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Identification and characterization of regulatory networks controlling the expression and activity of the alternative sigma factor PvdS in *Pseudomonas aeruginosa*

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Abstract

Identification and characterization of regulatory networks controlling the expression and activity of the alternative sigma factor PvdS in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most dreaded opportunistic pathogens in the hospital setting and represents the first cause of morbidity and mortality in cystic fibrosis patients. *P. aeruginosa* is able to produce several virulence factors; among them, the siderophore pyoverdine plays a critical role in *P. aeruginosa* pathogenicity. This siderophore is not only the main iron uptake system of *P. aeruginosa* but also a signaling molecule which promotes the expression of other virulence factors (i.e. exotoxin A, extracellular protease PrpL). The key role of pyoverdine in *P. aeruginosa* infections makes this siderophore a promising target for the development of anti-virulence drugs.

The antimicrobial agents currently used in clinical practice induce a strong selective pressure which causes the development and spreading of resistant bacteria. An alternative antibacterial strategy, which should reduce the emergence of resistance, is the development of anti-virulence drugs able to disarm pathogens without inhibiting their growth.

In the first part of this PhD thesis the use of a drug repurposing approach to search for anti-pyoverdine compounds is described. This approach is based on the search for side activities in old drugs already used in humans. A library of 1,120 marketed drugs was screened with a purpose-generated reporter strain, leading to the identification of a promising hit compound, the antimycotic drug flucytosine, which strongly reduced pyoverdine production *in vitro* without affecting bacterial growth. The anti-virulence activity of flucytosine was also confirmed *in vivo* in a mouse model of lung infection. This work provided the first evidence that pyoverdine inhibition is a suitable strategy for anti-virulence therapy against *P. aeruginosa*, and that drug repurposing is a cheap and rapid approach to search for novel anti-virulence compounds.

The prominent role of pyoverdine in *P. aeruginosa* virulence emphasized the importance of pyoverdine regulation for full understand *P. aeruginosa* pathogenicity. Previous transcriptomic studies suggested that the Gac system, which regulates the switch between the planktonic and biofilm lifestyles, could also influence pyoverdine gene expression in *P. aeruginosa*, although different studies obtained opposite results. In the second part of this PhD thesis the role

of Gac system in the regulation of pyoverdine production has been investigated. It has been demonstrated that the Gac system and high intracellular levels of the intracellular signaling molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) coordinately promote pyoverdine production. In more detail, these two systems indirectly control pyoverdine genes by modulating the expression of the operons encoding the enzymes involved in the synthesis of the two aggregative exopolysaccharides Pel and Psl. The deletion of both the *pel* and *psl* operons caused a strong reduction in the production of pyoverdine and pyoverdine-dependent virulence factors, irrespective of the Gac activation state or the intracellular c-di-GMP levels, indicating that these exopolysaccharides plays an important role in the regulation of this siderophore. We also found that the effect of Pel and Psl on pyoverdine production depends on their ability to support the formation of planktonic aggregates, rather than on the exopolysaccharides *per se*. Indeed, we observed that the simulation of aggregation in a Pel- and Psl-independent manner is able to restore pyoverdine production in the exopolysaccharide-null mutant. These results indicate that pyoverdine, and consequently, pyoverdine-dependent virulence factors are also controlled by a new regulatory system activated by the cellular aggregation.

In conclusion, this thesis shows that cell aggregation is not only important in the first stages of biofilm formation, but also in the regulation of virulence in *P. aeruginosa*. The formation of cellular aggregates depends on the production of the Pel and Psl exopolysaccharides, which are regulated by the Gac system. Therefore, given the role of Gac also in the activation of pyoverdine-dependent virulence, this system could represent a potential target to inhibit simultaneously biofilm formation and the production of several virulence factors. The drug repurposing approach was thus used to search for Gac inhibitors. Although the initial screening led to identify three promising anti-Gac compounds, subsequent experiments revealed that these molecules promote, rather than inhibit, biofilm formation in *P. aeruginosa*, in a Gac-independent manner. Consequently, they were not further investigated as potential anti-virulence drugs.

Riassunto

Identificazione e caratterizzazione dei sistemi di regolazione deputati al controllo del fattore sigma alternativo PvdS in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa è un batterio gram-negativo che rappresenta una delle principali cause di infezione in ambito ospedaliero e soprattutto nei pazienti affetti da fibrosi cistica. La patogenicità di *P. aeruginosa* dipende dalla sua capacità di resistere a molteplici classi di farmaci e di produrre numerosi fattori di virulenza. Tra i fattori di virulenza prodotti da *P.aeruginosa,* il sideroforo pioverdina è uno dei più importanti, in quanto non solo rappresenta il principale sistema di acquisizione del ferro del batterio, ma agisce anche da molecola segnale che attiva l'espressione dei geni coinvolti nella sintesi della pioverdina stessa e di altri fattori di virulenza. Il ruolo chiave svolto dalla pioverdina nelle infezioni causate da *P. aeruginosa* rende la via biosintetica e regolativa di questa molecola bersagli promettenti per lo sviluppo di farmaci anti-virulenza. I farmaci anti-virulenza, che mirano a disarmare il batterio senza inibirne la crescita, rappresentano una potenziale alternativa ai comuni farmaci antibatterici i quali, avendo un'azione generalmente battericida o batteriostatica, esercitano una forte pressione selettiva che favorisce l'insorgenza e la diffusione di resistenze. Nella prima parte della presente tesi di dottorato è stata descritta la ricerca di farmaci anti-pioverdina attraverso l'utilizzo di un approccio noto come "*drug repurposing*"*.* Tale approccio consiste nella ricerca, in farmaci comunemente utilizzati in clinica, di attività diverse rispetto a quelle per cui ciascun farmaco viene utilizzato. A tale scopo è stato generato un ceppo ricombinante di *P. aeruginosa* che permettesse di monitorare in maniera rapida l'espressione dei geni della pioverdina, la produzione di pioverdina stessa e la crescita batterica. L'effetto anti-pioverdina è stato saggiato in una collezione di 1,120 composti ed è stato selezionato un farmaco antimicotico, la 5-fluorocitosina, in grado di causare una forte riduzione della produzione di pioverdina e dei fattori di virulenza pioverdina-dipendenti senza però avere effetti rilevanti sulla crescita batterica. La riduzione della virulenza di *P. aeruginosa,* causata dall'inibizione della sintesi della pioverdina mediata dall'azione della 5-fluorocitosina, è stato confermata anche *in vivo* in un modello murino di infezione polmonare. Questo studio ha dimostrato che la pioverdina è un bersaglio eccellente per un'eventuale terapia finalizzata a ridurre la virulenza di *P. aeruginosa* e che il *drug repurposing* rappresenta una strategia rapida ed efficace per la ricerca di farmaci anti-virulenza.

Dato il ruolo chiave della pioverdina nella virulenza di *P. aeruginosa*, è stato essenziale approfondire i meccanismi che regolano la produzione di tale sideroforo, al fine di poter comprendere a pieno la patogenicità di *P. aeruginosa*. A tale scopo sono stati condotti degli studi, descritti nella seconda parte della presente tesi di dottorato, mirati a chiarire il ruolo del sistema Gac nella regolazione della pioverdina. Il sistema Gac è un sistema a due componenti coinvolto principalmente nella regolazione dell'espressione dei geni responsabili della sintesi dei polisaccaridi Pel e Psl, che rappresentano una componente essenziale della matrice del biofilm. Tali esopolisaccaridi sono regolati a livello trascrizionale anche dal bis-(3'-5')-diguanosina monofosfato ciclico (c-di-GMP), un secondo messaggero intracellulare che svolge un ruolo chiave nella formazione del biofilm. Nel presente lavoro di tesi è stato dimostrato che lo stato di attivazione del sistema Gac ed elevati livelli intracellulari di c-di-GMP promuovono la produzione di pioverdina. Questi due sistemi di regolazione controllano la produzione di pioverdina indirettamente, attraverso la modulazione dell'espressione dei geni per gli esopolisaccaridi Pel e Psl. La generazione di un doppio mutante negli operoni *pel* e *psl* ha consentito di scoprire che questi operoni svolgono un ruolo essenziale nella regolazione della pioverdina, in quanto la mancata produzione di entrambi gli esopolisaccaridi determina una drastica riduzione della sintesi del sideroforo e dei fattori di virulenza da esso regolati. I risultati ottenuti in questo studio suggeriscono fortemente che l'effetto degli esopolisaccaridi sulla produzione di pioverdina sia dovuto alla loro capacità di indurre l'aggregazione cellulare in P. *aeruginosa*. Simulando l'aggregazione in maniera indipendente dagli esopolisaccaridi è stato infatti possibile ripristinare la produzione di pioverdina nel doppio mutante *pel psl* a livelli paragonabili a quelli del ceppo parentale. I risultati ottenuti indicano quindi la presenza di un nuovo sistema di regolazione, mediato dall'aggregazione cellulare, che influenza la produzione di pioverdina e dei fattori di virulenza pioverdina-dipendenti.

Nel presente lavoro di tesi è stato quindi dimostrato che l'aggregazione cellulare non solo svolge un ruolo chiave nelle prime fasi della formazione del biofilm, ma anche nella regolazione della virulenza. L'aggregazione è mediata principalmente dagli esopolisaccaridi, i quali sono regolati dal sistema Gac. Pertanto, questo sistema di regolazione rappresenta un potenziale bersaglio per inibire al contempo la produzione di alcuni fattori di virulenza e la formazione del biofilm. E' stato quindi utilizzato l'approccio del *drug repurposing* per identificare composti anti-Gac. Sebbene siano

stati individuati tre derivati flavinici in grado di ridurre significativamente l'attivazione del sistema Gac, tali farmaci hanno mostrato un forte effetto di promozione della formazione del biofilm in *P. aeruginosa*, del tutto indipendente dal sistema Gac, e pertanto le loro potenzialità come farmaci anti-virulenza sono compromesse.

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Introduction and aims

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1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a non-spore forming, Gram-negative, rod-shaped γ-Proteobacterium measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. This bacterium grows aerobically but is capable of using nitrate or arginine as a final electron acceptor in the absence of oxygen, allowing anaerobic growth (Vander Wauven *et al.*, 1984; Haas *et al.*, 1992). Because of its remarkable adaptability, *P. aeruginosa* can be found in diverse environments such as soil, water, plants and animals (Rahme *et al.*, 1995; Mahajan-Miklos *et al.*, 2000). *P. aeruginosa* synthesizes 2-aminoacetophenone (2-AA), producing a fruity, grape-like smell. This feature, along with the fact that this bacterium produces the blue colored soluble pigment pyocyanin, greatly helps in identifying an unknown colony as *P. aeruginosa*. The complete genome of the widely used laboratory strain *P. aeruginosa* PAO1, a wound isolate (Holloway, 1955), was sequenced and published in 2000 (Stover *et al.*, 2000). The genome has a high G+C content (66.6 %) and consists of 6.3 million base pairs. The presence of 5,570 predicted open reading frames reflects the genetic complexity of the *P. aeruginosa* genome underlying the capability of this bacterium to grow in different ecological niches (Stover *et al.*, 2000). Consistent with its larger genome size and environmental adaptability, *P. aeruginosa* was argued to contain the highest proportion of regulatory genes observed for a bacterial genome (Stover *et al.,* 2000).

2 P. aeruginosa pathogenicity

P. aeruginosa is typical opportunistic pathogen which can cause a wide range of infections in different tissues, including lung, eyes, ears, urinary tract and burns (Lyczak *et al*., 2000). Airway infections, caused by this pathogen, could be classified in two different types: acute and chronic infections (Arancibia *et al*., 2002). The best example of an acute respiratory nosocomial infection is the ventilator associated pneumonia (VAP). VAP is generally a consequence of the damage of the airway from mechanical ventilation which is followed by the acute pneumonia. The high mortality rate of VAP (34-48%) appears to be related to dysregulated host-pathogen interaction with an excessive host response to pneumonia (William *et al*., 2010). If the acute infection is not fully eradicated, *P. aeruginosa* can adapt to the lung environment and can cause chronic infection. Chronic infections are generally associated with cystic

fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (Doring *et al*., 2011). CF is a congenital disease affecting 1:2,500 newborns in the Caucasian population; it is due to loss-of-function mutations in the cystic fibrosis membrane regulator (CFTR), which result in a dehydrated and thickened airway surface liquid that impairs the normal clearance of the airway. Chronic *P. aeruginosa* infections represent the main cause of morbidity and mortality in CF patients (www.cftr2.org). Chronic *P. aeruginosa* lung infections are also associated with people who have COPD. This pathology is caused by chronic inflammation of lung tissues leading to the restriction of airway passage. The incidence of *P. aeruginosa* infection in patients with COPD ranges from 4 to 15% (William *et al*., 2010) and the mortality rate due to exacerbation is high (22-49%).

Therapeutic options to treat acute and chronic *P. aeruginosa* infections are limited due to the ability of *P. aeruginosa* to resist to many antimicrobial agents. *P. aeruginosa* has developed many different mechanisms of resistance, that can be classified as intrinsic or acquired (Moore and Flaws, 2011). The intrinsic mechanisms by which *P. aeruginosa* exerts its ability to resist to many antimicrobial agents are mainly three. The first is the low permeability of the outer membrane, which reduces the entrance of the antimicrobial agents into the cell. Second, *P. aeruginosa* expresses many efflux pumps, which are proteins able to eject a wide range of antibiotics out of the cell (Benz and Hancock, 1981; Schweizer, 2003). The third intrinsic mechanism is the production of the beta lactmase AmpC which is localized in the periplasm; it is expressed at low levels but can be induced by sub-inhibitory concentrations of certain beta-lactams (Juan *et al*., 2005). It has been demonstrated that *P. aeruginosa* could also acquire antibiotic resistance by taking up resistance genes from other pathogens and/or by mutation in the antibiotic targets or in the regulatory systems which reinforce the intrinsic resistance (Breidestein *et al*., 2011). At present, few new drugs are available to fight *P. aeruginosa* infections and there has been a return of old drugs. In particular, colistin (a polymixin family drug), which was originally fallen out due to its toxic side effects, is now routinely administered via inhalation in CF patients suffering recurrent infections whit multi drug resistant strains of *P. aeruginosa* (Falagas and Kasiakou, 2006).

