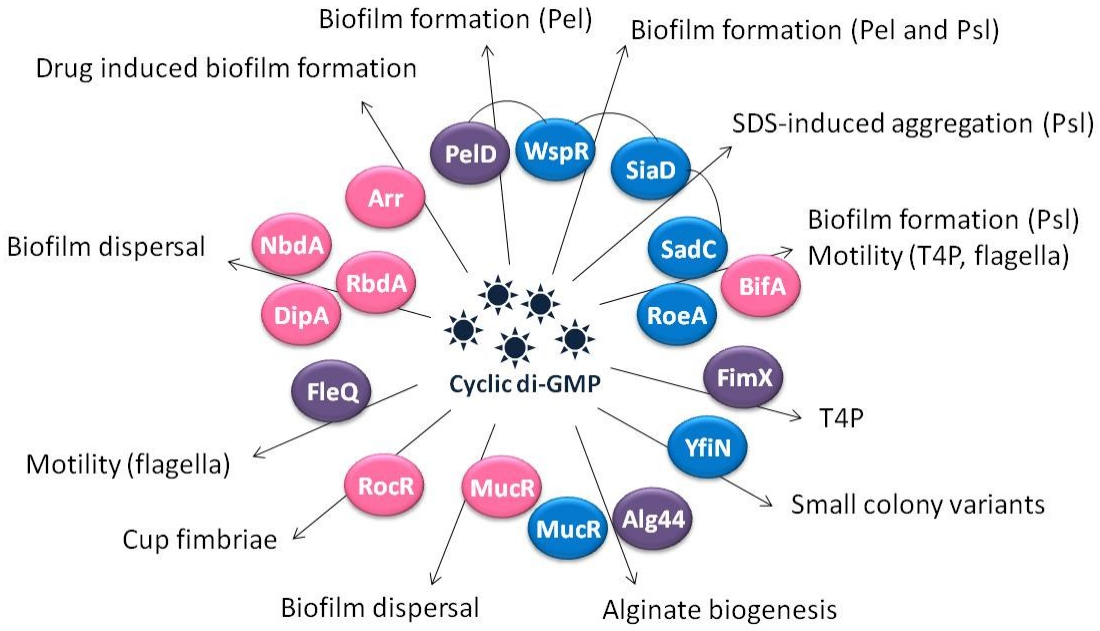

Figure 6.1 The c-di-GMP network operates at high specificity.

The *P. aeruginosa* proteins that have been related to c-di-GMP so far are represented. PDEs are indicated in pink, DGCs in blue and c-di-GMP binding proteins in purple.

Although in *P. aeruginosa* the bacterial lifestyle is generally associated with the mode of infection, with the sessile lifestyle being related to the establishment of chronic infections, the virulence of the bacterium correlates closely to the expression of different secretion systems. In acute infections, the T2SS and T3SS are up-regulated and secrete toxins into the environment and host cells, and in chronic infections the T6SS is active and important for the colonization of the host. Crucial for this inverse regulation of the secretion systems is the Gac system. This signalling cascade comprises the central two-component system GacAS which leads to the expression of two sRNAs that antagonize the translational repressor RsmA. The function of RsmA can be antagonized by deleting or overexpressing, respectively, the additional sensors RetS or LadS. A *retS* mutant is a hyperbiofilm former and has an up-regulated T6SS and down-regulated T3SS. As shown in Chapter 4, by artificially modulating the levels of c-di-GMP with the overexpression of a DGC or a PDE, it was possible to induce the switch between the different secretion systems. At high levels of c-di-GMP the T6SS was up-regulated, whereas at low levels of c-di-GMP the T3SS was up-regulated (Figure 4.2). Hence, the regulation by c-di-GMP goes beyond the lifestyle switch to modulate other important virulence factors like the T3SS/T6SS switch (Figure 6.2). Moreover, the observations that the hyperbiofilm *retS* mutant displays high levels of c-di-GMP (Figure 4.1) and that the c-di-GMP-dependent T6SS up-regulation relies on an active GacAS cascade and the production of the sRNAs (Figure 4.4), provide a direct evidence of the existence of a firm link between the Gac and c-di-GMP signalling networks.

This unprecedent link between c-di-GMP and the T3SS and T6SS regulation leaves open the question on how the c-di-GMP-dependent T3SS/T6SS switch occurs at the molecular level. The mechanisms by which c-di-GMP signals in bacteria have proven to be very diverse and the possibilities for this regulation are many. For instance, something similar to what happens with the c-di-GMP control of the swimming motility velocity in *E. coli* (Chapter 1, section 1.4) could be happening to one of the two secretion systems and for that reason it would be appropriated to check if c-di-GMP also affects the secretion of the different effector proteins. Ideally, the specific DGCs, PDEs and c-di-GMP binding proteins involved in the regulation of the two secretion systems should be identified and this could be done by using an approach similar to the screen performed with the PA14 transposon collection upon overexpression of *ladS* (Figure 4.7). To elucidate the full regulatory cascade, methods like bacterial two-hybrid or ChIP-seq could be applied to further look for interactions between proteins or between proteins and DNA.

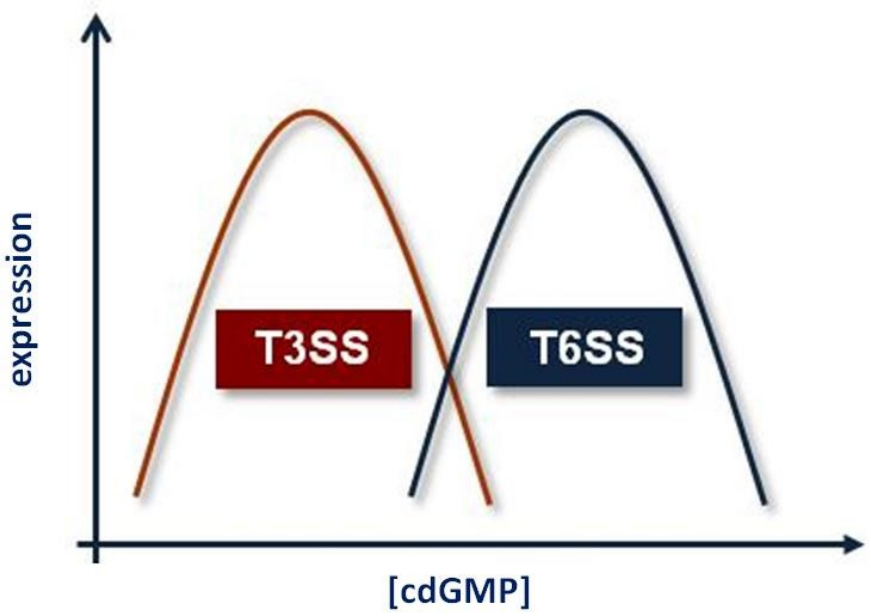

Figure 6.2 The c-di-GMP regulation of the T3SS/T6SS switch.

Low levels of c-di-GMP promote the expression of the T3SS whereas high levels of c-di-GMP favour the expression of the T6SS.

Finally, in Chapter 5 the DGC responsible for the elevated levels of c-di-GMP in the *retS* mutant was pinpointed. According to previously published papers, the genes PA0338 and *sadC* encoding proteins with a GGDEF domain were considered of interest for being, respectively, up-regulated in a *rsmA* mutant or identified as a suppressor mutation of the *retS* hyperbiofilm phenotype. A qRT-PCR screen confirmed that PA0338 is up-regulated in the *retS* mutant and no other proteins with a GGDEF domain showed a similar profile (Figure 5.1). Despite being up-regulated, PA0338 was found to not be responsible for the elevated levels of c-di-GMP in the *retS* mutant (Figure 5.3). Instead, SadC was identified as being central in the c-di-GMP-dependent regulation exerted by the Gac system (Figure 5.4-6). Not only was SadC required for the hyperbiofilm phenotype of *retS*, it was also indispensable for the phenotypes observed in the *rsmA* and *hptB* deletion mutants, and the strains overexpressing *ladS* and *hsbR*. In addition, the SadC downstream partner SadB, a protein of unknown biochemical function, was shown to be partially required for the *retS* and *hptB* mutants hyperbiofilm phenotype (Figure 5.7). Based on structural homology, it was hypothesized that the N-terminal HD domain of SadB is actually a HD-GYP domain and that SadB may act as a c-di-GMP binding protein, thus relaying SadC activity.

In order to elucidate this pathway further, the biochemical function of SadB needs to be determined. For instance, the DRaCALA assay could be used to assess the ability of SadB to bind c-di-GMP. In fact, the same applies for all the putative HD-GYP domains identified by the BackPhyre analysis. In addition, the PA4379 bacterial two-hybrid hit should be further validated by engineering a clean deletion of the gene in the *retS* and *hptB* mutants and assessing the impact of that deletion in biofilm formation. Since some of the other possible SadC interaction partners were proteins involved in T4P-mediated twitching motility, it would be interesting to make a careful analysis of this phenotype.

The exact mechanism by which SadC is induced to produce c-di-GMP in a *retS* or *rsmA* deletion background is still unknown. While the DGC PA0338 seems to be a direct target of RsmA, the same evidence was not provided for SadC and ultimately, the RsmA ability to bind both mRNAs should be investigated. Also, the possibility that the DGC PA3343 is down-regulated in the RsmA mutant and encoded in the same operon as HptB is an interesting one and deserves to be explored.

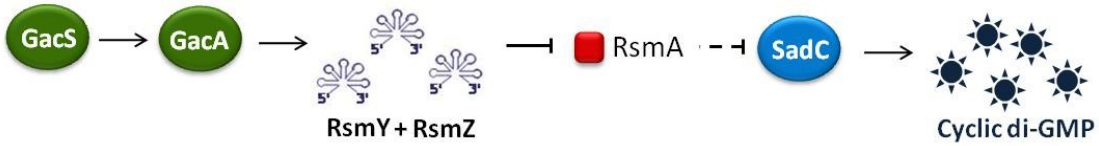

Figure 6.3 The Gac and c-di-GMP pathways are interlinked.

The Gac system acts upstream of SadC and this is responsible for the synthesis of c-di-GMP when the pathway is activated.

In summary, the Gac system and the c-di-GMP network have in common the regulation of several biological functions like the biofilm development and the production of different secretion machineries. At least in relation to biofilm formation, the two pathways seem to cooperate with the Gac system acting upstream of the c-di-GMP network (Figure 6.3). When the GacAS two-component system is active, the production of the sRNAs inhibits RsmA which, by a yet unknown mechanism, leads to the production of c-di-GMP by the DGC protein SadC.