2.1 Acute infection

Acute *P. aeruginosa* infections are characterized by the massive production of virulence factors and by a strong immune response. *P. aeruginosa* has the genetic

potential to produce an arsenal of virulence factors; some of them are present on the cell surface, such as type IV pili, flagella and type III secretion system (T3SS), while others are secreted, such as extracellular proteases, toxins, phospholipases (Fig 1), (Sadikot *et al*., 2005). The type IV pili and flagella are mainly involved in *P. aeruginosa* motility, but they also play a role in the pathogenesis by eliciting an inflammatory response (Kipnis *et al*., 2006). The type IV pili are the most important adhesins and the retractile properties of these structures make them responsible for the twitching motility, which enables bacterial cells to move on solid surfaces. They also facilitate the swarming motility (Mattick, 2002). Swarming is a special form of motility; it depends on flagella and type IV pili and allows bacteria to move across semi-solid surface. This type of motility is thought to be relevant to the movement of *P. aeruginosa* through mucus layers because the conditions that trigger swarming (intermediate viscosity and amino acids as a poor nitrogen source) exist in the lung (Overhage *et al*., 2008). *P. aeruginosa* is also able to swim in aqueous environments by using the flagellum which is a complex protein structure localized at the pole of the bacterium (Feldman *et al*., 1998). These motile surface appendances are responsible for bacterial motility and progression towards epithelial contact. The adhesion to epithelial cells mediated by pili and the flagellum represents the first step of colonization (Feldman *et al*., 1998). The contact with the host cells activates T3SS; it is a major determinant of virulence which allows to directly inject toxins into the host cell, through a syringe-like apparatus (Hauser *et al*., 1998). The toxins secreted by the *P. aeruginosa* T3SS are ExoU, ExoS, ExoT, ExoY. These toxins are variably expressed in different strains and all participate in the cytotoxicity. ExoT and ExoS have ADP-ribosyltrasferase activity which causes the destruction of the cytoskeleton of the host cell. ExoU has a phospholipase activity which destroys the eukaryotic cell membranes, while ExoY is an adenylate cyclase which causes the increase of the cAMP in the cytosol leading to increased epithelial barrier permeability in the lung (Hauser *et al*., 2009). Other virulence factors involved in the invasion and dissemination of *P. aeruginosa* are secreted through the type II secretion system (T2SS). They are the phospholipase C (PlpC), the exotoxin A (ToxA) and the proteases LasB, LasA, PrpL. The elastases LasA and LasB are not only involved in the damage of the respiratory epithelium through the destruction of the cellular junctions (tight junction) but they also induces an inflammatory response by the host (Azghani, 1996; Kon *et al*., 1999). The PlpC, also called the hemolytic phospholipase, targets the eukaryotic membrane phospholipids and participates to lung injury and inflammation (Konig *et al*., 1993). A

major role in *P. aeruginosa* virulence is played by the ToxA. This toxin inhibits eukaryotic protein synthesis by blocking the elongation factor 2 (EF-2), and induces cell death (Ochsner *et al*., 1996). ToxA and the protease PrpL are mainly expressed under iron-depleted conditions (Paragraph 2.1). The extracellular protease PrpL (or Protease IV) is an endoprotease which cleaves casein, lactoferrin, transferrin, elastin, and decorin, and was found to contribute to the ability of *P. aeruginosa* to persist in a rat chronic pulmonary infection model (Wilderman *et al*., 2001). Another important protease produced by *P. aeruginosa* during the infections is the alkaline protease AprA. This protease is a zinc metallo-protease released by the type I secretion system. It has been demonstrated that AprA degrades several components of the host immune system such as the cytokines INF-γ TNF-α and the protein C1q e C3 inhibiting the activation of the complement (Hong *et al*., 1992; Parmely *et al*., 1990). AprA also cleaves the iron-binding protein transferrin (Laarman *et al*., 2012). The proteolysis of transferrin causes the release of iron which could be taken up by the bacterium favoring its spreading (Kim *et al*., 2006). The damage caused by the above-mentioned virulence factors and the resulting strong inflammatory response make the acute infections by *P. aeruginosa* severe and often lethal (Gellantly *et al*., 2013)

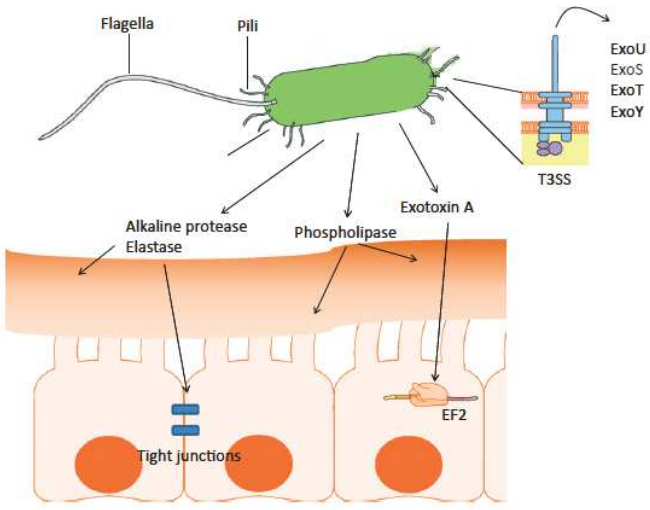

Fig 1. Virulence factors produced by *P. aeruginosa* during acute infections. Flagella, pili and T3SS are present on the cell surface. Pili and flagella represent the mains adhesions involved in the contact with the host epithelial cells. The contact activates the T3SS and consequently the injection of the cytotoxins (ExoU, ExoS, ExoT and ExoY) directly into the host cells . During the lung colonization *P. aeruginosa* also secretes several virulence factors such as the proteases, phospholipase C and exotoxin A (Modified from Gellantly and Hancock, 2013).

2.2 Chronic infection

CF, COPD or immune compromised patients are generally unable to completely eradicate the acute infections and it could evolves in chronic infection in which *P. aeruginosa* is completely adapt to the lung environment (Deretic *et al*., 1995). During the chronic infection *P. aeruginosa* grows and persists as a biofilm, which is a surface attached community of bacteria encased in a self-produced polymeric matrix (Bjarnsholt *et al*., 2009). The polymeric matrix consists of polysaccharides, nucleid acids, proteins and lipids and represents 50-90% of the biofilm volume. Biofilm formation is a multistep process. The first contact of bacterial cells with the surface is followed by a strong (irreversible) attachment. After the attachment bacteria grow as microcolonies and begin to produce the extracellular polymeric matrix. The last step relies on the maturation of the biofilm structure, characterized by the formation of mushroom shaped structures and of the fluid filled channels for the exchange of nutrients and waste products (Klausen *et al*., 2003). The microbial biofilm is a relevant problem in clinical setting, since it allows bacteria to persist in the human body and on medical devices such as catheters and endotracheal tubes. Bacteria growing as biofilm show enhanced tolerance to the host immune response and to the activity of antimicrobial agents. In particular, *P. aeruginosa* growing as a biofilm has a minimal inhibitory concentration (MIC) and a minimal bacterial concentration (MBC) for many antibiotics 100-1,000 fold higher than planktonic cells (Moskowitz *et al*., 2004). The increased tolerance of bacteria growing as biofilm likely depends on the limited penetration of the antimicrobial agents through the biofilm matrix and on the reduced metabolic activity of biofilm-forming bacterial cells (Walters *et al*., 2003; Chiang *et al*., 2013; Pamp *et al*., 2008). Many antibiotics target DNA replication, protein synthesis or cell wall biogenesis and are highly active on actively replicating planktonic bacteria; however, since the metabolic activity of bacteria growing as biofilm is much higher in the upper part of biofilm compared to the inner part, biofilm-forming bacterial cells present in the inner part of the biofilm are more tolerant to these classes of antimicrobial agents (Werner *et al*., 2004; Pump *et al*., 2008). The ability of *P. aeruginosa* growing as biofilm to persist on biotic and abiotic surfaces and to resist to antimicrobial agents makes chronic infections difficult to eradicate.

3 P. aeruginosa iron uptake: evidence of different strategies in different types of infections

Iron is an essential element in almost all living organisms. Although iron is the one of the most abundant elements on the earth, the concentration of biologically useful iron is generally extremely low. In aerobic inorganic environment, iron is mainly present in the oxidized ferric form Fe(III) which aggregates into insoluble oxy-hydroxide polymers and, consequently, it is not easily available to microorganisms. Once in the human host, pathogens such as *P. aeruginosa* are forced with the problem of iron acquisition. In human fluids iron is sequestered by proteins with high affinity for Fe(III), such as transferrin and lactoferrin, while in the cells iron is fastened in heme, iron-sulfur clusters and ferritins. The limited availability of iron is addressed by bacteria through the development of different systems able to actively acquire iron under depleted conditions (Ratledge and Dover 2000). *P. aeruginosa* has evolved different strategies to acquired iron, which are: (i) the production of low-molecular weight compounds which scavenge iron from various sources (siderophores); (ii) the uptake of siderophore and iron chelators which are not synthesized by *P. aeruginosa* itself, (iii) the uptake of heme molecule from host hemoproteins and (iv) the reduction and/or uptake of Fe(II) (Poole and McKay, 2003).

Except for the last one, these uptake systems require the presence of specific receptors on the outer membrane. These receptors need a complex of three proteins localized in the cytoplasmic membrane (TonB, ExbB and ExbD) called the "TonB complex". The TonB complex converts the transmembrane proton gradient in energy to internalize the iron carrier (siderophore or hemophore or hemoprotein) into the periplasm. Once in the periplasm the transport across the inner membrane to the cytoplasm is carried out by ABC permeases (Wandersman and Stojiljkovic, 2000).

The cytoplasmic concentration of iron is strictly regulated, and an overload of iron is toxic for bacterial cells because the reduced form Fe(II) activates the Fenton reaction generating reactive oxygen species which damage many biological macromolecules. In order to prevent an excessive iron uptake, in *P. aeruginosa*, as well as in many gram-negative bacteria, the systems involved in iron uptake are negatively regulated by the ferric uptake regulator Fur. Fur is a 15 KDa protein which has been proposed to be essential in *P. aeruginosa*, since deletion mutants in the *fur* gene were not obtained in this bacterium (Vasil and Ochsner, 1996). Fur acts as an iron intracellular sensor which represses directly or indirectly the expression of iron uptake genes under iron replete conditions. When Fur is loaded with Fe(II) it forms homodimers that bind the

regulatory elements of its target genes and prevent the binding of the RNA polymerase and hence transcription (Ochsner and Vasil, 1996). It has been proposed that *P. aeruginosa* could vary the iron uptake strategies according to the type of infection in order to best fulfill its needs without spending too much energy (Cornelis and Dingemans , 2013)

3.1 Siderophores: Pyoverdine and Pyochelin

Pyoverdine is a green fluorescent siderophore which represents the primary iron uptake system of *P. aeruginosa* (Meyer *et al*., 2000). This siderophore is composed by three parts: (i) a fluorescent dihydroxyquinoline chromophore, (ii) an acyl side chain bound to amino group of the chromophore and (iii) a variable peptide chain linked by an amino group to the C1 carboxyl group of the chromophore (Fig 2).

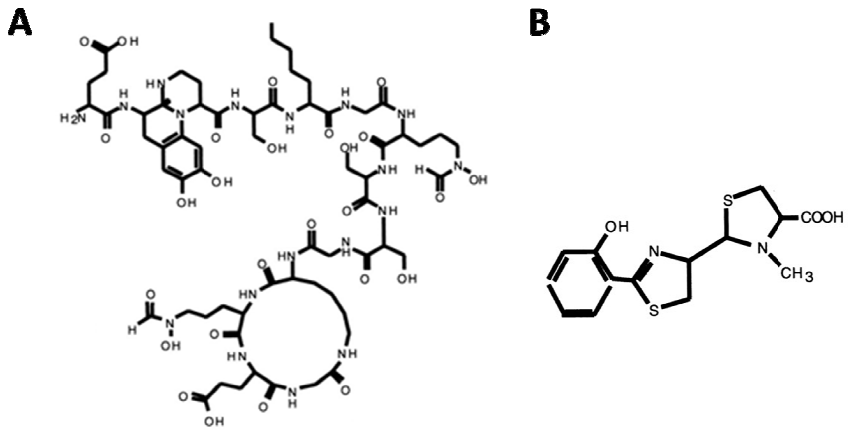

Fig 2. Structure of A) type I pyoverdine and B) pyochelin (Modified from Crosa and Walsh; 2002)

Each *P. aeruginosa* strain can produce one of three different pyoverdine types (type I, II or III; Bodilis *et al*., 2009). Pyoverdine is able to chelate Fe(III) with high affinity ($K_f \approx 10^{24} M^{-1}$) and a 1:1 stoichiometric ratio (Visca *et al*., 2007). The high affinity of pyoverdine for Fe(III) makes it capable to scavenge iron from host proteins such as transferrin and lactoferrin, allowing bacterial growth also in human serum (Ankenbauer *et al*., 1985; Sriyosachati and Cox, 1986).

The ability to acquire iron is not the only property of this siderophore; indeed, pyoverdine also acts as a signaling molecule involved in the regulation of virulence genes expression (Fig 3) (Lamont *et al*., 2002). The signaling mediated by this siderophore is called pyoverdine signaling and involves the TonB dependent receptor FpvA, the antisigma factor FpvR, which spans the cytoplasmic membrane through a single transmembrane helix, and the alternative sigma factors PvdS and FpvI. FpvA is

the pyoverdine outer membrane receptor; it has a beta barrel structure with a globular domain (plug domain) which occludes the interior of the barrel. Once pyoverdine is loaded with iron, it binds FpvA and induces a conformational change of the plug domain that enables the entry of the ferri-siderophore in the periplasm. The binding of ferri-pyoverdine to FpvA also promotes the interaction between FpvA and the periplasmic domain of the antisigma factor FpvR, and this interaction results in the transmission of a signal to the cytoplasmic domain of FpvR. This signal leads to the release of FpvI and PvdS from the FpvR antisigma, ultimately determining the activation of these two sigma factors. The alternative sigma factor FpvI drives the expression of the gene encoding the receptor FpvA, while PvdS controls the expression of almost 30 genes (Ochsner *et al*., 2002).

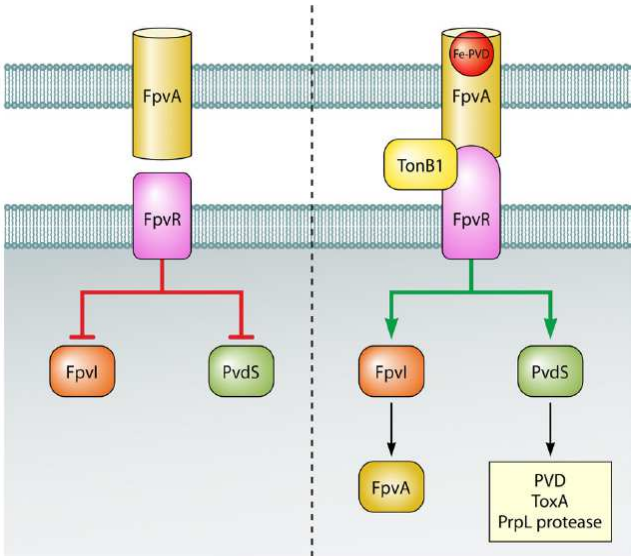

Fig 3. Schematic representation of the pyoverdine signaling cascade. In the absence of pyoverdine, FpvR represses the activity of the alternative sigma factors FpvI and PvdS. The binding of pyoverdine loaded with iron to its outer membrane receptor FpvA induces a signal through FpvR which leads to the release of the sigma factors and consequent activation of FpvI- and PvdS-dependent genes (Jimenez *et al*., 2012).

Among the genes regulated by PvdS there are genes encoding virulence factors which play a critical role in *P. aeruginosa* pathogenicity, such as pyoverdine, the protease PrpL and the exotoxin A (Lamont *et al*., 2002). The prominent role of pyoverdine in pathogenicity has been demonstrated in different mouse model of infections (Meyer *et al*., 1996; Takase *et al*., 2000). In particular, in the burned mouse model of infection used by Meyer and colleagues, mice were infected post-burned with 10^2 CFU (colony forming unit) of wild type strain or mutants unable to produce pyoverdine. The mortality of the mice infected with the wild type strain was almost 100% while the pyoverdine defective mutants appeared fully avirulent since no mice died. The

injection of pyoverdine into the infected mice restored the virulence of the pyoverdine defective mutants and increased the mortality of burned mice (Meyer *et al*., 1996).
The other experimental model in which was tested the virulence of pyoverdine defective mutant is the immunosuppressed mice. The wild type strain or the pyoverdine defective mutant (approximately 10^6) were inoculated intranasally in immunosuppressed mice. The virulence of pyoverdine defective mutant appeared to be attenuated because the mutant took somewhat longer to kill the mice then wild type strain (12 hours *versus* 48 hours post inoculum), (Takase *et al*., 2000). Given that pyoverdine is involved in the regulation of virulence factors important for the colonization and invasion of human host, it seems to be essential to cause acute infection (Cornelis and Dingemans, 2013). Interestingly, pyoverdine production is also important for biofilm formation which is a typical trait of chronic infection. It has been demonstrated that mutants which do not produce pyoverdine are unable to generate mature biofilms under iron limited conditions, but the addition of exogenous pyoverdine to the growth media restores the biofilm formation ability of pyoverdine defective mutants (Banin *et al*., 2005; Patriquin *et al*., 2008) (Fig 4). Although pyoverdine is involved in different biological processes, ranging from host invasion to biofilm formation, its production is extremely energy consuming for *P. aeruginosa*, given that its biogenesis occurs through non ribosomal peptide synthesis and requires the activity of many different enzymes (Visca *et al*. 2007).

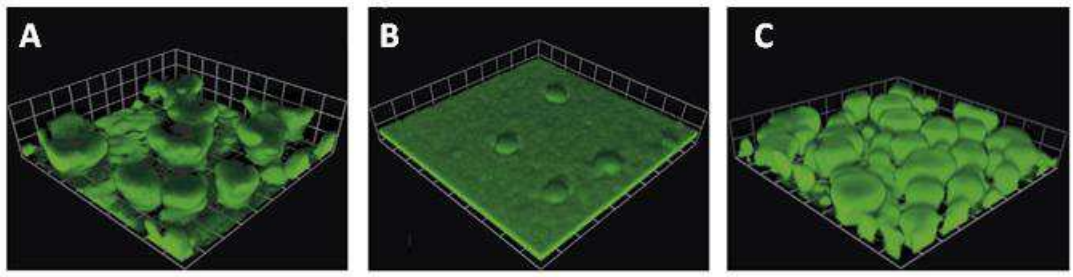

Fig 4. Biofilm development in flow cell of A) *P. aeruginosa* PAO1 (wild type), B) an isogenic mutant in the biosynthetic gene *pvdA* which is unable to produce pyoverdine, and C) the *pvdA* mutant in the presence of exogenous pyoverdine The image represent a 6 day biofilm on a glass surface in a flow of 1% of TSB (tryptic soy broth), (modified by Banin *et al*., 2005)

This could plausibly explain why PvdS activity and pyoverdine production are finely regulated by the pyoverdine signaling cascade (see above). Indeed, this regulatory network allows maximum siderophore production only when pyoverdine is effective in catching iron from the environment or from host proteins, given that PvdS activity is fully activated only when iron-loaded pyoverdine binds to the FpvA receptor.

In addition, *pvdS* expression is directly repressed at the transcriptional level by Fur-Fe(II) and, consequently, the production of pyoverdine is shut-off under iron replete conditions (Ochsner *et al*., 1996). More recently, other proteins have been described to positively influence *pvdS* expression, such as the LipA lipase and the transcriptional regulator CysB (Funken *et al*., 2011; Imperi *et al*., 2010): LipA is an extracellular lipolytic enzyme, and its absence causes a strong reduction in *pvdS* expression and pyoverdine production, although the mechanism is still unknown (Funken *et al*., 2011). CysB is a LysR-type transcriptional regulator which plays a central role in sulfur metabolism. It has been demonstrated that CysB binds to the promoter region of *pvdS* and positively controls its expression and, consequently, the production of PvdS-dependent virulence factors (Imperi *et al*., 2010).

The second siderophore produced by *P. aeruginosa* is pyochelin (Fig 2). Pyochelin is a condensation product of salicylate and two molecules of cysteine; it chelates iron with a 2:1 (pyochelin:iron) stoichiometry (Tseng *et al*., 2006). The affinity for iron of pyochelin ($k_f \approx 10^5 M^{-2}$) and the number of genes involved in its biosynthesis are lower than pyoverdine (Youard *et al*., 2011). The production of this siderophore is regulated by the cytoplasmic regulator PchR, which is a protein of the AraC/XylS family. These kind of regulators can act as transcriptional repressor or activator depending on the presence of specific effector molecules. In particular, the pyochelin biosynthetic genes are repressed by PchR in the absence of pyochelin, while they are induced when PchR is activated by pyochelin binding (Fig 5) (Michel *et al*., 2007). Thus, as in the case of pyoverdine, also pyochelin is only produced when it is effective in feeding the cell with iron. Moreover, pyochelin production is also subjected to the repression by Fur, which controls the expression of the genes involved in the synthesis (*pchDCBA*, *pchEFGHI*), regulation (*pchR*) and internalization (*fptA*) of this siderophore. Recently, it has been demonstrated that *P. aeruginosa* firstly produces pyochelin and then switches to pyoverdine when iron concentration becomes extremely low (Dumas *et al*., 2013). Given that the cost associated with the synthesis of pyochelin is much lower compared to that of pyoverdine, it has been proposed that this regulatory strategy could allow *P. aeruginosa* cells to save energy until the pyoverdine synthesis is absolutely required for growth (Dumas *et al*., 2013).

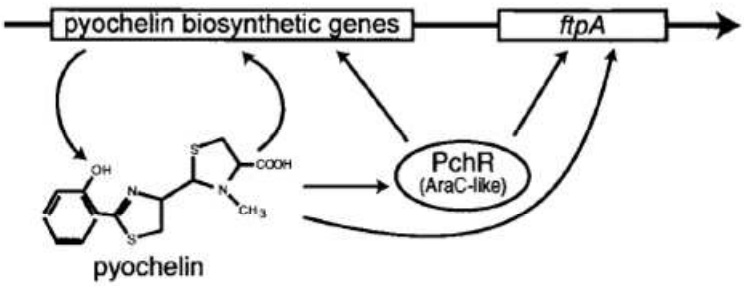

Fig 5. Schematic representation of the pyochelin genes regulation. In the presence of pyochelin, PchR triggers the transcription of the pyochelin biosynthetic genes and of the gene *ftpA*, encoding the outer membrane receptor for ferri-pyochelin (Modified from Crosa and Walsh, 2002)

3.2. Other iron uptake systems

Although endogenous siderophores, in particular pyoverdine, represent the main iron carriers of *P. aeruginosa*, this bacterium has evolved other strategies to acquire iron in different environments. During the infection, a possible source of iron is represented by the heme and hemoglobin (Wandersman and Stojiljkovic, 2000). Two different systems for heme uptake have been identified and characterized in *P. aeruginosa*, namely the Phu and Has systems. The Phu system consists of a TonB-dependent outer membrane receptor for heme (PhuR) which binds heme and transport it into the periplasm. Once in the periplasm, the periplasmic protein PhuS and the ATP-dependent permease system PhuTUV direct the transfer of heme into the cytoplasm (Ochsner *et al*., 2000). The other heme uptake system (Has) reminds the siderophore-dependent iron uptake, since this system is based on the activity of the haemophore HasA, an extracellular protein secreted by the bacterial cell that catches heme with high affinity and delivers it to its TonB-dependent receptor HasR (Letoffé *et al*., 1998; Takase *et al*., 2000). Once heme is in the cytoplasm, the heme oxygenase HemO degrades heme to form biliverdin, CO and Fe(II) (Barker *et al*., 2012).

During the polymicrobial infection, *P. aeruginosa* could be benefited from the ability to recognize and use siderophores produced by other species (xenosiderophores) or other exogenous iron chelators. For this reason, *P. aeruginosa* has the genetic potential to encode several TonB-dependent receptors for exogenous iron carriers, such as PfeA and PirA for the *E. coli* siderophore enterobactin (Dean and Poole, 1993), FoxB and FiuA for the uptake of ferioxamine and ferrichrome, respectively (Llamas *et al*., 2006), FecA for the Fe-citrate (Marshall *et al*., 2009), FemA for the utilization of mycobactin and carboxymycobactin (Llamas *et al*., 2008), ChtA for rhizobactin and

aerobactin (Cuiv *et al*., 2006) and FvbA for the uptake of vibriobactin (Elias *et al*., 2011).

The last iron uptake system used by *P. aeruginosa* is the Feo system, which internalizes Fe(II). Fe(II) is soluble and is mainly present under anaerobic conditions and/or in the presence of low pH (Andrews *et al*., 2003). The ferrous iron diffuses through the outer membrane through porins and is transported in the cytoplasm by FeoB. FeoB is a GTP-dependent transporter and it is associated with other two proteins of the inner membrane, FeoA and FeoC (Cartron *et al*., 2006). It has recently been proposed that the Feo system could play a role in iron uptake during lung chronic infections in CF patients, due to the ability of phenazines, which are secondary metabolites produced by *P. aeruginosa*, to reduce Fe(III) to Fe(II). Indeed, it has been demonstrated that phenazines and Fe(II) accumulate in the lung of CF patients during chronic *P. aeruginosa* infection (Hunter *et al*., 2012; Hunter *et al*., 2013)

4 The regulatory switch from acute to chronic infection

During chronic and acute infections *P. aeruginosa* expresses different sets of genes involved respectively in biofilm formation or in virulence factor production. The ability to modulate gene expression on the basis of the type of infection is due to the presence of intricate regulatory networks which can control, by repressing or activating, different sets of genes (Stover *et al*., 2000). Among the systems involved in the regulation of the switch between the planktonic and biofilm lifestyles, the most studied are the Gac system and the bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) intracellular signal.

4.1 Gac regulatory network

The Gac regulatory network (global activator of antibiotic and cyanide synthesis) is a two component system typically present in gamma proteobacteria (Fig 6). This system consists of the inner membrane sensor kinase GacS, the response regulator GacA, two small RNAs and the translational repressor RsmA (regulatory of secondary metabolism). In response to a still unknown signal, GacS phosphorylates and activates GacA, which in turn recognizes and binds the conserved Gac sequence ($TGTAAGN_6CTTACA$) present in the *rsmZ* and *rsmY* promoter regions, thereby promoting their transcription (Brencic *et al*., 2009). These small RNAs are the exclusive targets of the GacA regulator, and are part of an uncharacterized feedback

mechanism by which they inhibit their own transcription (Kay *et al*., 2006). Structural prediction of these small RNAs suggests the presence of multiple copies of a GGA motif in the single strand region of a steam loop; this motif is responsible for the binding of these sRNA to RsmA. RsmZ and/or RsmY binding to RsmA inhibits the translational repressor activity of this protein. RsmA is a member of the CsrA family of small translational regulatory proteins; it binds GGA motifs present within the 5' untranslated region of target mRNAs, occluding the ribosome binding site and consequently inhibiting translation (Lapouge *et al*., 2008). Additional regulators have been described to modulate the activity of the Gac system, namely the hybrid sensors RetS and LadS, which exert a negative and positive effect on *rsmY* and *rsmZ* transcription, respectively. While RetS (regulator of exopolysaccharides and T3SS) heterodimerizes with GacS, preventing its autophosphorylation and, consequently, GacA activation, LadS (lost adherence sensor) promotes the activation of GacA through a still unknown molecular mechanism (Ventre *et al*., 2006). The inhibitory effect of RetS on GacA activity is confirmed by the fact that *retS* mutants have a phenotype very similar to that of *rsmA* mutants (Goodman *et al*., 2004).

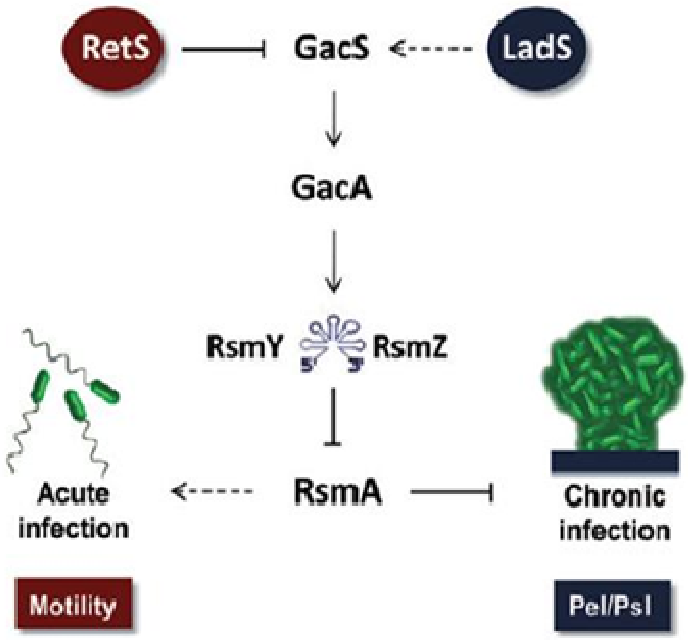

Fig 6. Schematic representation of the Gac system (modified from Moscoso *et al*., 2011).

The effect of *rsmA* deletion on *P. aeruginosa* physiology has been extensively characterized by several research groups through transcriptomic and phenotypic analyses. In particular, *rsmA* mutants show strongly increased biofilm formation,

reduced T3SS expression and cytotoxicity, and diminished twitching motility. This characteristic phenotype depends on the ability of RsmA to directly inhibit the translation of genes responsible in the biosynthesis of the exopolysaccharide Psl, and to indirectly promote the transcription of the genes involved in the production of the T3SS, type IV pili and flagella (Irie *et al*., 2010; Brencic and Lory 2009, Burrowes *et al*., 2006). Moreover transcriptomic analyses of *rsmA* mutants indicated that RsmA negatively influence the expression of the genes encoding the type VI secretion system and of the *pel* operon which encodes the enzymes involved in the synthesis of the exopolysaccaride Pel (Brencic and Lory 2009). Transcriptomic analyses also suggested that the Gac system influences the expression of several iron uptake genes, although different studies obtained opposite results (Brencic and Lory 2009; Burrowes *et al*., 2006). In one study, the deletion of *rsmA* in the *P. aeruginosa* reference strain PAO1 caused an increase in the transcription of some iron uptake genes (Burrowes *et al*., 2006), while in another study, performed on the *P. aeruginosa* strain PAK, the *rsmA* deletion resulted in a decrease in iron uptake gene expression (Brencic and Lory 2009). Additional studies are clearly required to clarify the role of the Gac system in the regulation of iron uptake in *P. aeruginosa*. Irrespective of its effect on iron uptake, the ability of the Gac system to coordinately control the expression of many virulence genes involved in the acute infection and those related to chronic infection makes the Gac system an important factor in *P. aeruginosa* pathogenicity, as it has been experimentally demonstrated in different models of infection. Indeed, deletion mutants in *gacA* showed a strongly reduced ability to cause infection in mice, plants, insects and nematodes (Rahme *et al*., 1995; Tan *et al*., 1999; Jander *et al*., 2000, Coleman *et al*., 2003). Accordingly it has been demonstrated that the deletion of *rsmA*, hence constitutive activation of Gac system, causes an increase in the ability of *P. aeruginosa* to persist in the mouse lung (Mulcahy *et al*., 2007). The enhanced persistence of the *P. aeruginosa rsmA* mutant could depend on the fact that in this mutant the production of the exopolysaccharides Pel and Psl is not repressed by RsmA, ultimately resulting in increased exopolysaccharide synthesis and biofilm formation compared to the wild type strain. Indeed, *in vitro* experiments have demonstrated that the deletion of *rsmA* causes a strong increase in the ability to form biofilms, while the deletion of the two small RNA genes *rsmY* and *rsmZ* or of *gacA* almost completely abolishes biofilm formation (Brencic *et al*., 2009).

Very recently, it has been proposed that the Gac system could also indirectly influence the transcription of the exopolysaccharide operons *pel* and *psl* and, therefore, biofilm

formation in *P. aeruginosa* by modulating the intracellular levels of the signaling molecule c-di-GMP. In particular it has been demonstrated that deletion of *retS*, which is the negative regulator of the activation state of Gac system, causes an increase of the intracellular level of c-di-GMP (Moscoso *et al*., 2011).

4.2 Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP)

The signaling molecule c-di-GMP has been identified in different bacteria and seems to take part in the control of the switch between the planktonic and biofilm lifestyles (Jenal *et al*., 2004). The intracellular levels of this molecule depend on the activity of two classes of enzymes which are the diguanilate cyclases (DGCs) and phospodiesterase (PDEs) (Fig 7).