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Appendices

Appendices

Research publications

Appendix 1

Moscoso, J.A., Mikkelsen, H., Heeb, S., Williams, P. & Filloux, A. (2011) The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretion via c-di-GMP signalling. *Environ Microbiol* 13, 3128-38

Appendix 2

Moscoso, J.A., Mikkelsen, H., Heeb, S., Williams, P. & Filloux, A. (2012)The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretion via c-di-GMP signalling. Environmental Microbiology 14, 1088-1089 (Corrigendum)

Book chapters

Appendix 3

Barraud, N., **Moscoso, J. A.**, Ghigo, J. M. & Filloux, A. (2013) "Methods for studying biofilm dispersal in Pseudomonas aeruginosa" in *Methods in Pseudomonas.* Springer (*in press*)

Appendix 1

environmental microbiology

Environmental Microbiology (2011) doi:10.1111/j.1462-2920.2011.02595.x

The *Pseudomonas aeruginosa* sensor RetS switches Type III and Type VI secretion via c-di-GMP signalling

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Summary

Acute bacterial infections are associated with motility and cytotoxicity via the type III secretion system (T3SS), while chronic infections are linked to biofilm formation and reduced virulence. In *Pseudomonas aeruginosa*, the transition between motility and sessility involves regulatory networks including the RetS/GacS sensors, as well as the second messenger c-di-GMP. The RetS/GacS signalling cascade converges on small RNAs, RsmY and RsmZ, which control a range of functions via RsmA. A *retS* mutation induces biofilm formation, and high levels of c-di-GMP produce a similar response. In this study, we connect RetS and c-di-GMP pathways by showing that the *retS* mutant displays high levels of c-di-GMP. Furthermore, a *retS* mutation leads to repression of the T3SS, but also upregulates the type VI secretion system (T6SS), which is associated with chronic infections. Strikingly, production of the T3SS and T6SS can be switched by artificially modulating c-di-GMP levels. We show that the diguanylate cyclase WspR is specifically involved in the T3SS/T6SS switch and that RsmY and RsmZ are required for the c-di-GMP-dependent response. These results provide a firm link between the RetS/GacS and the c-di-GMP pathways, which coordinate bacterial lifestyles, as well as secretion systems that determine the infection strategy of *P. aeruginosa*.

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Introduction

Pathogenic bacteria can adopt different lifestyles, both in nature and during infection. In the planktonic phase, they are motile and can cause acute infections associated with production of toxins via the Type III secretion system (T3SS). Conversely, bacteria in a biofilm tend to be less virulent, more resistant to antibiotics and cause chronic infections. *Pseudomonas aeruginosa* is a versatile bacterial pathogen (Lyczak *et al*., 2000) that produces many virulence factors, of which secretion systems are major weapons required for colonization, survival, cytotoxicity and evasion of the innate host immune system (Bleves *et al*., 2010). Such a versatile organism has a wide range of survival strategies and causes chronic as well as acute infections (Roy-Burman *et al*., 2001; Hauser *et al*., 2002). *Pseudomonas aeruginosa* chronic infections are a major burden and cause of mortality in people suffering from cystic fibrosis (CF). In the CF lungs, the bacteria establish as a biofilm (Singh *et al*., 2000), which is difficult to eradicate by the immune system or antibiotic therapy (Costerton *et al*., 1999). Previous reports suggest that *P. aeruginosa* chronic infections are associated with expression of the type VI secretion system (T6SS) (Potvin *et al*., 2003; Mougous *et al*., 2006).

It has been shown in *P. aeruginosa* that the decision-making process to adopt a chronic/biofilm or acute/planktonic lifestyle depends on a regulatory network involving two sensors, RetS and LadS (Goodman *et al*., 2004; Ventre *et al*., 2006; Bordi *et al*., 2010; Vincent *et al*., 2010; Mikkelsen *et al*., 2011). These sensors act antagonistically to inversely control motility and the T3SS versus biofilm formation and the T6SS (Goodman *et al*., 2004). The RetS sensor displays a unique mechanism of action that is based on modulating the activity of another sensor kinase, GacS (Goodman *et al*., 2009). GacS has long been known for its crucial role in *P. aeruginosa* virulence and biofilm formation (Tan *et al*., 1999; Parkins *et al*., 2001). Briefly, the formation of heterodimers between RetS and GacS prevents GacS autophosphorylation and thus phosphotransfer-dependent activation of the cognate response regulator GacA. The exclusive targets for GacA are the genes encoding two small regulatory RNAs (sRNAs), RsmY and RsmZ (Brencic *et al*., 2009). The function of these sRNAs is to sequester the post-transcriptional regulator RsmA, which profoundly

influences *P. aeruginosa* biology and lifestyle (Brencic and Lory, 2009). Among the transcripts that are directly repressed by RsmA are the *pel* and *psl* genes that are important in biofilm development (Irie *et al*., 2010). The *pel* genes are proposed to encode a yet uncharacterized exopolysaccharide (Coulon *et al*., 2010), while the *psl* gene products direct the synthesis of a repeating pentasaccharide containing D-mannose, D-glucose and D-rhamnose (Byrd *et al*., 2009). Both exopolysaccharides contribute to the biofilm architecture (Friedman and Kolter, 2004; Vasseur *et al*., 2005; Colvin *et al*., 2011). Other genes directly controlled by RsmA are encoding the T6SS (H1-T6SS) (Brencic and Lory, 2009). In contrast, the genes encoding the T3SS are positively regulated by RsmA, likely in an indirect manner (Brencic and Lory, 2009). These observations highlight the antagonistic regulation of the various components of the T3SS and the T6SS. Furthermore, the co-regulation of the T6SS and biofilm-related genes (*pel* and *psl*) support the notion that the T6SS is important in chronic infections.

In a number of bacterial species, including *P. aeruginosa*, the second messenger c-di-GMP is utilized as a signal for transition between the planktonic and sessile lifestyles (Tamayo *et al*., 2007; Jonas *et al*., 2009). High c-di-GMP levels promote biofilm formation and chronic infections, while low c-di-GMP levels favour swimming and virulence. The intracellular levels of c-di-GMP are controlled by the antagonistic activity of diguanylate cyclases, such as WspR, and phosphodiesterases, such as PA2133 (Hickman *et al*., 2005). The *P. aeruginosa* WspR is part of a chemosensory pathway that is involved in bacterial cell aggregation and wrinkly colony morphology (D'Argenio *et al*., 2002). Hickman and colleagues showed that WspR is an active diguanylate cyclase that is involved in upregulation of the *pel* genes with a concomitant decrease in flagellar motility (Hickman *et al*., 2005). In the same study, it was shown that a *P. aeruginosa* phosphodiesterase, PA2133, counteracts the activity of the *wsp* pathway by degrading c-di-GMP. This activity resulted in a drastic loss of biofilm formation.

In the present study, we show that the *P. aeruginosa* RetS/GacS regulatory network is linked to the c-di-GMP signalling network. We present evidence that c-di-GMP inversely controls the T3SS and the T6SS expression in a RetS-independent manner and show that this control is dependent on the WspR diguanylate cyclase and the two sRNAs, RsmY and RsmZ.

Results

The retS *mutant displays elevated levels of c-di-GMP*

A *P. aeruginosa retS* mutant is known to overproduce exopolysaccharides and has a hyperbiofilm phenotype as shown using a crystal violet staining assay (Fig. 1A). Considering that c-di-GMP has been associated with the switch between the planktonic and biofilm lifestyles, we hypothesized that the hyperbiofilm phenotype of a *retS* mutant could be linked to a variation in c-di-GMP. We compared the levels of intracellular c-di-GMP between the *P. aeruginosa* PAK wild-type strain and the isogenic *retS* mutant (PAKΔ*retS*) by LC-MS/MS (see *Experimental procedures*). The hyperbiofilm phenotype of PAKΔ*retS* coincided with a ~1.4-fold increase in c-di-GMP levels as compared with the parental strain (Fig. 1B). This observation is in agreement with the established notion that high c-di-GMP levels promote biofilm formation. To investigate whether the elevated level of c-di-GMP was directly attributable to the *retS* mutation, or a consequence of the development of a biofilm, the c-di-GMP concentration was

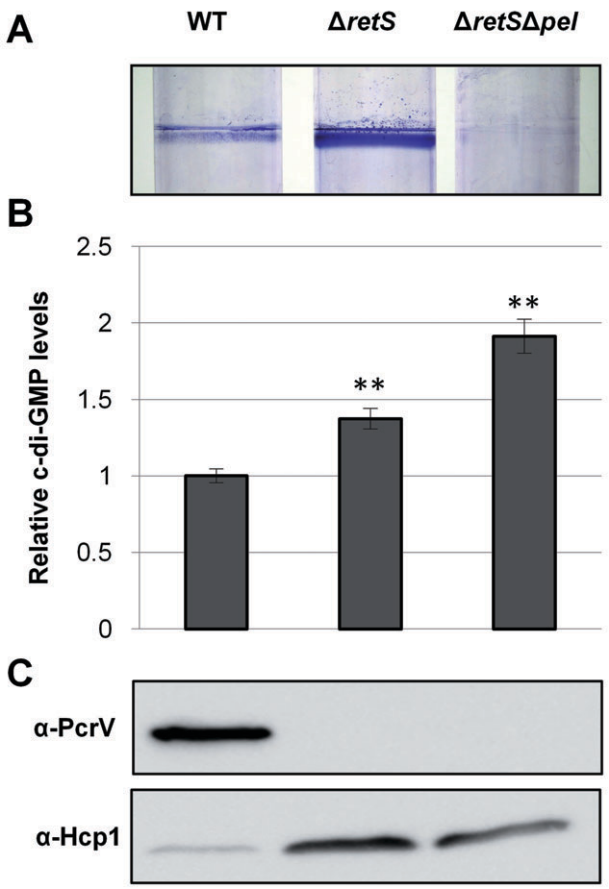

Fig. 1. Phenotypes of *P. aeruginosa* PAK and selected deletion mutants. Strains are indicated in (A).
A. Biofilm formation in static growth conditions visualized by crystal violet staining.
B. Intracellular c-di-GMP levels measured by LC-MS/MS. The levels of c-di-GMP in the wild-type were set to 1, and statistically significant changes (Student's *t*-test, $P < 0.01$) are indicated by asterisks. Statistical analysis is based on three replicates.
C. Western blot using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS).

determined in a *retS* mutant unable to form biofilms due to a *pel* gene mutation (PAKΔ*retS*Δ*pel*) (Fig. 1A). Interestingly, the c-di-GMP levels remained high (~twofold increase) in the double mutant (Fig. 1B). We therefore concluded that a *retS* mutation increases intracellular c-di-GMP levels, and that this is independent of biofilm formation.

RetS signalling antagonistically controls the type III and type VI secretion systems

In addition to repressing Pel and Psl exopolysaccharide production, RetS inversely regulates the genes encoding the T3SS and the T6SS (Goodman *et al*., 2004; Mougous *et al*., 2006). Since c-di-GMP signalling seems to play a role in the RetS pathway, we hypothesized that, if the regulation of biofilm formation by RetS is c-di-GMP-dependent, the same could apply to the T3SS and the T6SS. The production of these secretion systems can be monitored by the detection of specific structural components of the machineries: PcrV is located at the tip of the T3SS needle (Lee *et al*., 2007), whereas Hcp1 is the main component of the T6SS nanotube (Ballister *et al*., 2008). Western blot analysis using specific antibodies directed against these components showed that Hcp1 was readily detected in a *retS* mutant, whereas the parental strain displayed low Hcp1 levels (Fig. 1C). Conversely, PcrV production was observed in the parental strain but not in the *retS* mutant (Fig. 1C). Furthermore, the production of the secretion systems was shown to be independent of biofilm formation, since a *retS*/*pel* double mutant displayed the same profile as the *retS* mutant (Fig. 1C). Our data therefore confirmed that a *retS* mutation switches the activity of the T6SS and the T3SS, leading to the hypothesis of an inverse correlation between the secretion profile and c-di-GMP levels.

The type III and type VI secretion systems are controlled by c-di-GMP

Several studies have shown that overproduction of active diguanylate cyclases results in high intracellular levels of c-di-GMP and enhances biofilm formation. (Kulasakara *et al*., 2006). Of the 17 GGDEF domain-containing proteins encoded on the *P. aeruginosa* PAO1 genome, WspR is perhaps the most extensively characterized (Hickman *et al*., 2005; Ude *et al*., 2006; De *et al*., 2008). We first confirmed that *wspR* overexpression increased biofilm formation in *P. aeruginosa* PAK (Fig. S1A and B). The diguanylate cyclase activity of WspR is tightly feedback-controlled, but replacement of a critical residue in the I-site (WspR^{R242A}) renders the enzyme highly active (De *et al*., 2008). The corresponding mutation in *wspR* was therefore engineered (pBBR1MCS-4-*wspR*^{R242A}), and

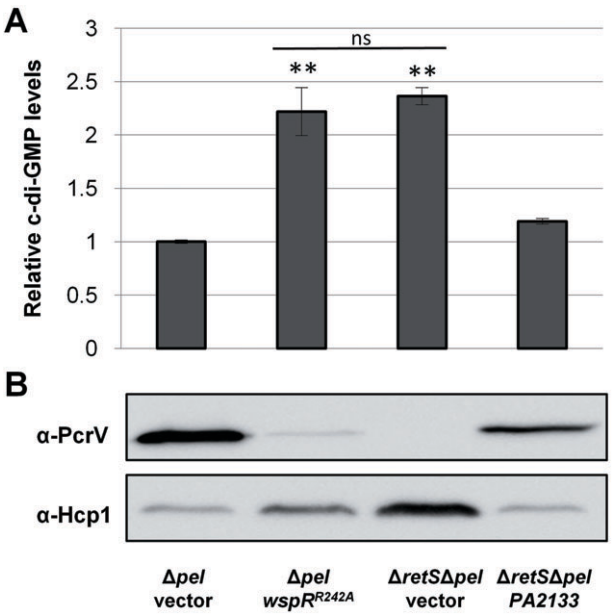

Fig. 2. c-di-GMP inversely controls the T3SS and the T6SS. Relationship between c-di-GMP levels and secretion systems in PAKΔ*pel* or PAKΔ*retS*Δ*pel* carrying either the empty vector, or overproducing proteins that modulate c-di-GMP levels as indicated in (B).
A. Relative intracellular levels of c-di-GMP measured by LC-MS/MS. The levels of c-di-GMP in PAKΔ*pel* carrying the vector control were set to 1, and statistically significant changes (Student's *t* test, $P < 0.01$) are indicated by asterisks. 'ns' indicates changes that are not statistically significant.
B. Western blot using antibodies directed against PcrV (T3SS) and Hcp1 (T6SS).

overproduction of WspR^{R242A} resulted in further elevated biofilm formation (Fig. S1A and B), enhanced polysaccharide production (Fig. S1C) and reduced swimming motility (Fig. S1D).

Since the control of biofilm formation by RetS is c-di-GMP-dependent, we hypothesized that artificial manipulation of c-di-GMP levels by overproducing WspR should not only impact biofilm formation but also the T3SS and the T6SS. We therefore used a *pel* mutant (PAKΔ*pel*) to test the influence of c-di-GMP variation on the T3SS/T6SS switch, independently of both biofilm formation and of a *retS* mutation. The recombinant plasmid overproducing WspR^{R242A} was introduced into PAKΔ*pel*, which resulted in a > twofold increase in c-di-GMP levels when compared with a strain carrying the empty vector (Fig. 2A). Interestingly, this increase was concomitant with a reduction in PcrV production (T3SS) and an increase in Hcp1 production (T6SS) (Fig. 2B).

Another diguanylate cyclase, PA1120, identified as YfiN (Malone *et al*., 2010) or TpbB (Ueda and Wood, 2009), has previously been shown to influence biofilm formation in *P. aeruginosa*. The PA1120 gene was also cloned into a broad host range vector (pBBR1MCS-4-PA1120) and

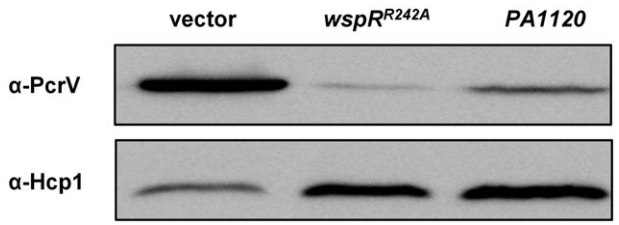

Fig. 3. The diguanylate cyclase PA1120 induces the T3SS/T6SS switch. Western blot analysis of PAKΔ*pel* carrying either empty vector or overproducing the diguanylate cyclases WspR^{R242A} or PA1120 as indicated. Antibodies are directed against PcrV (T3SS) and Hcp1 (T6SS).

introduced into PAKΔ*pel*. Overexpression of PA1120 in this strain resulted in a switch between the T3SS and the T6SS (Fig. 3), similar to the one observed upon overexpression of *wspR*^{R242A} (Fig. 2B).

These results show that an artificial increase in c-di-GMP levels, upon overproduction of an active diguanylate cyclase, controls the T3SS and the T6SS in an inverse manner. This T3SS/T6SS switch is similar to what was previously observed in a strain with a *retS* mutation (Fig. 1C).

Overproduction of the PA2133 phosphodiesterase reverts the T3SS/T6SS switch

Overproduction of phosphodiesterases has been shown to revert the biofilm-related phenotypes associated with high levels of c-di-GMP. Several putative phosphodiesterases encoded on the *P. aeruginosa* genome have been shown to be enzymatically active. One example of this is PA2133, the overproduction of which reduces biofilm formation in *P. aeruginosa* PAO1 (Hickman *et al*., 2005). We confirmed that overexpression of the PA2133 gene in PAKΔ*retS* reverted the hyperbiofilm and Congo red binding phenotype (Fig. S2) and hypothesized that reduction of c-di-GMP levels in a *retS* mutant would revert the expression profiles of the T3SS and the T6SS. Indeed, introduction of PA2133 into PAKΔ*retS*Δ*pel* resulted in a ~twofold decrease in c-di-GMP levels (Fig. 2A) and coincided with a switch in the production of secretion systems (Fig. 2B).

Taken together, these data show that artificial modulation of c-di-GMP levels leads to a reciprocal change in the production of the T3SS and the T6SS (Fig. 2). We therefore conclude that c-di-GMP is a central signalling molecule in the regulation of these two key protein secretion systems involved in *P. aeruginosa* virulence.

WspR is required for the switch between the T3SS and the T6SS

In this study it has been shown that the T3SS/T6SS switch that occurs in a *retS* mutant is dependent on c-di-GMP levels. This switch can also be observed by an artificial and global increase in c-di-GMP levels. We therefore assessed whether any specific diguanylate cyclases could be associated with the RetS/LadS/GacS signalling cascade. This investigation exploited the fact that *ladS* overexpression activates the Gac/Rsm cascade and gives the same phenotype as a *retS* mutation (Goodman *et al*., 2004; Ventre *et al*., 2006). Fifteen mutants in genes encoding diguanylate cyclases were selected from a previously published *P. aeruginosa* PA14 library (Kulasakara *et al*., 2006; Liberati *et al*., 2006) and the *ladS* gene carried on a broad host range plasmid (pBBR*ladS*) was introduced into these strains. The data revealed that overexpression of *ladS* in the PA14 wild-type induced the T6SS, compared with the vector control, which expressed the T3SS (Fig. 4, first two lanes). Furthermore, all the tested transposon mutants retained the ability to switch to the T6SS apart from one, namely the *wspR* mutant. Interestingly, although overexpression of the gene encoding PA1120 could induce the switch (Fig. 3), the mutant in this gene was not affected. This strongly supports the notion

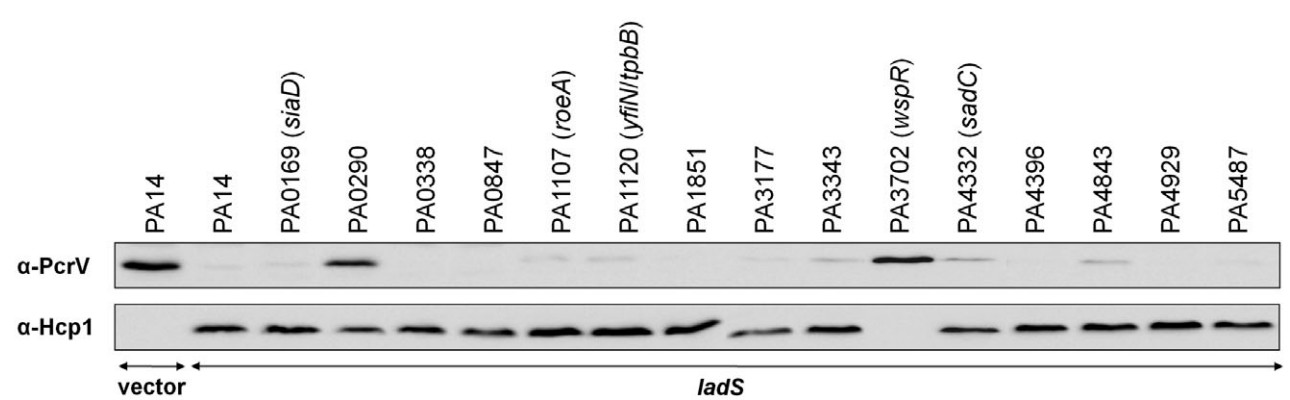

Fig. 4. WspR is required for the LadS-induced T3SS/T6SS switch. Western blot analysis of PA14 wild-type carrying the empty vector or overexpressing *ladS*, as well as transposon mutants in GGDEF-domain proteins, all overexpressing *ladS* as indicated. Antibodies are directed against PcrV (T3SS) and Hcp1 (T6SS).