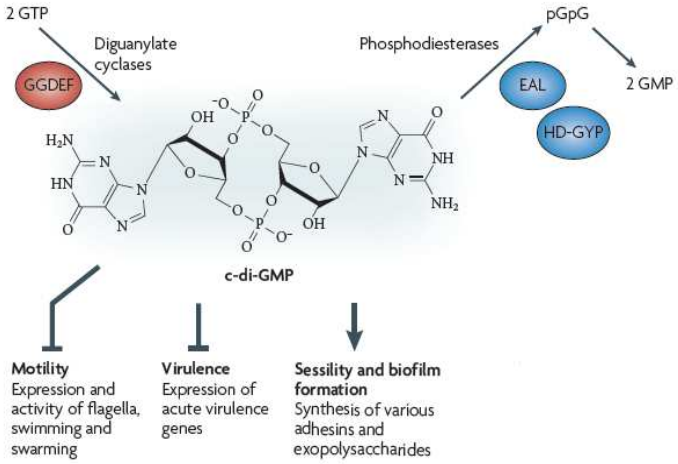

Fig 7. Structure and functions of c-di-GMP. The intracellular levels of c-di-GMP depend on the activity of diguanylate cyclases and phosphodiesterases. High levels of c-di-GMP promote biofilm lifestyle while inhibiting virulence gene expression and motility (Modified from Hengge, 2009)

The active DGC is a dimer of two subunits with a GGDEF domain, which is essential for the production of c-di-GMP from two molecules of GTP. The catalytic site is located at the interface between the two subunits. Each subunit binds one molecule of GTP, and point mutations in the GGDEF motif impair the catalytic activity (Malone *et al*., 2007). The PDE activity is associated with two different motifs: EAL and HD-GYP. The EAL-type PDE is a monomeric enzyme that linearizes c-di-GMP to 5'-pGpG which is then degraded by other cellular enzymes, while the HD-GYP proteins are able to directly degrade c-di-GMP to two molecules of GMP (Christen *et al*., 2005). Notably, some enzymes have both the domains EAL and GGDEF, although the function of these proteins remains unclear (Kulasakara *et al*., 2006). In *P. aeruginosa,* about 41 genes

have been predicted to encode proteins with DGC and/or PDE domains (Kulasakara *et al*., 2006). The high numbers of genes involved in the metabolism of c-di-GMP makes the signaling network mediated by this molecule difficult to characterize. One of the best characterized protein involved in the synthesis of c-di-GMP is the DGC WspR, belonging to the Wsp chemosensory system. The Wsp system is a multi component chemosensory system with homology to the well characterized chemotaxis pathway. This system consists of a putative methyl-accepting chemotaxis protein (WspA), an histidine kinsase (WspE), two adaptor proteins (WspB and WspD) predicted to link WspA with WspE, a methytransferase (WspC),a methyesterase (WspF) and a response regulator WspR (Fig 8). Likely in response to surface contact, WspA activates the histidine kinase WspE resulting in phosphorylation of WspR receiver domain and DGC activation (Guvener and Harwood, 2007). The methylesterase WspF is a negative regulator of Wsp system. The inactivation of WspF causes an increase in the intracellular levels of c-di-GMP which in turn promote cell aggregation and biofilm formation (Hickman *et al*., 2005). A typical feature of strains with high intracellular levels of c-di-GMP, as in the case of *wspF* mutant, is the small colony variant (SCV) phenotype (Hickman *et al*., 2005). These colonies appear wrinkly and it is due to the very high production of exopolysaccharides, which causes an increase in surface adherence and aggregation. The link between SCVs and intracellular levels of c-di-GMP has been demonstrated by different research groups through the analysis of the effect of mutations in the negative regulators of two different DGCs, WspR and YfiN (Hickman *et al*., 2005; Malone *et al*., 2010). The deletion of these inhibitors caused, in both cases, an increase in the intracellular level of c-di-GMP and the concomitant appearance of the SCV phenotype. The SCVs are characterized by enhanced levels of persistence and resistance to many different antimicrobial agents and represent a quite common morphotype of *P. aeruginosa* isolated from the sputum of chronically infected CF patient (von Götz *et al*., 2004, Drenkard and Ausubel, 2002; Starkey *et al*., 2009), suggesting that evolution towards increased c-di-GMP levels occurs during *P. aeruginosa* colonization of the CF lung.

As the Gac/Rsm system, c-di-GMP regulates biofilm formation and motility in opposite ways. High levels of c-di-GMP cause an increase in exopolysaccharide production and biofilm formation, but, at the same time, they result in the decreased expression of genes encoding the flagellum and the type IV pilus, involved in the swimming, swarming and twitching motilities (Choy *et al*., 2004; Hickman *et al*., 2005; Huang *et al*., 2003).

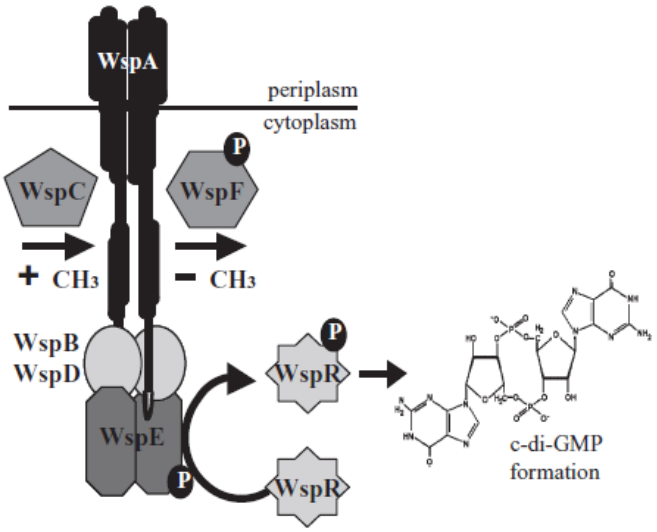

Fig 8. Schematic representation of Wsp system (modified from Guvener and Harwood, 2007)

In the last few years, an increasing number of systems involved in the regulation of intracellular levels of c-di-GMP has been discovered. However, the molecular mechanism by which c-di-GMP controls diverse cellular processes remains largely unknown. This is the case of the *psl* operon, encoding the enzymes for the synthesis one of the two aggregative exopolysaccharides produced during the biofilm formation; although transcriptomic analysis indicates that c-di-GMP positively controls Psl production, the underlying molecular mechanisms remain to be elucidated (Hickman *et al*., 2005). Recently, the mechanism by which c-di-GMP exerts its regulatory activity on the production of the Pel exopolysaccharide and flagella has been partly elucidated. The Pel exopolysaccharide is responsible for the pellicle formation and it is the only exopolysaccharide produced by *P. aeruginosa* strain PA14. It has been demonstrated that c-di-GMP controls the production of Pel at transcriptional and post transcriptional level (Baraquet *et al*., 2012). The expression of the operon encoding the enzymes involved in the synthesis of Pel is regulated by the transcriptional regulator FleQ. FleQ could act as a repressor or activator of *pel* operon expression depending on the intracellular levels of c-di-GMP. In the absence of c-di-GMP, two molecules of FleQ bind two different sites into the promoter region of *pel* operon and prevent RNA polymerase binding. The binding of c-di-GMP to FleQ induces a conformational change in the protein and in the DNA promoter region which promotes the binding of RNA polymerase and consequently transcription of the *pel* operon.
In addition to regulating *pel* genes for biofilm formation, FleQ has a second, better-known role as master regulator of flagella gene expression. In particular FleQ

activates the expression of genes involved in the regulation and assembly of the flagella export apparatus and basal body (Hickman and Harwood, 2008). FleQ contains an N-terminal domain, a central ATPase domain, and a C-terminal helix-turn-helix DNA-binding domain. In general, ATP hydrolysis, by the ATPase domain, provides energy for loading of the template strand of DNA into the active site of the RNA polymerase (Bose *et al*., 2008). Baraquet and colleagues have demonstrated that the binding of c-di-GMP inhibits FleQ ATPase activity and consequently causes a reduction in the FleQ ability to activate flagella gene expression (Baraquet and Harwood, 2013).

Thus, high intracellular levels of c-di-GMP promote the expression of *pel* operon and at the same time repress the expression of flagella genes. This signaling molecule could also be involved in the regulation of biofilm formation at post transcriptional level. Indeed, the production of Pel is also regulated by c-di-GMP during the generation of exopolysaccharide chain (Lee *et al*., 2007). It has been demonstrated that PelD, encoded by one of the genes within the *pel* operon, specifically binds c-di-GMP. PelD is predicted to be an inner membrane protein with four transmembrane helices and a large cytosolic region (Whitney *et al*., 2012). The expression of PelD is required for Pel polysaccharide production and mutations in the c-di-GMP binding site of PelD impair the ability of the *P. aeruginosa* strain PA14 to produce biofilm (Lee *et al*., 2007). Although the role of PelD in the synthesis of Pel is still unknown it is thought that it is involved in the transport of the saccharide polymer across the inner membrane (Franklin *et al*., 2011). The different types of regulation described above are emblematic examples of the versatility of c-di-GMP regulation; this molecule could be considered a transcriptional or post-transcriptional regulator depending on the role of its effector proteins (Hengge *et al*., 2009).

Recently another regulator has been proposed to play an important role in the modulation of the switch from the planktonic to the biofilm lifestyle: AmpR. This protein belongs to the LysR family of transcriptional regulators and was originally discovered as a regulator of the *ampC* gene (paragraph 1). AmpR seems to affect both the intracellular levels of c-di-GMP and the activation state of Gac system. In particular, AmpR negatively regulates RsmA activity by upregulating the expression of *ladS*, therefore promoting the planktonic lifestyle and acute infection (Balasubramanian *et al*., 2014). A proteomic study has demonstrated that AmpR also positively regulates the transcription of three different phosphodiesteres (PA4367,

PA4969 and PA4781) and, as a consequence, it causes a decrease in the intracellular c-di-GMP levels, again promoting the acute infection (Kumari *et al*., 2014).

5 Aims of the thesis

Pyoverdine plays a key role in *P. aeruginosa* pathogenicity. It is important for iron uptake and as a signaling molecule which promotes the expression of others virulence factors. The main aims of this PhD thesis are (1) to search for inhibitors of the synthesis or regulation of this siderophore and (2) to clarify the role of the Gac system in pyoverdine. Given the increasing emergence of *P. aeruginosa* resistant strains, mainly due to the strong selective pressure caused by the use of antibiotics, new therapeutic options are needed. A possible strategy is to disarm bacteria by inhibiting the regulatory networks involved in the regulation of virulence gene expression. To this aim, a drug repurposing approach has been used to search for inhibitors of pyoverdine production. Drug repurposing relies on the search for side activities in old drugs already approved for use in humans, and could represent a fast and cheap strategy to identify new uses for old drugs. The compounds identified with this strategy will then be tested *in vitro* and *in vivo* for their anti-virulence activity.
Given the crucial role of pyoverdine in the *P. aeruginosa* infection process, the characterization of the mechanisms which control the synthesis of this siderophore is essential to fully understand *P. aeruginosa* pathogenicity. Recently, two independent transcriptomic studies on the influence of the Gac system on pyoverdine production provided opposite results. To clarify the actual role of this regulatory network on pyoverdine gene regulation, deletion mutants in specific components of the Gac system has been generated and assessed for pyoverdine production and expression of pyoverdine genes. After having defined the role of the Gac system in pyoverdine regulation, the mechanism(s) by which this system controls pyoverdine production has been investigated, by verifying whether it acts through any of the already known regulators of pyoverdine production or through a still unexplored regulatory mechanism(s).

References

Andrews SC, Robinson AK, Rodríguez-Quiñones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev. **27**:215-37.

Ankenbauer R, Sriyosachati S, Cox CD (1985) Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infect Immun. **49**:132-40.

Arancibia F, Bauer TT, Ewig S, Mensa J, Gonzalez J, Niederman MS, Torres A (2002) Community-acquired pneumonia due to gram-negative bacteria and *Pseudomonas aeruginosa*: incidence, risk, and prognosis. Arch Intern Med. **162**:1849-58.

Azghani AO (1996) *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A Am J Respir Cell Mol Biol. **15**:132-40.

Balasubramanian D, Kumari H, Jaric M, Fernandez M, Turner KH, Dove SL, Narasimhan G, Lory S, Mathee K (2014) Deep sequencing analyses expands the *Pseudomonas aeruginosa* AmpR regulon to include small RNA-mediated regulation of iron acquisition, heat shock and oxidative stress response. Nucleic Acids Res. **42**:979-98.

Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci U S A. **102**:11076-81.

Baraquet C, Murakami K, Parsek MR, Harwood CS (2012) The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the pel operon promoter in response to c-di-GMP. NucleicAcids Res. **40**:7207-18.

Baraquet C, Harwood CS (2013) Cyclic diguanosine monophosphate represses bacterial flagella synthesis by interacting with the Walker A motif of the enhancer-binding protein FleQ. Proc Natl Acad Sci U S A. **110**:18478-83.

Barker KD, Barkovits K, Wilks A (2012) Metabolic flux of extracellular heme uptake in *Pseudomonas aeruginosa* is driven by the iron-regulated heme oxygenase (HemO). J Biol Chem. **287**:18342-50

Benz R, Hancock RE (1981) Properties of the large ion-permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. Biochim Biophys Acta. **646**:298-308.

Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N (2009) *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol. **44**:547-58.

Bodilis J, Ghysels B, Osayande J, Matthijs S, Pirnay JP, Denayer S, De Vos D, Cornelis P (2009) Distribution and evolution of ferripyoverdine receptors in *Pseudomonas aeruginosa*. Environ Microbiol. **11**:2123-35.

Bose D, Joly N, Pape T, Rappas M, Schumacher J, Buck M, Zhang X (2008) Dissecting the ATP hydrolysis pathway of bacterial enhancer-binding proteins. Biochem Soc Trans. **36**:83-8.

Breidenstein EB, de la Fuente-Núñez C, Hancock RE (2011) *Pseudomonas aeruginosa*: allroads lead to resistance. Trends Microbiol. **19**:419-26.

Brencic A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, Lory S (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. Mol Microbiol. **73**:434-45.

Brencic A, Lory S (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. Mol Microbiol.**72**:612-32.

Burrowes E, Baysse C, Adams C, O'Gara F (2006) Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. Microbiology. **152**:405-18.

Cartron ML, Maddocks S, Gillingham P, Craven CJ, Andrews SC (2006) Feo--transport of ferrous iron into bacteria. Biometals. **19**:143-57.

Chiang WC, Nilsson M, Jensen PØ, Høiby N, Nielsen TE, Givskov M,Tolker-Nielsen T (2013) Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother. **57**:2352-61.

Choy WK, Zhou L, Syn CK, Zhang LH, Swarup S (2004) MorA defines a new class of regulators affecting flagellar development and biofilm formation in diverse *Pseudomonas* species. J Bacteriol. **186**:7221-8.

Christen M, Christen B, Folcher M, Schauerte A, Jenal U (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allostericcontrol by GTP. J Biol Chem. **280**:30829-37.

Coleman FT, Mueschenborn S, Meluleni G, Ray C, Carey VJ, Vargas SO, Cannon CL,Ausubel FM, Pier GB (2003) Hypersusceptibility of cystic fibrosis mice to chronic *Pseudomonas aeruginosa* oropharyngeal colonization and lung infection. ProcNatlAcadSci U S A. **100**:1949-54.

Cornelis P, Dingemans J (2013) *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. Front Cell Infect Microbiol. **3**:75.

Crosa JH, Walsh CT (2002) Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev. **66**:223-49.

Cuív PO, Clarke P, O'Connell M (2006) Identification and characterization of an iron-regulated gene, chtA, required for the utilization of the xenosiderophores aerobactin, rhizobactin 1021 and schizokinen by *Pseudomonas aeruginosa*. Microbiology. **152**:945-54

Dean CR, Poole K (1993) Cloning and characterization of the ferric enterobactin receptor gene (pfeA) of *Pseudomonas aeruginosa*. J Bacteriol. **175**:317-24.

Deretic V, Schurr MJ, Yu H (1995) *Pseudomonas aeruginosa*, mucoidy and the chronic infection phenotype in cystic fibrosis. Trends Microbiol. **3**:351-6.

Döring G, Parameswaran IG, Murphy TF (2011) Differential adaptation of microbialpathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease. FEMS Microbiol Rev. **35**:124-46.

Drenkard E, Ausubel FM (2002) Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature. **416**:740-3.

Dumas Z, Ross-Gillespie A, Kümmerli R (2013) Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. Proc BiolSci. **280**:20131055

Elias S, Degtyar E, Banin E (2011) FvbA is required for vibriobactin utilization in *Pseudomonas aeruginosa*. Microbiology. **157**:2172-80.

Feldman M, Bryan R, Rajan S, Scheffler L, Brunnert S, Tang H, Prince A (1998) Roleof flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. InfectImmun. **66**:43-51.

Falagas ME, Kasiakou SK (2006) Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. Crit Care. **10**:R27.