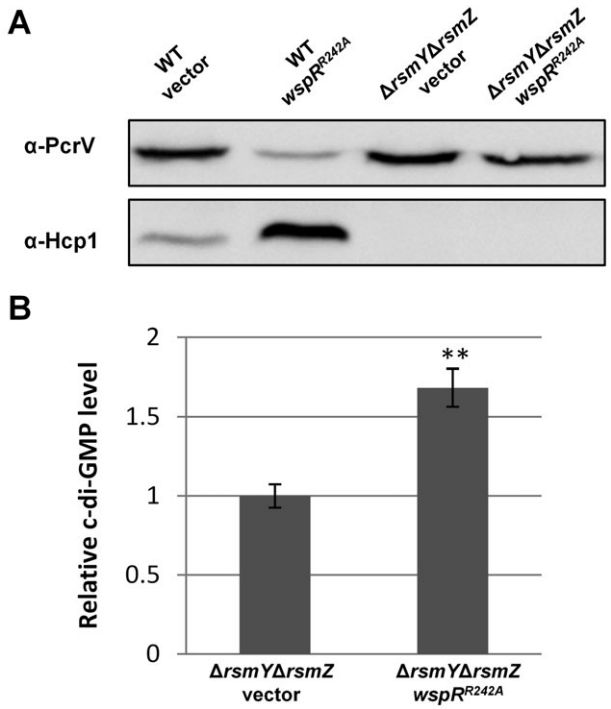

Fig. 5. The c-di-GMP-dependent T3SS/T6SS switch requires RsmY and RsmZ.
A. Western blot analysis of PAK or PAKΔ*rsmY*Δ*rsmZ* carrying either empty vector or overexpressing *wspR*^{R242A}. Antibodies are directed against PcrV (T3SS) and Hcp1 (T6SS).
B. Relative c-di-GMP levels of PAKΔ*rsmY*Δ*rsmZ* carrying either the empty vector or overexpressing *wspR*^{R242A} as indicated. The c-di-GMP level of the strain carrying the empty vector has been set to 1, and statistically significant changes (Student's *t* test, $P < 0.01$) are indicated by asterisks.

that the switch involves a specific set of enzymes, and that WspR is one of them. Interestingly, one of the other mutants (PA0290) was moderately affected in the switch and displayed low levels of both the T3SS and the T6SS. We concluded that there is a level of specificity in the role that various diguanylate cyclases play in the T3SS/T6SS switch, and that WspR is one of the major players.

RsmY and RsmZ are required for the c-di-GMP-dependent T3SS/T6SS switch

RetS is part of a signalling cascade that converges on the sRNAs, RsmY and RsmZ. Given the link between RetS and c-di-GMP levels, and the regulation of the T3SS/T6SS switch by both RetS and c-di-GMP, we investigated whether the two signalling networks act in parallel or whether there was a direct link between them. Whereas *wspR*^{R242A} overexpression in the wild-type resulted in a clear T3SS/T6SS switch, the same was not observed in a mutant that lacked the sRNAs RsmY and RsmZ (PAKΔ*rsmY*Δ*rsmZ*) (Fig. 5A). This was not due to a lack of increase in c-di-GMP, since *wspR*^{R242A} overexpression resulted in a ~1.6-fold increase in c-di-GMP levels in this strain as compared with the vector control (Fig. 5B). This is a clear indication that the c-di-GMP-dependent switch between the T3SS and the T6SS requires the presence of the sRNAs to counteract the activity of the post-transcriptional regulator RsmA.

Discussion

During colonization of a susceptible host, several bacterial species utilize complex regulatory pathways that control the switch between planktonic and biofilm lifestyles and consequently influence whether they cause acute or chronic infections. In *P. aeruginosa*, the regulatory cascade associated with the switch between the two infections strategies has been shown to involve the RetS and LadS signalling pathways (Goodman *et al*., 2004; Ventre *et al*., 2006). RetS and LadS are both orphan hybrid sensors that modulate the activity of the GacS/GacA two-component system to control the levels of the sRNAs RsmY and RsmZ. While RetS negatively influences the regulatory cascade by direct inhibition of the GacS sensor (Goodman *et al*., 2009), LadS has the opposite effect and activates *rsmY* and *rsmZ* expression via a currently unknown mechanism. When levels of RmsY and RsmZ are elevated, the post-trancriptional regulator RsmA is sequestered from target transcripts to allow translation.

Among the genes under direct negative control of RsmA are the *pel* and *psl* genes (Brencic and Lory, 2009; Irie *et al*., 2010), which are essential for biofilm formation, whereas genes involved in motility, such as type IV pili and flagellar genes, are positively regulated by RsmA (Brencic and Lory, 2009). In addition to the switch between biofilm formation and motility, the RetS/LadS/Gac/Rsm cascade tightly controls the production of virulence factors. A *retS* mutation is known to result in reduced mammalian cell cytotoxicity because of a down-regulation of the T3SS. This suggests that RsmA positively and indirectly influences the expression of these genes (Goodman *et al*., 2004; Brencic and Lory, 2009). The expression of the *P. aeruginosa* T6SS was shown to be directly and negatively controlled by RsmA (Brencic and Lory, 2009). Interestingly, the T6SS has been associated with CF patients chronically infected with *P. aeruginosa* (Mougous *et al*., 2006). Since *P. aeruginosa* very likely grows in biofilm communities in the CF lungs, this observation highlights the survival strategy of this organism by coordinating the expression of biofilm-related genes with those encoding the T6SS, both of which are associated with the chronic mode of infection. Collectively, these findings suggest that the RetS/LadS/Gac/Rsm signalling network plays a central role in a switch between acute virulence and chronic persistence.

Although RetS and LadS appear to be restricted to Pseudomonads (Records and Gross, 2010), GacS/GacA-like systems have been identified in several other bacteria. Different RsmA homologues have been identified in *Escherichia coli* (UvrY/BarA and CsrA) and in *Salmonella enterica* serovar Typhimurium (SirA/BarA and CsrA), and some of them are involved in the motility/sessility switch. In *E. coli*, CsrA represses the *pga* genes, which are involved in polysaccharide production (Wang *et al*., 2005), and also positively regulates the expression of flagellar genes (Wei *et al*., 2001). In *S. typhimurium*, CsrA downregulates the production of fimbriae, which are required for biofilm formation, and upregulates flagellar genes (Teplitski *et al*., 2006). In both bacteria, the activity of CsrA is antagonized by two sRNAs, CsrB and CsrC. Although only few direct CsrA targets are identified in *E. coli* and *S. typhimurium*, these proteins are likely to govern a central switch between motility and sessility.

In addition to the GacS/GacA signalling cascade, c-di-GMP has emerged as a widespread second messenger that controls the switch from motility to sessility in many bacteria (Hengge, 2009). This control is exerted on many different levels and has been shown to target a wide range of functions, including polysaccharide production and motility. c-di-GMP is known to influence the production of Pel exopolysaccharides in *P. aeruginosa* (Hickman *et al*., 2005; Lee *et al*., 2007; Starkey *et al*., 2009), cellulose and curli fimbriae production in *S. typhimurium* (Garcia *et al*., 2004; Romling and Amikam, 2006) and the VPS polysaccharide in *Vibrio cholera* (Casper-Lindley and Yildiz, 2004; Tischler and Camilli, 2004). This control occurs at least partly at the transcriptional level. Furthermore, c-di-GMP was shown to exert post-transcriptional control of the swimming velocity of *E. coli* and *Salmonella* by binding to YcgR, which acts as a molecular brake that interacts with proteins of the flagellar motor and slows down its rotation (Boehm *et al*., 2010; Paul *et al*., 2010).

The fact that both c-di-GMP and RetS signalling converge on *pel* gene expression prompted us to investigate whether the two pathways were linked by measuring intracellular c-di-GMP levels in a *retS* mutant. Indeed, our results showed that a *retS* mutant displays a 1.4-fold elevation in c-di-GMP levels compared with wild-type *P. aeruginosa*, which increased to a twofold elevation in a *retS*/*pel* double mutant that is unable to form biofilms. This overlap between the RetS and the c-di-GMP network was indirectly suggested by previous work, where Goodman and collaborators found that the *retS* phenotype could be suppressed by secondary mutations in the diguanylate cyclase-encoding gene *sadC* (PA4322) (Goodman *et al*., 2004; Merritt *et al*., 2007). In order to investigate whether the overlap of the two regulatory systems went beyond the *pel* genes, we used the diguanylate cyclase activity of $WspR^{R242A}$ and the phosphodiesterase activity of PA2133

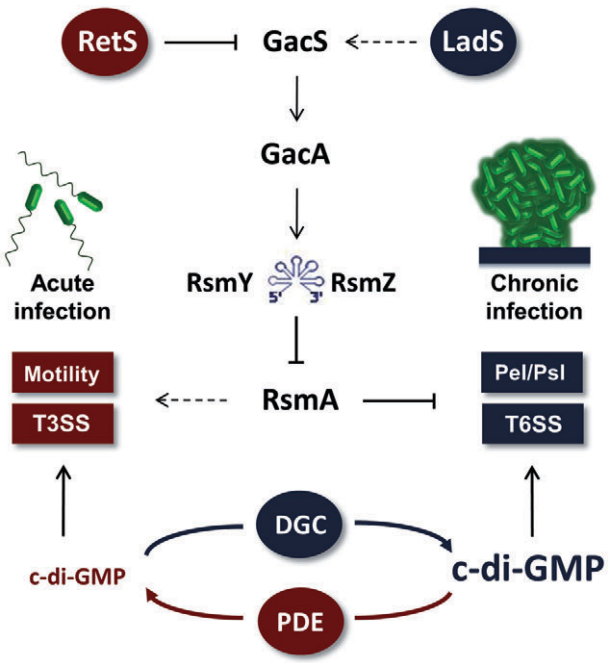

Fig. 6. Schematic representation of regulatory networks that switch *P. aeruginosa* lifestyles. The *retS* and c-di-GMP signalling pathways act on the same targets to inversely control functions involved in motility versus biofilm formation, or in acute versus chronic infection. The RetS/LadS signalling network converges on the post-transcriptional regulator RsmA to inversely regulate the T3SS and the T6SS. The same regulation can be achieved by artificially modulating c-di-GMP levels upon overproduction of a diguanylate cyclase (DGC) or a phosphodiesterase (PDE). The dotted arrows indicate an unknown mechanism of action.

to artificially modulate c-di-GMP levels in the parental strain and the *retS* mutant. We monitored the presence of the T3SS and T6SS and found that increased c-di-GMP levels caused the wild-type to switch from the T3SS to the T6SS, while decreased c-di-GMP levels caused the *retS* mutant to do the opposite and switch from the T6SS to the T3SS. The regulation of the T6SS and the T3SS by c-di-GMP therefore mimics the regulation by RetS, which further confirms the link between these two pathways and highlights that they have several target functions in common (Fig. 6).

Previous studies in other bacteria have also suggested a link between c-di-GMP and certain virulence functions. In *S. typhimurium* and *Dickeya dadantii* (formerly *Erwinia chrysantemi*), recent research has shown that increased c-di-GMP levels not only promote biofilm formation, but also downregulate the T3SS (Lamprokostopoulou *et al*., 2010; Yi *et al*., 2010). Furthermore, although no link to the Gac system was suggested in *D. dadantii*, CsrA in *S. typhimurium* has been shown to downregulate the diguanylate cyclases STM4551 and STM1987, as well as upregulate the phosphodiesterase STM3611 (Jonas *et al*., 2010). Similarly, CsrA in *E. coli* has been found to

directly repress the diguanylate cyclases YcdT and YcdE (Jonas *et al*., 2008). A connection between the two pathways might therefore be suggested in these bacteria.

While the inverse control exerted by RsmA on the T3SS and the T6SS in *P. aeruginosa* has been well established (Brencic and Lory, 2009), the c-di-GMP-dependent switching of these secretion systems is a novel finding. It is clear that overproduction of $WspR^{R242A}$ or PA1120 results in a substantial increase in c-di-GMP levels that might saturate many c-di-GMP-dependent processes in a non-specific manner. This has been observed in *S. typhimurium* upon overproduction of the diguanylate cyclase AdrA (Simm *et al*., 2004). We therefore investigated the specificity of this switch in a system that does not require overexpression of diguanylate cyclases or phosphodiesterases. To achieve this, we took advantage of the fact that *ladS* overexpression activates the Gac/Rsm signalling cascade and gives the same phenotype as a *retS* mutation (i.e. a switch from the T3SS to the T6SS). We then took fifteen PA14 transposon mutants in selected diguanylate cyclases-encoding genes from a previously published library (Liberati *et al*., 2006) and overexpressed *ladS in trans* in all these strains. In these conditions, the expected switch occurred in all strains apart from the *wspR* mutant, suggesting that WspR is required for the switch to occur. Conversely, mutations in the previously mentioned PA4322 (*sadC*), or in PA0338, which has been identified as an RsmA target (Brencic and Lory, 2009), did not prevent the switching, suggesting that these components are not required for the process in this experimental set-up. However, the mutant in PA0290 was moderately affected and displayed reduced levels of both secretion systems, suggesting that WspR might not be the only player involved.

We next addressed the question whether c-di-GMP-variation influences the Gac/Rsm signalling cascade, or whether it is a consequence of its activity. As discussed above, diguanylate cyclases and phosphodiesterases are targets of RsmA/CsrA in a number of bacteria. However, in the case of *P. aeruginosa*, the known RsmA targets were shown to not be required for the c-di-GMP-dependent switch between the secretion systems, at least in our experimental set-up. In order to further investigate the hierarchy between these regulatory systems, we tested whether the presence of the Gac/Rsm signalling cascade was required for c-di-GMP-dependent switching to occur. This was done by overexpressing $wspR^{R242A}$ in an *rsmY*/*rsmZ* double mutant. Although c-di-GMP levels were twofold higher in this strain than in the vector control, it still produced the T3SS and not the T6SS (Fig. 5). This clearly indicates that c-di-GMP alone cannot relieve RsmA repression in the absence of the sRNAs.

The c-di-GMP-dependent switching from motility and T3SS to sessility and T6SS therefore requires the presence of the Gac/Rsm signalling cascade. However, the exact level at which c-di-GMP influences the cascade is still unclear. One hypothesis could be that c-di-GMP serves as a signal influencing the activity of one of the three sensors, RetS, LadS or GacS. Alternatively, the second messenger could be a co-regulator influencing the activity of the sRNAs. A third option is that the activity of the cascade depends on a pool of available c-di-GMP, and that this pool is partly controlled by direct RsmA-targets (e.g. PA0388) and partly by other diguanylate cyclases/phosphodiesterases that respond to alternative signals. This may be the reason for the 'half switch' observed in the PA0290 mutant (Fig. 4).

A number of different mechanisms of action for c-di-GMP have so far been demonstrated in bacteria. These have proven to be very versatile, and well known examples range from transcriptional regulation (e.g. FleQ in *P. aeruginosa*) (Hickman and Harwood, 2008), translational regulation (riboswitches in *V. cholerae* and *Bacillus subtilis*) (Sudarsan *et al*., 2008), regulation of protein activity (*alg44* in *P. aeruginosa* and the flagellar brake in *E. coli* and *Salmonella*) (Boehm *et al*., 2010; Paul *et al*., 2010), and cross-envelope signalling (e.g. LapD in *Pseudomonas fluorescens*) (Navarro *et al*., 2011; Newell *et al*., 2011). With this in mind, there are multiple possibilities of c-di-GMP control of the switch between the T3SS and the T6SS, and these could involve direct control of activity, as well as production of machinery components, as observed in this study.

In conclusion, there is growing evidence that versatile pathogens are able to switch their lifestyle or infection mode by sensing environmental conditions. The switch is driven by sophisticated regulatory networks, which often modulate the levels of small molecules that in turn influence bacterial gene expression and physiology. The present work demonstrates that, in *P. aeruginosa*, two types of such molecules, c-di-GMP and sRNAs, function in a concerted manner to coordinate a switch between two secretion machineries, the T3SS and the T6SS. This study therefore clearly demonstrates that c-di-GMP plays a major role in controlling secretion machineries that are essential in *P. aeruginosa* pathogenicity. Future work will aim to further elucidate the molecular mechanisms of this regulation and the specific components involved. This will provide important information on perhaps the most important regulatory cascade controlling the virulence strategies of *P. aeruginosa* in the face of dwindling efficiency of currently available antibiotics.

Experimental procedures

Strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. Bacteria were cultured at 37°C with

shaking in Luria–Bertani (LB) medium or M9 minimal medium containing 22 mM glucose, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$. Culture media were supplemented with antibiotics at the following concentrations, when appropriate. For *E. coli*: 50 μg ml^{-1} ampicillin (Ap) and 50 μg ml^{-1} kanamycin (Km). For *P. aeruginosa*: 500 μg ml^{-1} carbenicillin (Cb) for selection of transconjugants or 300 μg ml^{-1} Cb for maintenance of plasmids and 15 μg ml^{-1} gentamicin (Gm). The T3SS was induced by adding 5 mM EGTA-20 mM $MgCl_2$ to the growth medium in the experiments shown in Figs 1, 2 and 5. Congo red staining assay was performed at 30°C on tryptone (10 g l^{-1}) agar (1%) plates supplemented with 40 μg ml^{-1} Congo red and 20 μg ml^{-1} Coomassie brilliant blue. Swimming plates were prepared using 0.3% agar and incubated at 30°C as described previously (Mikkelsen *et al.*, 2009). *Escherichia coli* OmniMAX and TOP10 were used for standard genetic manipulations.

The *wspR* gene was amplified by PCR using primers WspRF (CGTGGAACAGCATTCAATTT) and WspRR (AA AGATACCCCCGAATGGTC). The PA2133 gene was amplified using primers PA2133F (TCACACAGGAAACTACA GTGAACGGTTCCCCAC) and PA2133R (GGAAGGCTG ATTGCTCTGTT). The PA1120 gene was amplified using primers PA1120F (TTCACACAGGAAACCGTGATGAACCG TCGTC) and PA1120R (AACAGCACCTTGCTCGACAT). PCR products were cloned into pCR2.1-TA and sub-cloned into pBBR1MCS-4.

The R252A-*wspR* allele was generated in pCR2.1 by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the supplier's protocol. Primers used were WspRmutR242F (CGAGGGC TGCAGTGCCTCCTCGGACCTG) and WspRmutR242AR (CAGGTCCGAGGAGGCACTGCAGCCCTCG). All constructs were confirmed by sequencing.

Transfer of plasmids into *P. aeruginosa* strains was carried out by triparental mating using the conjugative properties of plasmid pRK2013. Transconjugants were isolated on *Pseudomonas* Isolation Agar (Difco) supplemented with appropriate antibiotics.

Biofilm assays

Visualization of biofilm formation was carried out in 14 ml borosilicate tubes. Briefly, LB (3 ml) supplemented with appropriate antibiotics was inoculated to a final OD_{600} of 0.1 and incubated at 37°C. Biofilms were stained with 0.1% crystal violet (CV) and tubes were washed with water to remove unbound dye. Quantification of biofilm formation was performed in 24-well polystyrene microtiter plates. M9 medium (1 ml per well) supplemented with appropriate antibiotics was inoculated to a final OD_{600} of 0.01 and incubated at 37°C with shaking for 8 h. Biofilms were stained and washed as above, and CV was solubilized in 96% ethanol before measuring the absorbance at 600 nm. For each biofilm assay three independent experimental repetitions were performed.

Western blots

Cultures were inoculated into LB medium to an OD_{600} of 0.1 and incubated at 37°C with shaking for 6 h. Whole-cell lysates were loaded (0.1 OD_{600} equivalent unit) on SDS-PAGE and proteins transferred on nitrocellulose membranes. Primary antibodies, anti-Hcp1 and anti-PcrV were used at dilutions of 1:500 and 1:1000 respectively. Secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) was used at 1:5000 dilution. Visualization was achieved using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo) and a LAS3000 Imaging System (Fuji). For each Western blot three independent experimental repetitions were performed.

Extraction and measurement of c-di-GMP levels

Intracellular c-di-GMP levels were determined by LC-MS/MS as previously described (Simm *et al.*, 2009), with the following modifications. Bacterial pellets from 100 ml overnight liquid cultures (M9 medium supplemented with nutrients and appropriate antibiotics) were resuspended in 0.19% ice-cold formaldehyde at a concentration of 300 mg wet weight per ml, incubated on ice for 10 min and centrifuged at 10 000 *g* for 15 min. The pellet was then resuspended in 300 μl of water and when required, c-di-GMP (BIOLOG LifeScience Institute, Bremen, Germany) was spiked at a concentration of 1.7 μM. After the ethanol extraction of nucleotides (Simm *et al.*, 2009), samples were dried under vacuum, reconstituted in 50 μl H_2O by vigorous vortexing and sonication for 5 min, centrifuged for 15 min at 10 000 *g* and the supernatants were analysed using an Agilent 1200 HPLC system with aC18 ACE 3 AQ 150 × 2.1 mm column with a matching guard maintained at 55°C. The mobile phase consisted of 0.1% formic acid run in an H_2O-acetonitrile gradient at a flow rate of 300 μl min^{-1}, reaching 50% acetonitrile in 14 min. MS analysis was then performed by negative ion electrospray from the Agilent HPLC system into a Bruker HCT Plus ion trap in multiple reaction mode. Using SmartFrag (HyStar software), the trap was set to isolate from full scan and then fragment ions at m/z 688.9, with a monitored mass range of m/z 450–700. DataAnalysis v3.3 was used to interrogate the acquisitions, and extracted ion chromatograms of m/z 538 were produced from the negative ion MS/MS of m/z 688.9. Retention times and peak spectra were matched to the standard injected at intervals throughout the run. Peak areas and relative c-di-GMP levels were normalized to the levels obtained with the reference strains. Each extraction and quantification was performed in triplicate.