Franklin MJ, Nivens DE, Weadge JT, Howell PL (2011) Biosynthesis of the *Pseudomonas aeruginosa* Extracellular Polysaccharides, Alginate, Pel, and Psl. Front Microbiol. **2**:167.

Funken H, Knapp A, Vasil ML, Wilhelm S, Jaeger KE, Rosenau F (2011) The lipase LipA (PA2862) but not LipC (PA4813) from *Pseudomonas aeruginosa* influences regulation of pyoverdine production and expression of the sigma factor PvdS. J Bacteriol. **193**:5858-60.

Gellatly SL, Hancock RE (2013) *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis. **67**:159-73.

Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. Dev Cell. **7**:745-54.

Güvener ZT, Harwood CS (2007) Subcellular location characteristics of the Pseudomonas aeruginosa GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. Mol Microbiol. **66**:1459-73.

Haas D, Gamper M & Zimmermann A (1992) Anaerobic control in *Pseudomons aeruginosa*. (Galli E, Silver S & Witholt B). 177-187. American Society for Microbiology, Washington, D.C.

Hauser AR, Fleiszig S, Kang PJ, Mostov K, Engel JN (1998) Defects in type III secretion correlate with internalization of *Pseudomonas aeruginosa* by epithelial cells. Infect Immun.**66**:1413-20.

Hauser AR (2009) The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat Rev Microbiol. **7**:654-65.

Hickman JW, Tifrea DF, Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A. **102**:14422-7.

Hickman JW, Harwood CS (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. Mol Microbiol. **69**:376-89.

Holloway BW (1955) Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* **13**: 572-581

Hong, Y. Q., and B. Ghebrehiwet (1992) Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin. Immunol. Immunopathol. **62**: 133–138.

Huang B, Whitchurch CB, Mattick JS (2003) FimX, a multidomain protein connecting environmental signals to twitching motility in *Pseudomonas aeruginosa*. J Bacteriol. **185**:7068-76.

Hunter RC, Klepac-Ceraj V, Lorenzi MM, Grotzinger H, Martin TR, Newman DK (2012) Phenazine content in the cystic fibrosis respiratory tract negatively correlates with lung function and microbial complexity. Am J Respir Cell Mol Biol. **47**:738-45.

Hunter RC, Asfour F, Dingemans J, Osuna BL, Samad T, Malfroot A, Cornelis P, Newman DK (2013) Ferrous iron is a significant component of bioavailable iron in cystic fibrosis airways. MBio. **4**: e00557-13.

Kay E, Humair B, Dénervaud V, Riedel K, Spahr S, Eberl L, Valverde C, Haas D (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. J Bacteriol. **188**:6026-33

Kim SJ, Park RY, Kang SM, Choi MH, Kim CM, Shin SH (2006) *Pseudomonas aeruginosa* alkaline protease can facilitate siderophore-mediated iron-uptake via the proteolytic cleavage of transferrins. Biol Pharm Bull. **29**:2295-300.

Kipnis E, Sawa T, Wiener-Kronish J (2006) Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Med Mal Infect. **36**:78-91.

Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S,Tolker-Nielsen T (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagellaand type IV pili mutants. MolMicrobiol. **48**:1511-24

Kon Y, Tsukada H, Hasegawa T, Igarashi K, Wada K, Suzuki E, Arakawa M, Gejyo F (1999) The role of *Pseudomonas aeruginosa* elastase as a potent inflammatory factor ina rat air pouch inflammation model. FEMS Immunol Med Microbiol.**25**:313-21.

König B, Pedersen SS, König W (1993) Effect of *Pseudomonas aeruginosa* alginate on Escherichia coli- and Staphylococcus aureus-induced inflammatory mediator releasefrom human cells. Int Arch Allergy Immunol. **100**:144-50.

Kulasakara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S, Lee DG, Neely AN, Hyodo M, Hayakawa Y, Ausubel FM, Lory S (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proc Natl Acad Sci U S A. **103**:2839-44.

Kumari H, Murugapiran SK, Balasubramanian D, Schneper L, Merighi M, Sarracino D, Lory S, Mathee K (2014) LTQ-XL mass spectrometry proteome analysis expands the *Pseudomonas aeruginosa* AmpR regulon to include cyclic di-GMP phosphodiesterases and phosphoproteins, and identifies novel open reading frames. J Proteomics. **96**:328-42.

Imperi F, Tiburzi F, Fimia GM, Visca P (2010) Transcriptional control of the *pvdS* iron starvation sigma factor gene by the master regulator of sulfur metabolism CysB in *Pseudomonas aeruginosa*. Environ Microbiol. **12**:1630-42.

Irie Y, Starkey M, Edwards AN, Wozniak DJ, Romeo T, Parsek MR (2010) *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. Mol Microbiol. **78**:158-72.

Jander G, Rahme LG, Ausubel FM (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J Bacteriol. **182**:3843-5.

Jenal U (2004) Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? Curr Opin Microbiol. **7**:185-91.

Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ (2012) The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev. **76**:46-65

Juan C, Maciá MD, Gutiérrez O, Vidal C, Pérez JL, Oliver A (2005) Molecularmechanisms of beta-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. Antimicrob Agents Chemother. **49**:4733-8.

Laarman AJ, Bardoel BW, Ruyken M, Fernie J, Milder FJ, van Strijp JA, Rooijakkers SH (2012) *Pseudomonas aeruginosa* alkaline protease blocks complement activation via the classical and lectin pathways. J Immunol. **188**:386-93.

Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A. **99**:7072-7.

Lapouge K, Schubert M, Allain FH, Haas D (2008) Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. Mol Microbiol. **67**:241-53.

Lee VT, Matewish JM, Kessler JL, Hyodo M, Hayakawa Y, Lory S (2007) A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Mol Microbiol. **65**:1474-84.

Létoffé S, Redeker V, Wandersman C (1998) Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. Mol Microbiol. **28**:1223-34.

Llamas MA, Sparrius M, Kloet R, Jiménez CR, Vandenbroucke-Grauls C, Bitter W (2006) The heterologous siderophores ferrioxamine B and ferrichrome activate signaling pathways in *Pseudomonas aeruginosa*. J Bacteriol. **188**:1882-91.

Llamas MA, Mooij MJ, Sparrius M, Vandenbroucke-Grauls CM, Ratledge C, Bitter W (2008) Characterization of five novel *Pseudomonas aeruginosa* cell-surface signalling systems. Mol Microbiol. **67**:458-72.

Lyczak JB, Cannon CL, Pier GB (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. **2**:1051-60.

Mahajan-Miklos S, Rahme LG, Ausubel FM (2000) Elucidating the molecular mechanisms of bacterial virulence using non-mammalian hosts. Mol Microbiol. **37**:981-8.

Malone JG, Williams R, Christen M, Jenal U, Spiers AJ, Rainey PB (2007) The structure-function relationship of WspR, a *Pseudomonas fluorescens* response regulator with a GGDEF output domain. Microbiology. **153**:980-94.

Malone JG, Jaeger T, Spangler C, Ritz D, Spang A, Arrieumerlou C, Kaever V, Landmann R, Jenal U (2010) YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in Pseudomonas aeruginosa. PLoS Pathog. **6**:e1000804

Marshall B, Stintzi A, Gilmour C, Meyer JM, Poole K (2009) Citrate-mediated iron uptake in *Pseudomonas aeruginosa*: involvement of the citrate-inducible FecA receptor and the FeoB ferrous iron transporter. Microbiology. **155**:305-15.

Mattick JS. Type IV pili and twitching motility (2002) Annu Rev Microbiol.**56**:289-314.

Meyer JM, Neely A, Stintzi A, Georges C, Holder IA (1996) Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infect Immun. **64**:518-23.

Meyer JM (2000) Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. Arch Microbiol. **174**:135-42.

Michel L, Bachelard A, Reimmann C (2007) Ferripyochelin uptake genes are involved in pyochelin-mediated signalling in *Pseudomonas aeruginosa*. Microbiology. **153**:1508-18.

Moore NM, Flaws ML (2011) Treatment strategies and recommendations for *Pseudomonas aeruginosa* infections. Clin Lab Sci. **24**:52-6.

Moscoso JA, 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.

Moskowitz SM, Foster JM, Emerson J, Burns JL (2004) Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis. J Clin Microbiol. **42**:1915-22.

Mulcahy H, O'Callaghan J, O'Grady EP, Maciá MD, Borrell N, Gómez C, Casey PG, Hill C, Adams C, Gahan CG, Oliver A, O'Gara F (2008) *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization,virulence, persistence, and pulmonary inflammation. Infect Immun. **76**:632-8.

Ochsner UA, Johnson Z, Lamont IL, Cunliffe HE, Vasil ML (1996) Exotoxin A productionin *Pseudomonas aeruginosa* requires the iron-regulated pvdS gene encoding an alternative sigma factor. MolMicrobiol. **21**:1019-28.

Ochsner UA, Vasil ML (1996) Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc Natl Acad Sci U S A.**93**:4409-14.

Ochsner UA, Johnson Z, Vasil ML (2000) Genetics and regulation of two distinct haem-uptake systems, phu and has, in *Pseudomonas aeruginosa*. Microbiology. **146**:185-98.

Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML (2002) GeneChip expression analysis of the iron starvation response in Pseudomonas aeruginosa: identification of novel pyoverdine biosynthesis genes. Mol Microbiol. **45**:1277-87.

Overhage J, Bains M, Brazas MD, Hancock RE (2008) Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J Bacteriol. **190**:2671-9.

Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T (2008) Tolerance to theantimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked tometabolically active cells, and depends on the pmr and mexAB-oprM genes. MolMicrobiol. **68**:223-40.

Parmely, M., A. Gale, M. Clabaugh, R. Horvat, and W. W. Zhou (1990) Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. Infect. Immun. **58**: 3009–3014.

Patriquin GM, Banin E, Gilmour C, Tuchman R, Greenberg EP, Poole K (2008)Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. J Bacteriol. **190**:662-71.

Poole K, McKay GA (2003) Iron acquisition and its control in *Pseudomonas aeruginosa*: many roads lead to Rome. Front Biosci. **8**:d661-86.

Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM (1995) Commonvirulence factors for bacterial pathogenicity in plants and animals. Science.**268**:1899-902.

Ratledge C, Dover LG (2000) Iron metabolism in pathogenic bacteria. Annu Rev Microbiol. **54**:881-941.

Takase H, Nitanai H, Hoshino K, Otani T (2000) Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. Infect Immun. **68**:1834-9.

Takase H, Nitanai H, Hoshino K, Otani T (2000) Requirement of the *Pseudomonas aeruginosa* tonB gene for high-affinity iron acquisition and infection. Infect Immun. **68**:4498-504.

Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM (1999) *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. ProcNatlAcadSci U S A. **96**:2408-13

Tseng CF, Burger A, Mislin GL, Schalk IJ, Yu SS, Chan SI, Abdallah MA (2006) Bacterial siderophores: the solution stoichiometry and coordination of the Fe(III) complexes of pyochelin and related compounds. J Biol Inorg Chem. **11**:419-32.

Sadikot RT, Blackwell TS, Christman JW, Prince AS (2005) Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. Am J RespirCrit Care Med. **171**:1209-23.

Schweizer HP (2003) Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet Mol Res.**2**:48-62.

Sriyosachati S, Cox CD (1986) Siderophore-mediated iron acquisition from transferring by *Pseudomonas aeruginosa*. Infect Immun. **52**:885-91.

Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A, Palacios S, Manoil C, Kirisits MJ, Starner TD, Wozniak DJ, Harwood CS, Parsek MR (2009) *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. J Bacteriol. **191**:3492-503.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature. **406**:959-64.

Vander Wauven C, Piérard A, Kley-Raymann M, Haas D (1984) *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. J Bacteriol. **160**:928-34.

Ventre I, Goodman AL, Vallet-Gely I, Vasseur P, Soscia C, Molin S, Bleves S,Lazdunski A, Lory S, Filloux A (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. Proc Natl Acad Sci U S A. **103**:171-6.

Visca P, Imperi F, Lamont IL (2007) Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. **15**:22-30.

von Götz F, Häussler S, Jordan D, Saravanamuthu SS, Wehmhöner D, Strüssmann A, Lauber J, Attree I, Buer J, Tümmler B, Steinmetz I (2004) Expression analysis of a highly adherent and cytotoxic small colony variant of *Pseudomonas aeruginosa* isolated from a lung of a patient with cystic fibrosis. J Bacteriol.**186**:3837-47.

Wandersman C, Stojiljkovic I (2000) Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. Curr Opin Microbiol. **3**:215-20.

Walters MC 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions ofantibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin.Antimicrob Agents Chemother. **47**:317-23.

Werner E, Roe F, Bugnicourt A, Franklin MJ, Heydorn A, Molin S, Pitts B,Stewart PS (2004) Stratified growth in *Pseudomonas aeruginosa* biofilms. Appl EnvironMicrobiol. **70**:6188-96.

White PM (1971) *Pseudomonas aeruginosa* in a skin hospital. *Br. J. Dermatol.* **85**: 412-417.

Whitney JC, Colvin KM, Marmont LS, Robinson H, Parsek MR, Howell PL (2012) Structure of the cytoplasmic region of PelD, a degenerate diguanylate cyclase receptor that regulates exopolysaccharide production in *Pseudomonas aeruginosa*. J Biol Chem. **287**:23582-93.

Wilderman PJ, Vasil AI, Johnson Z, Wilson MJ, Cunliffe HE, Lamont IL, Vasil ML (2001) Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. Infect Immun. **69**:5385-94.

Williams BJ, Dehnbostel J, Blackwell TS (2010) *Pseudomonas aeruginosa*: host defence in lung diseases. Respirology. **15**:1037-56.

Youard ZA, Wenner N, Reimmann C (2011) Iron acquisition with the natural siderophore enantiomers pyochelin and enantio-pyochelin in *Pseudomonas* species. Biometals. **24**:513-22.

Chapter 2

Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity

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Chapter 2
Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity

Traditional antibiotics used for the treatment of infectious diseases either kill bacteria or inhibit their growth. These type of antimicrobial agents induce a strong selective pressure resulting in the emergence and spreading of multidrug resistance bacteria. An alternative strategy to reduce the emergence of resistance is the development of anti-virulence drugs, which should disarm pathogens rather than kill them (Rasko and Sperandio , 2010). Many bacteria have evolved numerous regulatory networks controlling the expression of different virulence factors in response to cellular and environmental signals. *P. aeruginosa*, which is renowned for its resistance, is also a paradigmatic example of complex regulation of virulence factor production (Lee *et al*., 2006). A smart strategy to disarm this pathogen could be to hit one of the regulatory networks controlling the expression of several virulence genes. One of the best characterized regulatory system controlling the expression the major *P. aeruginosa* virulence factors is the pyoverdine signaling (Chapter 1). Given the relevant role of pyoverdine in *P. aeruginosa* virulence gene expression, we hypothesized that it could represent a promising target to disarm this bacterium. In the present work, we searched for pyoverdine inhibitors by using a "drug repurposing" approach. This strategy relies on the search for side activities in drugs already approved for use in humans. The advantages of this strategy is the significant reduction of the time and cost generally associated with the standard drug discovery processes (Wermuth *et al*., 2006). The drug repurposing approach allowed us to identify a promising anti-virulence activity in the antimycotic drug flucytosine. The promising results obtained with flucytosine, both *in vitro* and *in vivo*, demonstrate not only that pyoverdine is a good target to disarm *P. aeruginosa*, but also that drug repurposing is a rapid and cheap strategy to identify anti-virulence proprieties in old drugs.

Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity

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Although antibiotic resistance represents a public health emergency, the pipeline of new antibiotics is running dry. Repurposing of old drugs for new clinical applications is an attractive strategy for drug development. We used the bacterial pathogen *Pseudomonas aeruginosa* as a target for the screening of antivirulence activity among marketed drugs. We found that the antimycotic agent flucytosine inhibits the expression of the iron-starvation σ-factor PvdS, thereby repressing the production of major *P. aeruginosa* virulence factors, namely pyoverdine, PrpL protease, and exotoxin A. Flucytosine administration at clinically meaningful dosing regimens suppressed *P. aeruginosa* pathogenicity in a mouse model of lung infection. The in vitro and in vivo activity of flucytosine against *P. aeruginosa,* combined with its desirable pharmacological properties, paves the way for clinical trials on the anti-*P. aeruginosa* efficacy of flucytosine in humans.

antivirulence drug | cystic fibrosis | drug repositioning | iron uptake | selective optimization of side activities (SOSA) approach

Only 70 y after the introduction of antibiotics in the clinical practice, the development and spread of resistance among pathogenic bacteria are limiting the therapeutic efficacy of these magic bullets. Inhibition of bacterial virulence, rather than growth, is an alternative approach to the development of new antimicrobials. Antivirulence drugs disarm rather than kill pathogens. In principle, they combat bacterial infections without exerting the strong selective pressure for resistance imposed by conventional antibiotics, with no predictable detrimental effect on the host microbiota (1). In the last decade, many antivirulence strategies have been proven effective in animal models of infection (reviewed in ref. 2), although no antivirulence compound has yet been tested in large-scale clinical trials.

A shortcut to the development of new drugs is searching for side activities in old drugs already approved for use in humans and for which safety issues have extensively been considered (3). This drug-repurposing strategy has a high probability of yielding safe and bioavailable hit compounds, which can move straightforward into clinical trials or be used as leads for drug optimization programs (3).

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most dreaded nosocomial pathogens and the leading cause of chronic lung infection in patients with cystic fibrosis (CF) (4). Multidrug-resistant *P. aeruginosa* has become increasingly frequent in healthcare settings and poses a tremendous challenge to traditional antibiotic therapy (5). Because *P. aeruginosa* has a large armamentarium of virulence factors (6), inhibition of master regulatory networks controlling its pathogenicity, rather than individual virulence traits, is more likely to cause an overall attenuation of virulence (7).

The siderophore pyoverdine represents a promising target for antivirulence compounds. Pyoverdine is not only the primary iron carrier during *P. aeruginosa* infection and biofilm formation (8, 9) but also, a master signal molecule that controls virulence gene expression through a mechanism called surface signaling (10). Interaction of iron-loaded pyoverdine with its cognate outer membrane receptor FpvA triggers a signal through the inner membrane-spanning anti–σ-factor FpvR, leading to full activation of the alternative σ-factor PvdS, which is responsible for expression not only of pyoverdine genes but also, key virulence factors (i.e., exoproteases and exotoxin A) (10). Pyoverdine synthesis is stimulated by iron deficiency, a nutritional condition characterizing the biological fluids of infected mammals (11), whereas negative control of pyoverdine synthesis is exerted by the global regulator of bacterial iron homeostasis Fur, which represses *pvdS* transcription under high-iron conditions (12). Although the role of pyoverdine in pathogenicity has been known for years, this system has so far been ignored as a target for antivirulence drugs. Only recently, an enzymatic screening assay allowed the identification of two compounds inhibiting the in vitro activity of PvdQ, a periplasmic hydrolase that is required for pyoverdine maturation (13). However, the antipyoverdine activity of these inhibitors has not been tested in bacterial cultures or in vivo.

The aim of the present work was to apply a drug-repurposing approach to identify antipyoverdine compounds that could represent good candidates for in vivo use as antivirulence drugs against *P. aeruginosa*. By using a specific biosensor for pyoverdine inhibitors, we screened a chemical library of marketed drugs and identified a promising US Food and Drug Administration-approved compound that resulted effective in suppressing *P. aeruginosa* virulence in vitro and in an animal model of pulmonary infection.

Results and Discussion

Identification of a Pyoverdine Synthesis Inhibitor. A screening system for pyoverdine inhibitors, based on a *P. aeruginosa* PAO1 reporter strain carrying a transcriptional fusion between the PvdS-dependent *pvdE* promoter (P*pvdE*) and the *luxCDABE* operon inserted at a neutral chromosomal site, was constructed (Fig. S1). This system was used to screen a commercial library of 1,120 chemical compounds with known biological activities selected for their high chemical and pharmacological diversity and safety in humans (Prestwick Chemicals). Blind screening led to the identification of one compound that reproducibly reduced bioluminescence and pyoverdine production by the reporter strain under iron-depleted conditions. This compound was decoded as flucytosine [5-Fluorocytosine (5-FC)], a synthetic fluorinated pyrimidine used as an antimycotic drug with the brand name of Ancobon.

To confirm the antipyoverdine activity of 5-FC, the compound was purchased from a different supplier (Sigma-Aldrich) and used for additional investigation. Although 5-FC did not affect *P. aeruginosa* growth (Fig. 1*A*) (minimum inhibitory concentration >

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10 mM), it had a very high inhibitory activity on P*pvdE*-dependent bioluminescence emission and pyoverdine production (Fig. 1*B*), with IC_{50} values of 2 and 3 μM, respectively. 5-FC also showed a similar inhibitory effect on the transcription of other pyoverdine biosynthetic genes (Fig. S2*A*), suggesting that 5-FC negatively affects the expression of the entire pyoverdine biosynthesis machinery. As a control, no variation in the expression of the housekeeping gene *proC* was observed in the presence of 5-FC (Fig. S2*B*).

5-FC Inhibits Pyoverdine Synthesis in Diverse *P. aeruginosa* Strains. Because *P. aeruginosa* strains can produce one of three different pyoverdine types (I, II, or III), which are recognized by cognate FpvA receptor variants (14), we assessed the effect of 5-FC on prototypic *P. aeruginosa* strains producing different types of pyoverdine and carrying the P*pvdE*::*lux* reporter construct. The inhibitory activity of 5-FC on both pyoverdine production and *pvdE* transcription was similar among *P. aeruginosa* strains producing type I, II, or III pyoverdine (Fig. 1 *B* and *C*), indicating that the antipyoverdine activity of 5-FC is independent of the chemical nature of the pyoverdine molecule and the structure of the ferripyoverdine receptor.

5-FC–dependent pyoverdine inhibition was also tested on a small collection of *P. aeruginosa* CF isolates ($n = 20$), including clonal variants isolated from the same CF patients during a period of more than 15 y (15) (Table S1). The IC_{50} values of 5-FC for CF isolates were comparable with or even lower than the IC_{50} determined for the laboratory strain PAO1 (Fig. 1*D*), suggesting that susceptibility to 5-FC is conserved in CF isolates.

5-FC Inhibits *pvdS* Gene Expression. To investigate the effect of 5-FC on pyoverdine signaling, we determined the antipyoverdine activity of 5-FC on a set of *P. aeruginosa* mutants impaired in different steps of the pyoverdine signaling cascade (Fig. 2*A*). The FpvA- and PvdA-deficient mutants are impaired in pyoverdine uptake and synthesis, respectively, and they are, therefore, unable to activate the PvdS σ-factor through pyoverdine-mediated signaling (signaling-off mutants). In contrast, the FpvR-deficient mutant cannot suppress PvdS activity in the absence of pyoverdine signaling, thus resulting in signaling-insensitive up-regulation of PvdS-dependent genes (constitutive signaling-on mutant) (Fig. 2*A*). 5-FC inhibited pyoverdine production and *pvdE* gene expression in all mutants tested (Fig. 2*A*), indicating that pyoverdine signaling is not the target of 5-FC. However, 5-FC seemed to be slightly less effective against the *fpvR* mutant (Fig. 2*A*). Because the constitutively active state of pyoverdine signaling in the *fpvR* mutant results in maximal activation of the PvdS intracellular pool (16), the lower activity of 5-FC in the *fpvR* background suggests that intracellular levels and/or activity of PvdS are critical for the inhibitory activity of 5-FC.

The effect of 5-FC on *pvdS* gene expression was investigated in *P. aeruginosa* carrying a transcriptional fusion between the *pvdS* promoter and the β-gal gene. 5-FC reduced *pvdS* promoter activity in a dose-dependent manner (Fig. 2*B*), suggesting that 5-FC acts as an inhibitor of *pvdS* transcription and consequently, reduces PvdS intracellular levels, expression of pyoverdine genes, and ultimately, pyoverdine production. To verify this hypothesis, the effect of 5-FC on pyoverdine production was assessed in a *pvdS*-deficient *P. aeruginosa* mutant carrying the *pvdS* coding sequence on a multicopy plasmid under the control of a constitutive promoter. Although 5-FC inhibited pyoverdine production by the WT strain carrying the empty vector, it had no effect on the strain constitutively expressing PvdS (Fig. 2*C*). Interestingly, 5-FC also repressed the transcription of the Fur-Fe^{2+}–regulated gene *pchR* (Fig. 2*D*), encoding an AraC/XylS-like transcriptional regulator essential for production of the second *P. aeruginosa* siderophore pyochelin (17). Accordingly, the expression of the PchR-regulated gene *pchE* and production of pyochelin were also reduced in the presence of 5-FC (Fig. 2*D*). 5-FC also inhibited the transcription of additional iron-repressible genes, namely *feoA* and *foxA*

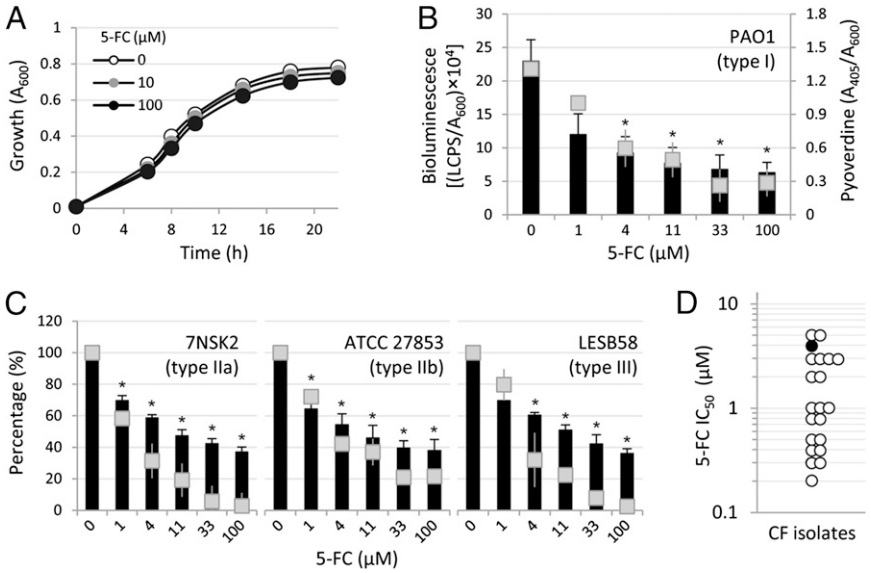

Fig. 1. 5-FC inhibits pyoverdine production in *P. aeruginosa*. (*A*) *P. aeruginosa* PAO1 growth curve in TSBD in the presence of different 5-FC concentrations (0–100 μM). (*B*) Dose–response effect of 5-FC (0–100 μM) on bioluminescence (black bars, left *y* axis) and pyoverdine production (gray squares, right *y* axis) by PAO1 P*pvdE*::*lux* at 14 h of growth in TSBD. (*C*) Dose–response effect of 5-FC (0–100 μM) on bioluminescence (black bars) and pyoverdine production (gray squares) at 14 h of growth in TSBD by *P. aeruginosa* strains producing different pyoverdine types and pyoverdine receptors (IIa, IIb, or III) and carrying the P*pvdE*::*lux* fusion. Values are normalized to A_{600} of bacterial cultures and expressed as percentage of the corresponding untreated control values. Values represent the mean (± SD) of at least three independent assays. The specific pyoverdine receptor type expressed by each strain is indicated in *B* and *C*. (*D*) IC_{50} values (micromolar) of 5-FC for pyoverdine production in 20 *P. aeruginosa* CF isolates (white circles) compared with the PAO1 reference strain (black circle). *Statistically significant differences ($P < 0.01$, ANOVA) in both bioluminescence and pyoverdine production with respect to the corresponding untreated controls.

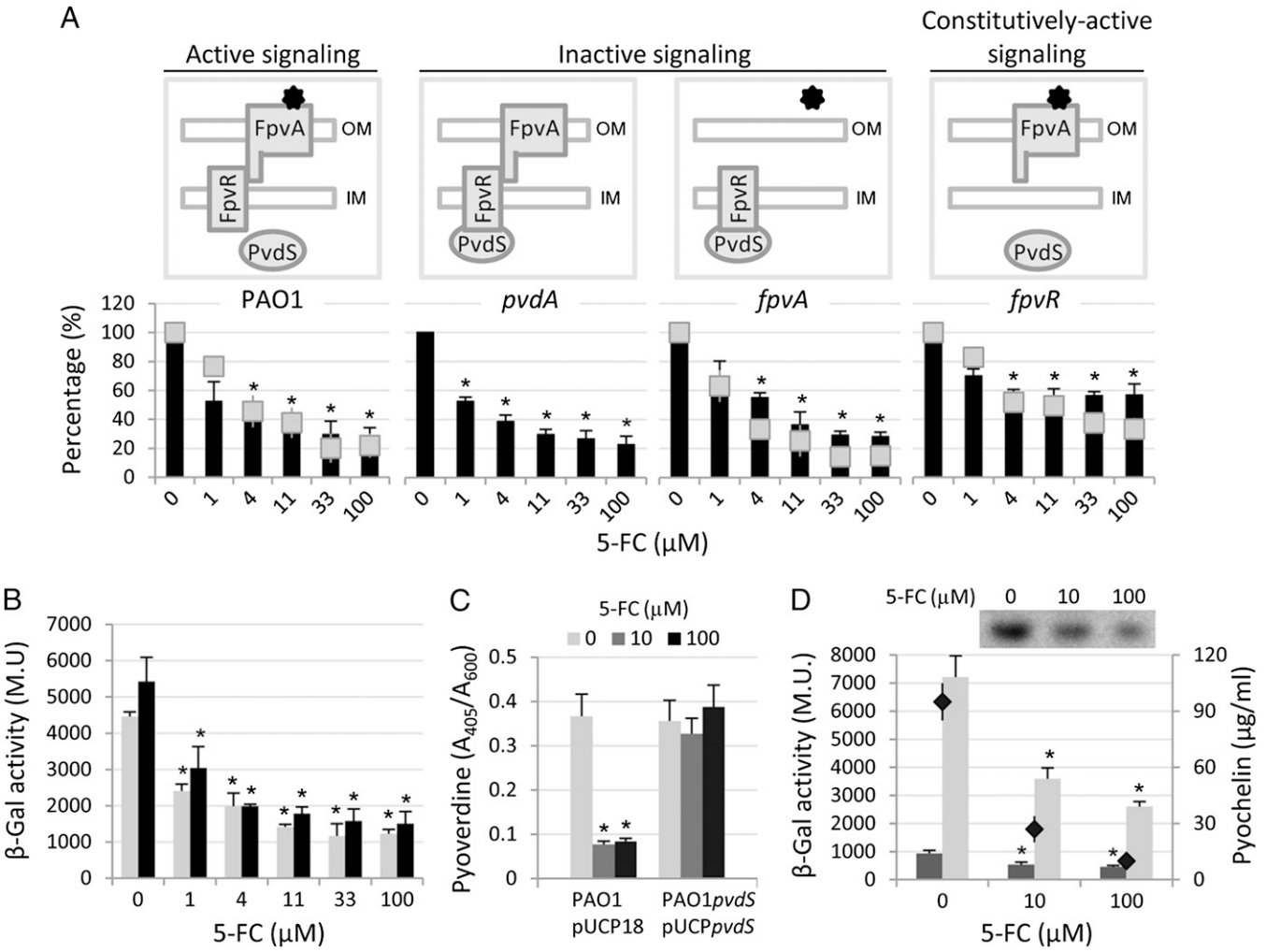

Fig. 2. 5-FC down-regulates *pvdS* transcription. (*A*) Dose–response effect of 5-FC (0–100 μM) on bioluminescence emission (black bars) and pyoverdine production (gray squares) at 14 h of growth in TSBD by *P. aeruginosa* WT and mutant strains defective in different steps of the pyoverdine signaling cascade and carrying the P*pvdE*::*lux* reporter fusion. Values are normalized to the cell density of the bacterial cultures and expressed as percentage of the corresponding untreated control values. The different behavior of mutants with respect to pyoverdine signaling is illustrated in *Upper* (black stars represent pyoverdine). (*B*) Dose–response effect of 5-FC (0–100 μM) on β-gal expression by PAO1 P*pvdS*::*lacZ* during exponential (gray bars) and stationary phase of growth in TSBD (black bars). (*C*) Effect of 5-FC (0–100 μM) on pyoverdine production at 8 h of growth in TSBD by *P. aeruginosa* PAO1 carrying the empty vector (pUCP18) and its isogenic *pvdS* mutant constitutively expressing PvdS (pUCP*pvdS*). (*D*) Effect of 5-FC (0–100 μM) on β-gal expression by *P. aeruginosa* PAO1 P*pchR*::*lacZ* (dark gray histograms, left *y* axis) and PAO1 P*pchE*::*lacZ* (light gray histograms, left *y* axis) and pyochelin production by PAO1 WT (black diamonds, right *y* axis) after 14 h of growth in TSBD. Values represent the mean (± SD) of three independent assays. *Inset* shows ferripyochelin yields following separation of PAO1 culture extracts on a representative TLC plate. *Statistically significant differences ($P < 0.01$, ANOVA) with respect to the corresponding untreated controls.