Acknowledgements

The authors wish to thank Mary Bruce (School of Molecular Medical Sciences, University of Nottingham) for c-di-GMP measurements and Arne Rietsch for the gift of antibodies directed against PcrV. Alain Filloux is supported by the Royal Society and the BBSRC grant No. BB/F019645/1. Joana Moscoso is supported by the 'Fundação para a Ciência e a Tecnologia' (FCT) and Helga Mikkelsen is supported by the BBSRC grant No. BB/F019645/1.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Overexpression of *wspR* or *wspR*^{R242A} influences biofilm-related phenotypes. PAK wild type carrying either empty vector or overexpressing *wspR* or *wspR*^{R242A}, as indicated in (A).
A. Biofilm formation in static growth conditions visualized by crystal violet staining.
B. Biofilm formation in shaking growth conditions quantified by crystal violet staining.
C. Bacterial colony on Congo red agar.
D. Swimming motility determined in 0.3% agar.
Fig. S2. Overproduction of a phosphodiesterase suppresses the hyperbiofilm phenotype of a *retS* mutant. Biofilm formation in glass tubes, visualized by crystal violet staining (left panel), and Congo red binding (right panel) of PAKΔ*retS* carrying either empty vector or overproducing the phosphodiesterase PA2133.
Table S1. Strains and plasmids used in this study.

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Appendix 2

environmental microbiology

Environmental Microbiology (2012) **14**(4), 1088–1089 doi:10.1111/j.1462-2920.2012.02723.x

Corrigendum

The *Pseudomonas aeruginosa* sensor RetS switches Type III and Type VI secretion via c-di-GMP signalling

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In the article 'The *Pseudomonas aeruginosa* sensor RetS switches Type III and Type VI secretion via c-di-GMP signalling' (Moscoso *et al*., 2011), we showed that a *P. aeruginosa* PAKΔ*retS* mutant displays increased levels of c-di-GMP, which explains, at least in part, why it has a hyperbiofilm phenotype. We further showed that expression of the type VI secretion system (T6SS) and type III secretion system (T3SS), which are antagonistically controlled by RetS, could clearly be switched in a RetS-independent manner upon manipulation of c-di-GMP levels by over-expressing the diguanylate cyclase-encoding genes, *wspR* or *PA1120* in a wild-type strain. We also showed that overexpression of these diguanylate cyclases (DGC) in an *rsmYZ* mutant, although increasing levels of c-di-GMP is not sufficient to induce the T3SS/T6SS switch indicating that an increase in c-di-GMP alone cannot relieve RsmA-mediated repression.

In the last part of the paper, and in an attempt to demonstrate DGC specificity, we tried to demonstrate that the switch involved a specific DGC. We showed that overexpression of *ladS* results in the same phenotype as a *retS* mutant with respect to the T3SS/T6SS switch. Our strategy has thus been to use the PA14 collection of transposon mutants (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi) and to overexpress *ladS* in the individual DGC mutants available from the collection. By doing so we observed that the *wspR* mutant no longer responded to *ladS* overexpression and no T3SS/T6SS switch was observed (fig. 4 in Moscoso *et al*., 2011).

We have now reconstructed a clean *wspR* deletion mutant (see *Experimental procedures*) and observed that upon overexpression of *ladS*, the T3SS/T6SS switch is perfectly effective (see Fig. 1), i.e. the T6SS is highly expressed whereas the T3SS is downregulated. This result rules out our initial observation that WspR is specifically involved in the T3SS/T6SS switch and leaves open the notion of specificity. Whereas none of the tested single mutations in genes encoding DGCs affects the T3SS/T6SS switch, it remains a possibility that the switch could only be affected when multiple DGC-encoding genes are deleted together.

We apologize for the confusion resulting from the use of two different *wspR* mutant strains.

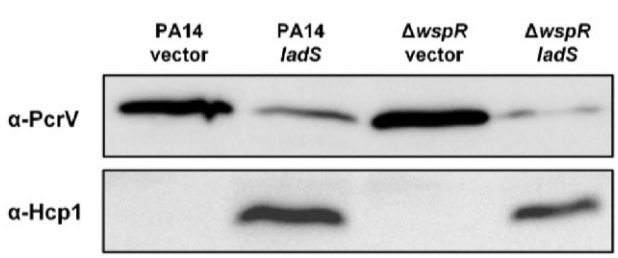

Fig. 1. Western blot analysis of *P. aeruginosa* PA14 and PA14Δ*wspR* mutant (indicated as Δ*wspR*) carrying the empty vector (pBBR1-MCS-4 indicated as vector) or overexpressing *ladS* (pBBR1-MCS-4-*ladS* indicated as *ladS*). The antibodies used are directed against PcrV (T3SS) and Hcp1 (T6SS) as indicated on the left of each panel.

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Experimental procedures

The PA14Δ*wspR* strain was obtained by using the suicide vector pKNG101 carrying a mutator fragment homologous to the flanking regions of *wspR*. The mutator fragment was obtained using primers 5′-GCAGCATGTCGACGAGGTAT-3′, 5′-TCAGGCCCAGTTGTGCATGTTTCTCTCCGG-3′, 5′-ATGCACAACTGGGCCTGATGGAACAGCCG-3′ and 5′-GATGTCC AGGTAGGCGTTGT-3′. Clones in which the double recombination events occurred, resulting in the deletion of *wspR*, were selected on sucrose-containing plates as described elsewhere (Kaniga *et al*., 1991; Vasseur *et al*., 2005).

Deletion of *wspR* was confirmed using the external primers 5′-GAGGATCGGAATCGTCAATG-3′ and 5′-CTGCATTT CCAGCTCGTACA-3′.

The *ladS* gene carried on a broad-host-range plasmid (pBBR*ladS*) or the cloning vector was introduced into the wild-type or *wspR* mutant strains (see Moscoso *et al*., 2011). Bacterial growth, preparation of whole cell extracts and Western blot analysis were performed as previously described.

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Appendix 3

Chapter 49

Methods for studying biofilm dispersal in *Pseudomonas aeruginosa*

Nicolas Barraud, Joana A. Moscoso, Jean-Marc Ghigo and Alain Filloux

Summary

Biofilm dispersal is the last and least understood stage of the biofilm life cycle. Several recent studies have characterised dispersal events in response to various cues and signals. Here we describe a range of methods useful for the investigation of dispersal in the biofilm model organism and opportunistic pathogen *Pseudomonas aeruginosa*.

Key words: Biofilm, dispersal, environmental cues, nitric oxide, batch culture, crystal violet staining, continuous culture, microfermenters.

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1. Introduction

In most environments bacteria adopt a biphasic life cycle, with a sessile surface-attached biofilm phase and a motile free-living planktonic phase. Biofilm bacteria, compared to their planktonic counterparts, often show increased resistance to environmental stress and antimicrobials. While the mechanisms for attachment of bacteria on surfaces and the onset of biofilm formation have been well documented in many species, the reverse transition, at the end of the biofilm development that involves the release into the bulk liquid of cells that were once strongly attached is still poorly understood. Biofilm dispersal appears to be a programmed, highly regulated process, which can be triggered by specific cues such as nutrients, oxygen and nitric oxide (NO) levels as well as bacterial derived signal molecules. The

population of dispersal bacteria often comprises high levels of phenotypic and genetic variants, which are thought to provide an ecological advantage by facilitating colonisation of new surfaces ***(1)***.

Elucidating the mechanisms of biofilm dispersal is of primary importance in order to better understand the maintenance, evolution and dissemination of biofilm infections. It may also lead to the identification of potential new targets for the control of biofilm by inducing their natural detachment in a non-toxic, non-resistant manner. The successful study of this phenomenon presents several challenges: (i) one needs to grow a functional, mature biofilm, (ii) then the biofilm needs to be exposed to a dispersal cue in a controlled fashion, and (iii) finally the remaining biofilm needs to be analysed for evidence of dispersal events. Since nutrients and oxygen availability can trigger dispersal, sudden changes in growth conditions during biofilm treatment or analysis may result in an uncontrolled detachment of portions of biofilm, compromising interpretation of the data. This paper describes two complementary systems that are useful to study dispersal in *Pseudomonas aeruginosa* ***(2)***. First, a microtitre-based batch system was designed for high throughput screening of dispersal in response to various cues. Second, a microfermenter-based system allows for varying a wide range of parameters, with minimum uncontrolled disturbance to the biofilm. These procedures can be useful to screen for compounds that may induce dispersal or for mutant strains that may be defective in dispersal. The use of microfermenters, by generating abundant biomass and allowing for the extraction of nucleic acids and proteins from the attached biofilm bacteria, can also support the examination of cellular changes that regulate dispersal. Further, because the dispersal population is readily collected in the biofilm runoff effluent, it is also possible to study the phenotypic and genotypic changes associated with biofilm dispersal.

2. Materials

2.1. Microtitre plate assay

1. Luria Bertani (LB) medium with 1% NaCl.
2. Modified M9 minimal medium (freshly made, *see* **Note 1**): M9 1X salts consisting of 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, pH 7.2, supplemented with the nutrients 2 mM $MgSO_4$, 100 µM $CaCl_2$ and glucose at 2 mM or 20 mM for continuous or batch culture experiments, respectively.
3. Tissue-culture-treated 24-well plates (BD).
4. Dispersal agents. For NO donors: sodium nitroprusside (SNP, Sigma) solution in water made fresh on the day; or NONOate disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (Proli, Cayman Chemicals) solution in 10 mM NaOH and stored at -20 ºC for no more than one week.
5. Vacuum station made using a vacuum pump (Fisherbrand) connected to a side arm flask with a venting cap/stopper linked to a Pasteur pipette.
6. Repeater pipettor (Eppendorf).
7. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
8. Crystal violet stain 0.2% crystal violet prepared by diluting with PBS a 2% crystal violet solution in 1.9% ethanol and 0.08% ammonium oxalate. Alternatively, a commercial stain, Gram crystal violet 0.3% in 1:1:18 isopropanol:ethanol/methanol:water (BD), may be used.

9. 100% ethanol.
10. Sonication bath (e.g. 150 W, Unisonics).

2.2. Microfermenter assay

11. Microfermenters equipped with glass spatula (Institut Pasteur, Paris).
12. Silastic silicone tubing internal diameter (ID)-outside diameter (OD) 1.0-2.6 mm (Cole Parmer) for upstream sterile medium flow; Tygon tubing ID-OD 2.4-4.0 mm (Saint Gobain) for peristaltic pump; silicone microbore tubing ID-OD 1.0-2.9 mm (Cole Parmer) for downstream biofilm effluent runoff flow.
13. Polypropylene adapters and connectors (Cole Parmer): female luer × 1/8" hose barb; male luer × 1/8" hose barb; barbed reducer 1/8" × 1/16"; barbed T-connector 4 mm.
14. Polypropylene 10-20 l carboys autoclavable equipped with a filling/venting cap (Cole Parmer).
15. Peristaltic pump (e.g. model 205S, Watson Marlow).
16. Water bath consisting of a 5-6 l tank and a thermostat with circulator (Cole Parmer).
17. Source of compressed air at 40 kPa.
18. Sterile filters with 0.2 μm pore size (Millipore).

---

3. Methods

2.3. Batch culture microtitre plate assay

In the batch assay, *P. aeruginosa* PAO1 wild type biofilms are grown in microtitre plates and dispersal is assessed as a concomitant increase in planktonic biomass and a decrease in biofilm biomass (**Fig. 1**).

1. Grow a *P. aeruginosa* PAO1 culture overnight in LB medium to an OD_{600} of 2.5.
2. Make a 1:200 dilution in M9 minimal medium with 20 mM glucose and aliquot 1 ml per well of a 24-well plate.
3. Put the plate on a platform shaker at 37 °C and incubate with shaking at 180 rpm (*see* **Note 2**).
4. After 6 h growth (*see* **Note 3**), induce dispersal by adding 10 µl per well of a solution at the appropriate concentration in less than 1 min per plate (*see* **Note 4**). For instance, add 10 µl of a 10 mM solution of NO donor Proli to make a final concentration of 100 µM (**Fig. 1C** and **1D**). Dispersal can also be induced by reducing the shaking speed to 60 rpm (**Fig. 1A** and **1B**), which is known to induce a decrease in oxygen tension in the culture within minutes ***(3)***.
5. Incubate for a further 15 min to 1 h.
6. The planktonic biomass is assessed by measuring the OD_{600} or colony-forming units (CFU) count of the supernatant.
7. Finally the biofilm biomass is assessed by performing either crystal violet staining ***(4)*** or CFU count of biofilm cells.

2.4. Crystal violet staining

Crystal violet provides a fast and highly reproducible method to quantify biofilm biomass.

1. Use the vacuum device for suction of the supernatant liquid from the wells.
2. To remove loosely attached biofilm cells, wash each well once by adding 1 ml PBS using a repeater pipettor and then removing the liquid using the vacuum device.

3. Stain with 1 ml crystal violet solution for 20 min.
4. Using the same vacuum device, remove the crystal violet dye from each well.
5. Wash twice with 1 ml PBS as in step 2.
6. Photograph the distribution of crystal violet dye on the walls of microtitre wells by using a digital camera (**Fig. 1B** and **1D**).
7. Add 1 ml of 100% ethanol to each well to re-elute the crystal violet dye and shake gently until all crystal violet is dissolved.
8. Measure the OD_{550}. The absorbance of an uninoculated well serves as a negative control and should be subtracted from the value of the inoculated wells.

2.5. CFU enumeration of biofilms

CFU count is a sensitive method that allows for detection of much lower numbers of biofilm bacteria compared to crystal violet staining. Further, CFU count is useful to assess the efficacy of biofilm control measures when dispersal treatments are combined with bactericidal agents.

1. Equip the vacuum device with a sterile Pasteur pipette and suction liquid from the cultures, briefly flaming the Pasteur pipette between each well.
2. Wash each well twice with 1 ml of sterile PBS. Drain all the wells.
3. Add 1 ml sterile PBS and resuspend biofilms on the bottom and walls of the well by swabbing with a sterile cotton bud and incubating in a sonication bath for 2 min.
4. Perform a serial dilution of resuspended biofilm cells and plate on LB agar.

5. Enumerate CFU after 24 h incubation at 37 °C. After 6 h incubation under these growth conditions, control biofilms that were not treated harboured 19.6 ± 2.6 × 10^6 CFU / cm^2.

2.6. Continuous culture microfermenter assay

In the continuous flow assay, *P. aeruginosa* PAO1 biofilms are grown under flow-through conditions in glass microfermenters ***(5)*** and dispersal is assessed by monitoring the biofilm effluent (**Fig. 3**). At the end of the assay, the remaining biofilm can be quantified or further analysed for gene or protein expression.

1. Prepare the microfermenter system as described in **Fig. 2**, and sterilise all components by autoclaving. Autoclave the Tygon pump tubing separately with both extremities free of any connector and wrapped in aluminium foil.
2. Prepare a 10-20 l M9 1X salts solution and sterilise by autoclaving at 121 ºC, 15 psi for 45 min instead of the usual 15 min. After cooling down, add 2 mM $MgSO_4$, 100 µM $CaCl_2$ and 2 mM glucose to the M9 1X salts solution, preferably under sterile conditions. Mix well to dissolve any calcium precipitate.
3. Connect the microfermenter to the medium inlet via a piece of Tygon tubing pinched to the roller of a peristaltic pump. Put the microfermenter upright in a water bath heated at 37 ºC. Close the air supply line with a clamp and rinse the whole system by pumping through each microfermenter 200 ml of fresh sterile M9 medium.
4. Stop the medium flow, open the microfermenter cap under sterile conditions and in each microfermenter containing 40 ml of M9 medium inoculate 1 ml of

an overnight culture of *P. aeruginosa* PAO1 grown in LB at 37 ºC. Close the cap tightly.

5. Allow cells to attach with medium flow and airflow turned off for 1 h.
6. Turn on liquid medium flow at a rate of 0.8 ml / min. Unclamp the air supply line and turn on the aeration at 40 kPa, 100 ml / min (*see* **Note 5**). When air and medium flows are turned on, the volume of medium inside the microfermenter is ~20 ml, thus making a residence time of 25 min.
7. Grow for 24 h without interruption or disturbance (*see* **Note 6**).
8. After 24 h, switch the medium inlet to separate containers bearing fresh media with specific treatment; e.g. NO donors SNP at 1 mM or $NaNO_2$ at 10 mM (**Fig. 3**), no carbon source (starvation), or 20 mM glucose (nutrient increase). For oxygen depletion-induced dispersal, switch aeration to nitrogen or argon gas (*see* **Note 7**). This procedure ensures minimal physical disruption of the biofilm growing inside the microfermenters.
9. Collect the biofilm effluent in separate 15 ml Falcon tubes (BD) every 10 min and monitor the release of dispersal cells by measuring the OD_{600} (*see* **Note 8**).
10. To assess the remaining biofilm biomass after dispersal has been induced, remove the spatula aseptically and resuspend the biofilm cells in 5 ml PBS by swabbing with a sterile cotton bud. Measure the OD_{600} of the suspension. This procedure could also be used to extract proteins or nucleic acids from biofilm cells after dispersal was induced.
11. For microscopy analysis of biofilm structures, remove the spatula and rinse once by dipping briefly in sterile PBS. Add an appropriate stain (e.g. live/dead bacterial viability kit, Invitrogen), put a cover slip on top and visualise under a microscope.

4. Notes

4.1. For the batch microtitre plate assay:

1. Complete M9 medium needs to be made fresh on the day by adding $MgSO_4$ (from 1 M stock autoclaved), glucose (from 20 % stock sterilised by passing through a 0.2 µm-filter) and $CaCl_2$ (from 1 M stock autoclaved) to the 1X salts solution. Autoclave the 1X salts solution beforehand (this can be kept on the bench for long periods).
2. To minimize variations in biofilm growth due to heaters and air fans that may induce variable evaporation/aeration (edge effect), put plates on the same row/level, and preferably in the middle of the shaker. To check for possible variations, include control wells (not dispersed with any treatment) both on the edge and in the middle of the plate.
3. Incubation time and inoculum may have to be adjusted according to the strain being used. For instance, when growing *P. aeruginosa* PA14 biofilms under these conditions, a 1:1,000 initial dilution in M9 and incubation for 18 h are recommended.
4. Do not stop or open the incubator during the experiment and work quickly every time the plate is taken out of the incubator. Use only 12 wells in the 2 middle rows of the 24-well plate in order to keep low number of wells, which should allow for processing each plate quickly enough every time the plate is removed from the shaker-incubator, i.e. for addition of treatments, quantification of supernatants, washing and crystal violet staining. If assessing biofilm biomass by crystal violet analysis rather than CFU count, the system does not need to be strictly sterile at the time of adding dispersal treatments,

i.e. 15 min to 1 h before the end of incubation. It is recommended to start by performing the oxygen-induced dispersal assay in order to better grasp the dynamics of dispersal that may be potentially induced when the plate is taken out of the incubator.

4.2. For the continuous flow microfermenter assay:

5. Always apply a positive pressure to medium and air supplies or the supernatant will flow back, contaminate the growth medium and block air filters. Therefore, to turn the system on, first tighten the microfermenters caps, then switch on the air pump and finally the peristaltic pump. To turn off, first switch off the peristaltic pump, then the air pump and finally loosen the caps.
6. Incubation time may have to be adjusted according to the strain being used. For instance, under these growth conditions *P. aeruginosa* PA14 form visible biofilms only after 3 days. Increased volumes of media may be necessary.
7. Use three-way valves for aeration of microfermenters in order to allow for switching aeration with minimum disruption. Alternate aeration lines should be equipped with pressure gauge and flow meters as well as fine regulators to adjust the pressure quickly after switching the aeration source.
8. Using a microbore tubing (e.g. 1 mm internal diameter) for the biofilm effluent ensures minimising biofilm growth in areas other than the microfermenter that could potentially interfere with the analysis of cells in the biofilm effluent that are expected to be exclusively released from the biofilm inside the microfermenter. A microbore tubing presents a small surface area and the flow velocity and shear stress are high.

Acknowledgement

This work was supported by grants from the Australian Research Council.

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Figure legends

Fig. 1. Microtitre plate assay for biofilm dispersal. (A) Oxygen-depletion induced dispersal. *P. aeruginosa* PAO1 biofilms were grown for 6 h with shaking at 200 rpm, then at t = 0, the shaking speed was reduced to 60 rpm to reduce oxygen tension. At various time points, the planktonic biomass was assessed by measuring the OD_{600} of the supernatant and the biofilm biomass by performing crystal violet staining. (B) Stained biofilms before and after 5 min of incubation at a reduced shaking speed. (C) NO-induced dispersal. *P. aeruginosa* biofilms were grown for 6 h with shaking at 200 rpm in the absence of NO donor. Then 10 µl of a Proli solution were added to the wells to make a final concentration of 100 µM and control wells were left untreated. Plates were incubated for a further 15 min before assessing dispersal by measuring the OD_{600} of the supernatant and performing crystal violet staining of the biofilm. (D) Stained biofilms untreated or treated with Proli for 15 min.

Fig. 2. (A) Illustration of the microfermenter flow-through culture set up: (i) 10-20 l fresh medium autoclavable carboy equipped with venting cap; (ii) 40 cm-long Silastic tubing (in a system where multiple microfermenters are used, this tubing may be diverted by using T-connectors) connected with male and female luer lock connectors to a 30 cm-long Tygon pump tubing which is pinched to the roller of a peristaltic pump and connected on the other side via a luer connection to a 20 cm-long Silastic tubing; (iii) T-connector; (iv) Silastic tubing with 0.2 µm-filter connected to a source of compressed air at 40 kPa; (v) glass microfermenter with unused capillaries sealed and equipped with a removable glass spatula; (vi) 3 cm-long Silastic effluent tubing connected with reducing connector to 80 cm-long microbore effluent tubing

connected with reducing connector to 3 cm long Silastic tubing; (vii) waste collection carboy capped with aluminium foil. (B) Microfermenters in a water bath. (C) Biofilm formed on the glass surfaces of microfermenter and spatula. (D) Theoretical dilution curve of a solute introduced at a concentration of 1 in the microfermenter at a time indicated by the arrow (illustrating a sudden dispersal event) and evacuated over time with the flow of fresh medium.