(Fig. S2 *C* and *D*), which are directly and indirectly controlled by Fur-Fe^{2+}, respectively (12). However, 5-FC–dependent suppression of pyoverdine production was also observed in a *P. aeruginosa* PAO1 *fur* mutant (Fig. S2*E*), suggesting that 5-FC could repress iron uptake genes through a Fur-independent mechanism.

5-FC Down-Regulates PvdS-Dependent Expression of Virulence Genes. The finding that 5-FC inhibits *pvdS* transcription implies that this compound could also affect the expression of PvdS-regulated virulence factors other than pyoverdine. To verify this hypothesis, we investigated the effect of 5-FC on the expression of two major virulence factors of *P. aeruginosa*, the endoprotease PrpL and exotoxin A, which are directly and indirectly regulated by PvdS, respectively (12). PrpL and exotoxin A were monitored at the expression level using *PprpL::lacZ* and *PtoxA::lacZ* fusions and at the protein level using antiexotoxin A Western blot analysis and a PrpL enzyme activity assay. As for pyoverdine, the expression of *toxA* and *prpL* genes was down-regulated in 5-FC–treated cultures with respect to untreated controls (Fig. 3*A*), consistent with the strongly reduced ToxA and PrpL levels in culture supernatants (Fig. 3 *B* and *C*).

5-FC Suppresses *P. aeruginosa* Pathogenicity in Vivo. The promising antivirulence activity of 5-FC in vitro led us to investigate the efficacy of 5-FC as an anti-*P. aeruginosa* drug in a mouse model of pulmonary infection. Mice were infected intratracheally with *ca.* 10^6 *P. aeruginosa* PAO1 cells embedded in agar beads and then treated two times daily with i.p. administration of either a therapeutic dose of 5-FC (30 mg/kg per day) or the placebo (saline). As a control, mice were also infected with an isogenic *pvdS* mutant and treated with saline. Although 75% of placebo-treated mice were killed within 4 d of PAO1 infection, 5-FC treatment almost completely protected mice from the *P. aeruginosa* lethal challenge (Fig. 4*A*). Notably, all mice infected with the *pvdS* mutant survived the challenge (Fig. 4*A*), highlighting the importance of PvdS as a major pathogenicity determinant in *P. aeruginosa* pulmonary

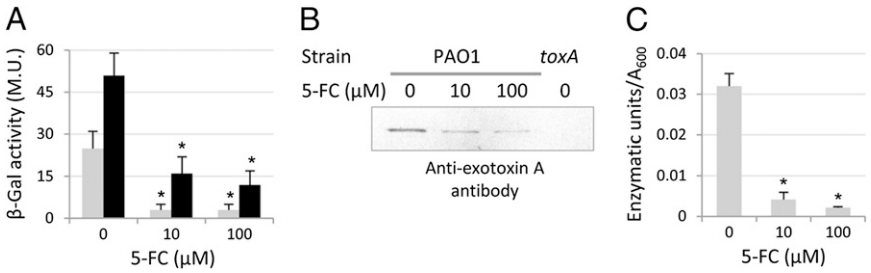

Fig. 3. 5-FC inhibits PvdS-dependent virulence gene expression. Effect of 5-FC (0–100 μM) on (*A*) β-gal expression by PAO1 P*toxA*::*lacZ* (gray bars) and PAO1 P*prpL*::lacZ (black bars) and (*B*) exotoxin A levels and (*C*) PrpL enzymatic activity in 10 μL PAO1 culture supernatants after 8 h of growth in TSBD. Values represent the mean (± SD) of three independent assays, whereas the Western blot is representative of two independent experiments giving similar results. *Statistically significant differences ($P < 0.01$, ANOVA) with respect to the corresponding untreated controls.

infection. After 6 d of infection, the bacterial load in lungs of surviving mice was comparable between mice infected with PAO1 and PAO1*pvdS* as well as between 5-FC–treated and -untreated mice (Fig. S3), confirming that 5-FC inhibits virulence rather than cell viability. Moreover, lung histopathology revealed that lesions and inflammation in bronchi and pulmonary parenchyma were similarly reduced in both 5-FC–treated and PAO1*pvdS*-infected mice compared with untreated mice infected with WT PAO1 (Fig. 4*B*).

Antivirulence Activity of 5-FC Requires Metabolic Conversion to 5-Fluorouracil. The antimycotic compound 5-FC is a prodrug that is taken up by fungi through one or more cytosine permeases, deaminated to 5-fluorouracil by a cytosine deaminase, and subsequently, converted to 5-fluoro-UMP and 5-fluoro-dUMP, ultimately causing perturbation of DNA and protein synthesis (18). Although 5-FC by itself is not toxic, 5-fluorouracil is highly cytotoxic. Therefore, the direct use of 5-fluorouracil in medicine is restricted to the treatment of solid tumors (19).

All *P. aeruginosa* genomes sequenced so far contain homologs of the *codA* and *codB* genes of *Escherichia coli* (www.pseudomonas.com), encoding a cytosine deaminase and a cytosine permease, respectively (20). To assess whether conversion to 5-fluorouracil is essential for the antipyoverdine activity of 5-FC, we tested 5-FC against individual *P. aeruginosa codA* and *codB* deletion mutants. Inhibition of pyoverdine production and *pvdS* gene expression by 5-FC was strongly reduced in the PAO1*codB* mutant and completely abrogated in the PAO1*codA* mutant, indicating that 5-FC uptake and conversion to 5-fluorouracil are essential for 5-FC activity in *P. aeruginosa* (Fig. 5). Interestingly, a very high 5-FC concentration (1 mM) retained some activity against the PAO1*codB* mutant (Fig. 5), suggesting that 5-FC can also enter *P. aeruginosa* cells through low-affinity secondary systems or by passive diffusion.

Conclusions

This work represents proof that the pyoverdine system is a suitable target for the development of antivirulence compounds against *P. aeruginosa*. We showed that the antimycotic drug 5-FC inhibits the production of critical virulence factors, like pyoverdine, exotoxin A, and protease PrpL, by down-regulating *pvdS* gene expression. 5-FC also suppressed *P. aeruginosa* pathogenicity in a mouse model of lung infection, consistent with the essential role played by PvdS during pulmonary infection (Fig. 4). The molecular mechanisms by which 5-FC inhibits *pvdS* transcription are unknown at the moment, although we showed that (*i*) 5-FC has an inhibitory effect on the expression of iron uptake genes and (*ii*) 5-FC uptake and metabolic conversion to 5-fluorouracil are essential steps for 5-FC activity. Cytosine deaminase is typically produced by microorganisms and has no counterpart in higher eukaryotes, including mammals. These features confer to 5-FC selective activity on those species capable of assimilating and activating the prodrug.

Our results gain additional relevance if the pharmacological properties of 5-FC are taken into account. 5-FC is currently used combined with other antifungal agents for the treatment of systemic mycoses and fungal pneumonias (21, 22). Orally administered 5-FC is almost completely adsorbed, reaches peak concentrations in serum within 1–2 h, and easily reaches most body sites (21). 5-FC is also well-tolerated and has very low toxicity as long as serum concentrations are maintained below 50 μg/mL (388 μM) (21, 23). This serum level is almost 40-fold higher than the 5-FC concentration (10 μM) able to exert the maximal inhibitory effect in vitro on *P. aeruginosa* virulence gene expression (Figs. 1, 2, and 3). 5-FC has also been successfully used to treat fungal infections in CF patients, including a case of pulmonary candidiasis, without causing side effects (24, 25). These issues raise the possibility that currently recommended 5-FC dosing regimens would also be effective as antivirulence therapy against *P. aeruginosa*. We hope that our findings will foster clinical investigations aimed at verifying the efficacy of 5-FC in the treatment of *P. aeruginosa* infections, offering the unique chance of assessing the clinical impact of an antivirulence drug.

Materials and Methods

Bacteria, Media, and Chemicals. Bacterial strains and plasmids used in this work are listed in Table S2. *P. aeruginosa* CF isolates are described in Table S1. Bacteria were grown in LB (26) for general genetic procedures, whereas they were grown in the low-iron media trypticase soy broth dialysate (TSBD) (27) or M9 minimal medium supplemented with succinate (26) for specific assays. 5-FC was purchased from Sigma-Aldrich. Exogenous pyoverdine was added as pyoverdine-conditioned medium (8).

General Genetic Procedures. *E. coli* was routinely used for recombinant DNA manipulations. The P*pvdE*::*lux* construct was generated by cloning in plasmid mini–CTX-lux (28) the SalI-HindIII DNA fragment encompassing the *pvdE* promoter region excised from pMP190::P*pvdE* (29). The P*pvdE*::*lux* construct was integrated into the genome of *P. aeruginosa* strains as described (30). The PAO1*pvdS* mutant was generated by replacement of the entire *pvdS* coding sequence with a Gm^R cassette using a previously described strategy (31). The in-frame deletion mutants PAO1*codA* and PAO1*codB* were generated using the suicide vector pDM4 as described (32). The complementing plasmids pUCP*codA* and pUCP*codB* were generated by cloning the *codA* and *codB* coding sequence, including their putative ribosome binding site, downstream to the *lac* promoter in the pUCP18 plasmid (Table S2). The P*pchR*::*lacZ* and P*feoA*::*lacZ* transcriptional fusions were generated by cloning a PCR-amplified DNA fragment encompassing the entire promoter region of *pchR* and *feoA* genes, respectively, into the promoter probe plasmid pMP220 (Table S2). Primers and restriction enzymes used for cloning of PCR products are listed in Table S3.

Screening for Pyoverdine Inhibitors. Overnight cultures of PAO1 P*pvdE*::*lux* were diluted to $A_{600} = 0.003$ in the iron-poor TSBD medium, and growth at 37 °C in microtiter plates in the presence or absence of 50 or 5 μg/mL each Prestwick compound (200 μL final volume) was monitored for up to 20 h. A_{600} and bioluminescence light counts per second (LCPS) were measured in

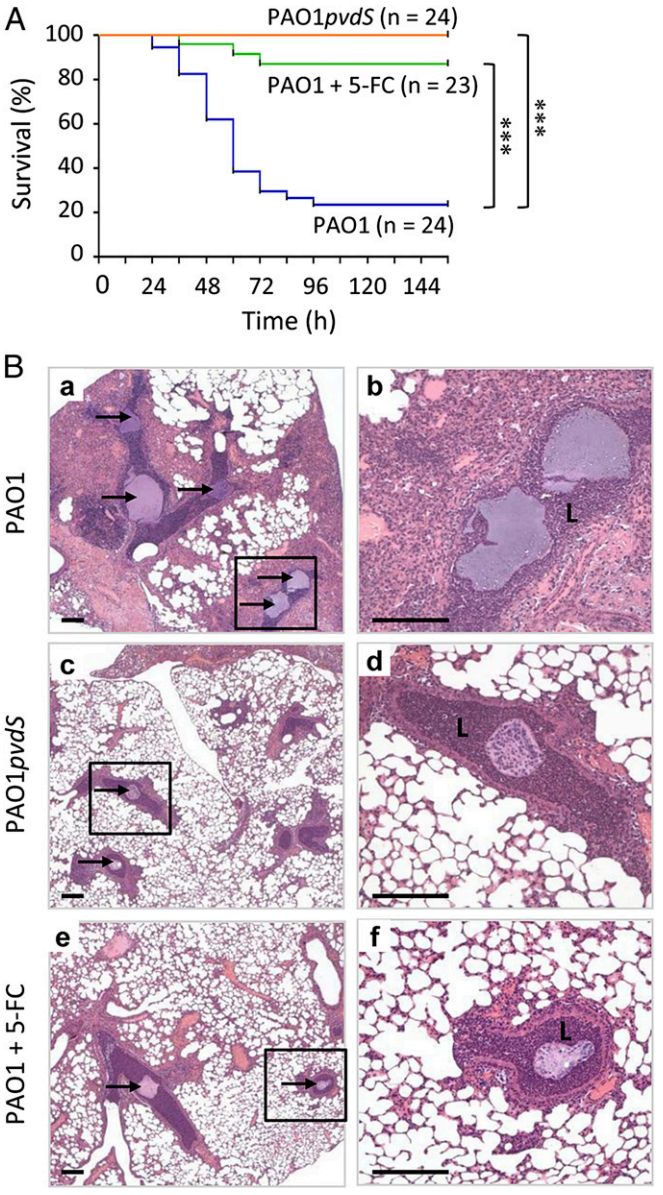

Fig. 4. 5-FC suppresses *P. aeruginosa* virulence in vivo. (*A*) Effect of 5-FC on *P. aeruginosa* PAO1 lethality in a mouse model of pulmonary infection. Mice were infected intratracheally with *P. aeruginosa* PAO1 embedded in agar beads and treated with i.p. administrations of 30 mg/kg per day 5-FC (green lines) or saline (blue lines). As control, mice infected with PAO1*pvdS* and treated with saline were used (orange lines). Data were pooled from two independent experiments (*n* indicates the total number of mice). ***$P <$ 0.0001 (Mantel–Cox test). (*B*) Murine lung histology. Four additional mice per group were infected with *P. aeruginosa* PAO1 or PAO1*pvdS* embedded in agar beads, treated with 5-FC or saline as described in *A*, and euthanized at day 2 postinfection (10). Lung sections were stained with H&E. PAO1-infected mice showed a massive bronchiolitis and huge interstitial/alveolar inflammation. In PAO1*pvdS*- and PAO1-infected mice treated with 5-FC (+5-FC), the inflammation was focal, and most of alveolar spaces were spared. Beads, indicated by arrows, are visible in the bronchial lumen (L), and *P. aeruginosa* macrocolonies can be observed into the beads. b, d, and f are enlargements of the boxed areas in a, c, and e. (Scale bars: 200 μm.)

a Victor^{3}V plate reader (Perkin-Elmer) as described (30). Pyoverdine fluorescence was assessed as emission at 460 nm after excitation at 405 nm (33). Luminescence and fluorescence values were normalized by the cell density and subtracted of untreated PAO1*pvdA* P*pvdE*::*lux* values. Criteria used for the selection of hit compounds were (*i*) ≥50% inhibition of normalized bioluminescence emission and/or pyoverdine-specific fluorescence and (*ii*) ≤20% alteration of growth relative to the untreated control. Criterion *ii* was aimed at avoiding any unspecific effect of altered growth on bioluminescence and/or pyoverdine production. For promising compounds, pyoverdine was also quantified in diluted cell-free culture supernatants (see below).