Fig. 3. Dispersal of *P. aeruginosa* PAO1 biofilms in response to NO and oxygen depletion. (A) One day-old biofilms grown in microfermenters were treated (arrow) with the NO donor SNP at 1 mM (white circles), 10 mM sodium nitrite (white diamonds) or 1 mM potassium ferricyanide (FCN, black circles), the SNP 'backbone' that does not release NO. To deplete the biofilm of oxygen, aeration was switched to nitrogen gas (white squares). To monitor dispersal events, OD_{600} of the biofilm effluent was measured. (B) After 2.5 h, biofilm cells remaining on the microfermenter spatula were resuspended in PBS and quantified by measuring the OD_{600}.

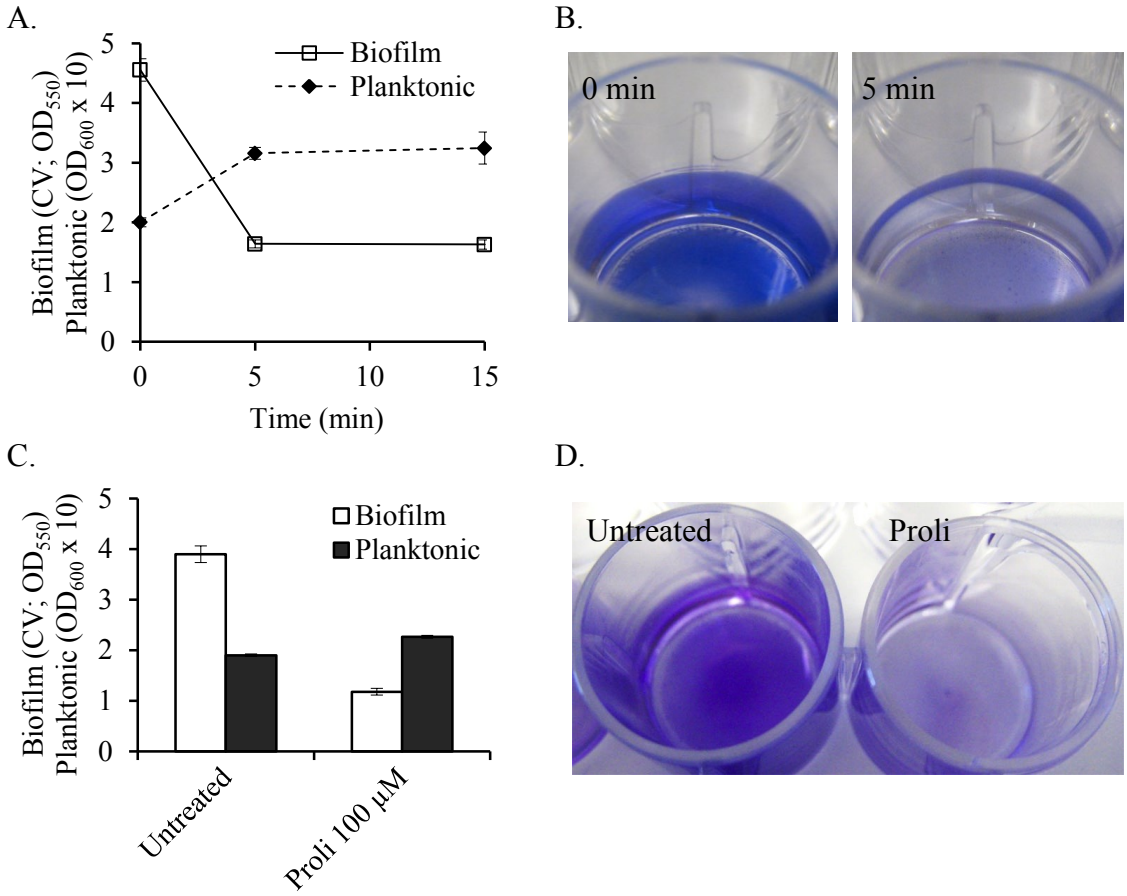


Fig. 1. Microtitre plate assay for biofilm dispersal. (A) Oxygen-depletion induced dispersal. *P. aeruginosa* PAO1 biofilms were grown for 6 h with shaking at 200 rpm, then at t = 0, the shaking speed was reduced to 60 rpm to reduce the oxygen tension. At various time points, the planktonic biomass was assessed by measuring the OD_{600} of the supernatant and the biofilm biomass by performing crystal violet staining. (B) Stained biofilms before and after 5 min of incubation at a reduced shaking speed. (C) NO-induced dispersal. *P. aeruginosa* biofilms were grown for 6 h with shaking at 200 rpm in the absence of NO donor. Then 10 µl of a Proli solution were added to the wells to make a final concentration of 100 µM and control wells were left untreated. The plates were incubated for a further 15 min before assessing dispersal by measuring the OD_{600} of the supernatant and performing crystal violet staining of the biofilm. (D) Stained biofilms untreated or treated with Proli for 15 min.

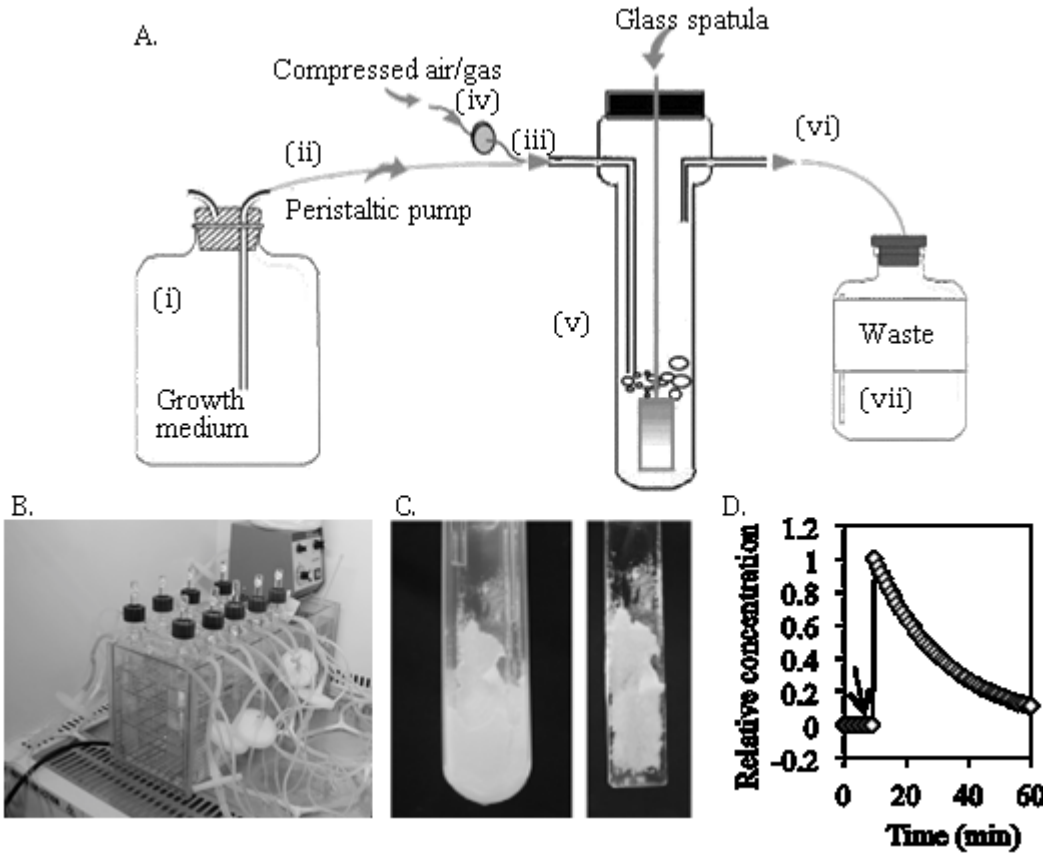
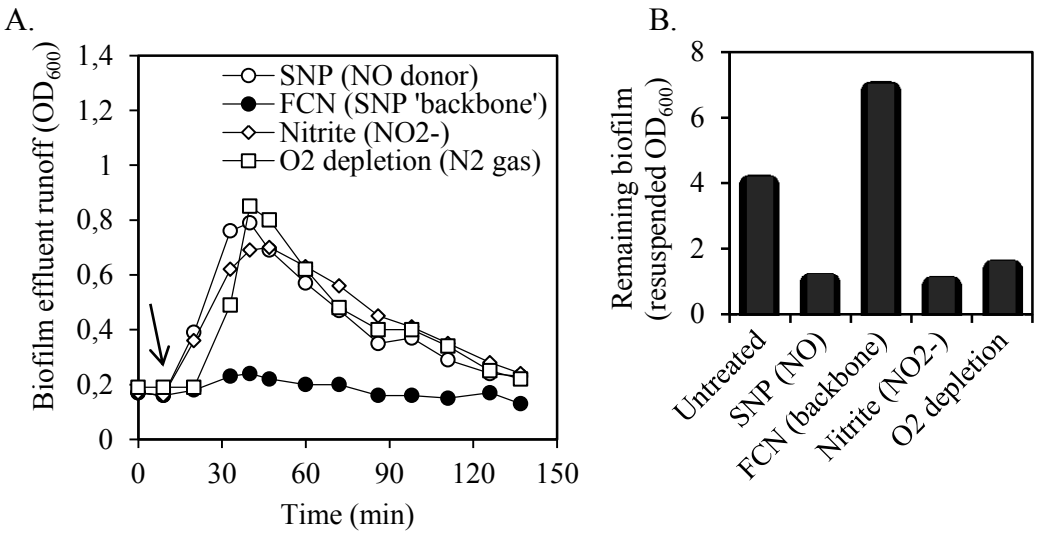


Fig. 2. (A) Illustration of the microfermentor flow-through culture set up: (i) 10-20 l fresh medium autoclavable carboy equipped with venting cap; (ii) 40 cm-long Silastic tubing (in a system where multiple microfermentors are used, this tubing may be diverted by using T-connectors) connected with male and female luer lock connectors to a 30 cm-long Tygon pump tubing which is pinched to the roller of a peristaltic pump and connected on the other side via a luer connection to a 20 cm-long Silastic tubing; (iii) T-connector; (iv) Silastic tubing with 0.2 µm-filter connected to a source of compressed air at 40 kPa; (v) glass microfermentor with unused capillaries sealed and equipped with a removable glass spatula; (vi) 5 cm-long Silastic effluent tubing connected with reducing connector to 80 cm-long microbore effluent tubing; (vii) waste collection carboy capped with aluminium foil. (B) Microfermentors in a water bath. (C) Biofilm formed on the glass surfaces of the microfermentor and spatula. (D) Theoretical dilution curve of a solute introduced at a concentration of 1 in the microfermentor at a time indicated by the arrow (illustrating a sudden dispersal event) and evacuated over time with the flow of fresh medium.



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