Miscellaneous Assays. Pyoverdine levels in culture supernatants were measured as A_{405} in 100 mM Tris·HCl (pH 8) and normalized by the cell density (A_{600}) of the bacterial cultures (33). Exotoxin A was detected in 10 μL culture supernatants by SDS/PAGE followed by Western blot with a polyclonal antiexotoxin A antibody (Sigma-Aldrich). PrpL and β-gal enzymatic activities were determined as previously described (34, 35). Pyochelin was isolated by ethyl acetate extraction of acidified culture supernatants, resuspended in methanol, and resolved by TLC on silica gel (36). Pyochelin was detected by spraying with 0.1 M $FeCl_3$ and quantified by A_{520} readings of ferripyochelin eluted with methanol from TLC plates (37). Anti-PvdA Western blot analysis was performed using the 3H6D12 monoclonal antibody as described (38).

Mouse Model of *P. aeruginosa* Lung Infection. C57BL/6 male mice (Charles River) were infected intratracheally with 10^6 *P. aeruginosa* viable cells embedded in agar beads as described (15), except for the use of TSBD agar instead of TSB agar for beads preparation. Mice were treated two times daily (starting 2 h postinfection) by i.p. administration of 50 μL 50 mM 5-FC in saline or 50 μL saline as control. Two 50-μL doses/d of 50 mM 5-FC correspond to a daily dosage of about 30 mg/kg (mouse weight was 20–22 g), which is within or below the dosage range recommended for humans; the dosage ranges for humans are 25–100 mg/kg per day for infants (<1 mo) and 50–150 mg/kg per day for children and adults (http://www.drugs.com/dosage/flucytosine.html). Mortality was monitored for a 6-d time period. Surviving mice were killed at day 6 postinfection, and lungs were excised, homogenized, and plated to determine the number of viable cells per lung. Four additional mice per group were infected with *P. aeruginosa* PAO1 or PAO1*pvdS* and treated with 5-FC or saline as described above, and they were euthanized at day 2 postinfection for lung histology. Lungs were removed *en bloc*, fixed in 4% (wt/vol) paraformaldehyde/PBS, and processed for paraffin

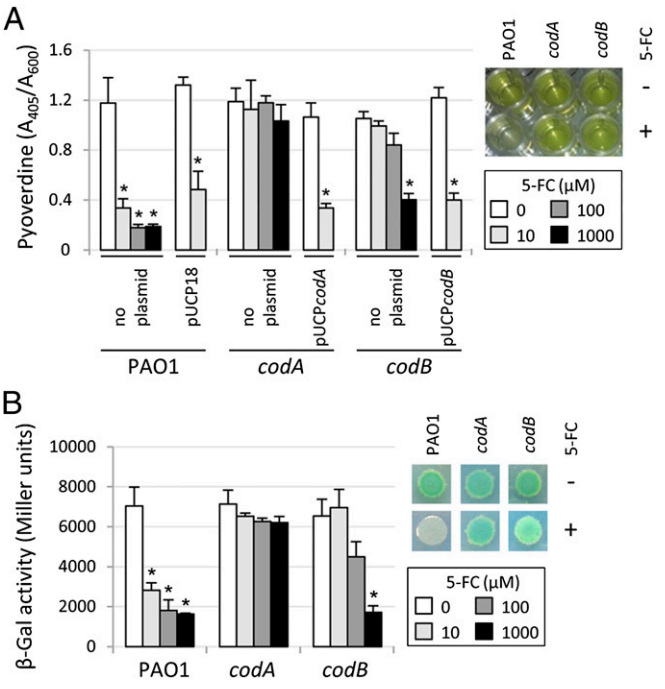

Fig. 5. Enzymatic conversion to 5-fluorouracil is essential for the anti-pyoverdine activity of 5-FC. Effect of 5-FC (0–1,000 μM) on (*A*) pyoverdine production by PAO1, PAO1*codA* (*codA*), and PAO1*codB* (*codB*) containing or not containing the plasmid pUCP18, pUCP*codA*, or pUCP*codB* as indicated and (*B*) β-gal activity by the same strains containing the P*pvdS::lacZ* fusion construct grown for 14 h in TSBD. *Insets* show (*A*) pyoverdine production in M9 medium (green fluorescence) and (*B*) β-gal activity (blue color) in M9 agar plates containing the chromogenic substrate X-gal after 14 h of growth in the absence (–) or presence (+) of 100 μM 5-FC. *Statistically significant differences ($P < 0.01$, ANOVA) with respect to the corresponding untreated controls.

embedding. Longitudinal sections of 5 μm taken at regular intervals were obtained using a microtome from the middle of the five lung lobes and stained with H&E. Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute Institutional Animal Care and Use Committee.

Statistical Analysis. Statistical analysis was performed with the software GraphPad Instat using one-way ANOVA. Survival curves for the mouse infection assay were analyzed using the log-rank Mantel–Cox test.

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1. Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ (2008) The biology and future prospects of antivirulence therapies. *Nat Rev Microbiol* 6(1):17–27.
2. Rasko DA, Sperandio V (2010) Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* 9(2):117–128.
3. Wermuth CG (2006) Selective optimization of side activities: The SOSA approach. *Drug Discov Today* 11(3–4):160–164.
4. Driscoll JA, Brody SL, Kollef MH (2007) The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* 67(3):351–368.
5. Page MG, Heim J (2009) Prospects for the next anti-*Pseudomonas* drug. *Curr Opin Pharmacol* 9(5):558–565.
6. Kipnis E, Sawa T, Wiener-Kronish J (2006) Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. *Med Mal Infect* 36(2):78–91.
7. Lee DG, et al. (2006) Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol* 7(10):R90.
8. Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci USA* 102(31):11076–11081.
9. Visca P, Imperi F, Lamont IL (2007) Pyoverdine siderophores: From biogenesis to biosignificance. *Trends Microbiol* 15(1):22–30.
10. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonasaeruginosa*. *Proc Natl Acad Sci USA* 99(10):7072–7077.
11. Nairz M, Schroll A, Sonnweber T, Weiss G (2010) The struggle for iron—a metal at the host-pathogen interface. *Cell Microbiol* 12(12):1691–1702.
12. Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML (2002) GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: Identification of novel pyoverdine biosynthesis genes. *Mol Microbiol* 45(5):1277–1287.
13. Drake EJ, Gulick AM (2011) Structural characterization and high-throughput screening of inhibitors of PvdQ, an NTN hydrolase involved in pyoverdine synthesis. *ACS Chem Biol* 6(11):1277–1286.
14. Bodilis J, et al. (2009) Distribution and evolution of ferripyoverdine receptors in *Pseudomonas aeruginosa*. *Environ Microbiol* 11(8):2123–2135.
15. Bragonzi A, et al. (2009) *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am J Respir Crit Care Med* 180(2):138–145.
16. Tiburzi F, Imperi F, Visca P (2008) Intracellular levels and activity of PvdS, the major iron starvation sigma factor of *Pseudomonas aeruginosa*. *Mol Microbiol* 67(1):213–227.
17. Michel L, González N, Jagdeep S, Nguyen-Ngoc T, Reimmann C (2005) PchR-box recognition by the AraC-type regulator PchR of *Pseudomonas aeruginosa* requires the siderophore pyochelin as an effector. *Mol Microbiol* 58(2):495–509.
18. Edlind TD, Katiyar SK (2010) Mutational analysis of flucytosine resistance in *Candida glabrata*. *Antimicrob Agents Chemother* 54(11):4733–4738.
19. Pan X, et al. (2011) Development of 5-Fluorouracil derivatives as anticancer agents. *Curr Med Chem* 18(29):4538–4556.
20. Danielsen S, Kilstrup M, Barilla K, Jochimsen B, Neuhard J (1992) Characterization of the *Escherichia coli codBA* operon encoding cytosine permease and cytosine deaminase. *Mol Microbiol* 6(10):1335–1344.
21. Vermes A, Guchelaar HJ, Dankert J (2000) Flucytosine: A review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *J Antimicrob Chemother* 46(2):171–179.
22. Yamada H, Kotaki H, Takahashi T (2003) Recommendations for the treatment of fungal pneumonias. *Expert Opin Pharmacother* 4(8):1241–1258.
23. Stamm AM, et al. (1987) Toxicity of amphotericin B plus flucytosine in 194 patients with cryptococcal meningitis. *Am J Med* 83(2):236–242.
24. Jenner BM, Landau LI, Phelan PD (1979) Pulmonary candidiasis in cystic fibrosis. *Arch Dis Child* 54(7):555–556.
25. Radike K, Kunzmann S, Abele-Horn M, Beer M, Hebestreit H (2011) Osteoarticular infection by *Candida albicans* in an infant with cystic fibrosis. *J Med Microbiol* 60(Pt 10):1542–1545.
26. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), 2nd Ed.
27. Ohman DE, Sadoff JC, Iglewski BH (1980) Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: Isolation and characterization. *Infect Immun* 28(3):899–908.
28. Becher A, Schweizer HP (2000) Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal lacZ and lux gene fusions. *Biotechniques* 29(5):948–950, 952.
29. Cunliffe HE, Merriman TR, Lamont IL (1995) Cloning and characterization of *pvdS*, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. *J Bacteriol* 177(10):2744–2750.
30. Massai F, et al. (2011) A multitask biosensor for micro-volumetric detection of N-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal. *Biosens Bioelectron* 26(8):3444–3449.
31. Cipollone R, et al. (2007) Involvement of *Pseudomonas aeruginosa* rhodanese in protection from cyanide toxicity. *Appl Environ Microbiol* 73(2):390–398.
32. Rampioni G, et al. (2010) Transcriptomic analysis reveals a global alkyl-quinolone-independent regulatory role for PqsE in facilitating the environmental adaptation of Pseudomonas aeruginosa to plant and animal hosts. *Environ Microbiol* 12(6):1659–1673.
33. Imperi F, Tiburzi F, Visca P (2009) Molecular basis of pyoverdine siderophore recycling in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 106(48):20440–20445.
34. Imperi F, Tiburzi F, Fimia GM, Visca P (2010) Transcriptional control of the *pvdS* iron starvation sigma factor gene by the master regulator of sulfur metabolism CysB in *Pseudomonas aeruginosa*. *Environ Microbiol* 12(6):1630–1642.
35. Miller JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Plainview, NY).
36. Zhang S, et al. (2005) Comparative signature-tagged mutagenesis identifies *Pseudomonas* factors conferring resistance to the pulmonary collectin SP-A. *PLoS Pathog* 1(3):259–268.
37. Cox CD, Graham R (1979) Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. *J Bacteriol* 137(1):357–364.
38. Imperi F, et al. (2008) Membrane-association determinants of the omega-amino acid monooxygenase PvdA, a pyoverdine biosynthetic enzyme from *Pseudomonas aeruginosa*. *Microbiology* 154(Pt 9):2804–2813.

Supporting Information

Imperi et al. 10.1073/pnas.1222706110

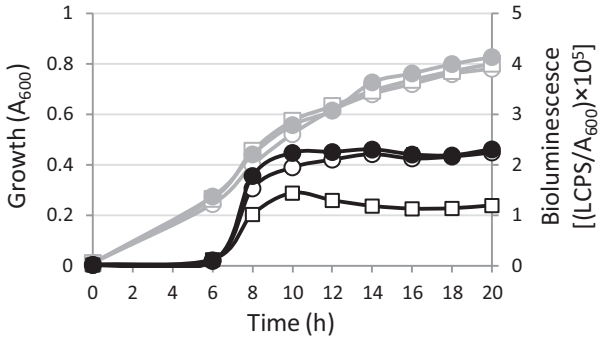

Fig. S1. Response of the PvdS-dependent *pvdE* promoter (P*pvdE*) *::lux* reporter to pyoverdine signaling. Bacterial growth (gray lines, left *y* axis) and bioluminescence emission (black lines, right *y* axis) of PAO1 P*pvdE::lux* (filled circles), PAO1*pvdA* P*pvdE::lux* (open squares), and PAO1*pvdA* P*pvdE::lux* plus 10 μM exogenously added pyoverdine (open circles) cultured in TSBD medium at 37 °C in microtiter plates. Values represent the mean of five independent assays (SD < 9% of the mean values).

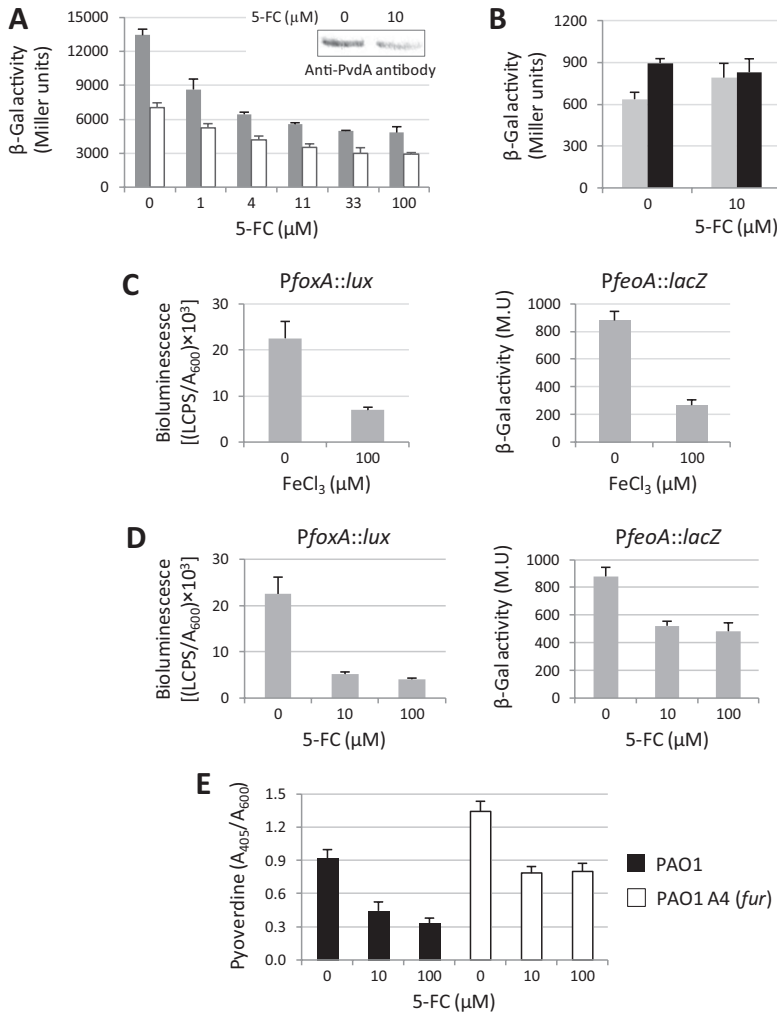

Fig. S2. 5-Fluorocytosine (5-FC) specifically inhibits transcription of iron-dependent genes. (*A*) Effect of 5-FC (0–100 μM) on β-gal expression by PAO1 P*pvdA*::*lacZ* (gray bars) and PAO1 P*pvdD*::*lacZ* (white bars) at 14 h of growth in TSBD. *Inset* shows a representative anti-PvdA Western blot on whole-cell lysates from 5-FC–treated or -untreated *Pseudomonas aeruginosa* PAO1 cells grown for 14 h in TSBD. (*B*) Effect of 5-FC (0–100 μM) on β-gal expression by PAO1 P*proC*::*lacZ* at 8 and 14 h of growth (gray and black bars, respectively) in TSBD. Values represent the mean (± SD) of at least three independent assays. (*C*) Bioluminescence emission by PAO1 P*foxA*::*lux* (*Left*) and β-gal expression by PAO1 P*feoA*::*lacZ* (*Right*) at 14 h of growth in TSBD supplemented or not supplemented with 100 μM $FeCl_3$. (*D*) Effect of 5-FC (0–100 μM) on bioluminescence emission by PAO1 P*foxA*::*lux* (*Left*) and β-gal expression by PAO1 P*feoA*::*lacZ* (*Right*) at 14 h of growth in TSBD. (*E*) Effect of 5-FC (0–100 μM) on pyoverdine production by PAO1 and the *fur* mutant PAO1 A4 expressing a Fur variant, which is strongly impaired in iron-dependent gene repression (1), at 14 h of growth in TSBD. Values represent the mean (± SD) of at least three independent assays.

1. Barton HA, Johnson Z, Cox CD, Vasil AI, Vasil ML (1996) Ferric uptake regulator mutants of Pseudomonas aeruginosa with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. *Mol Microbiol* 21(5):1001–1017.

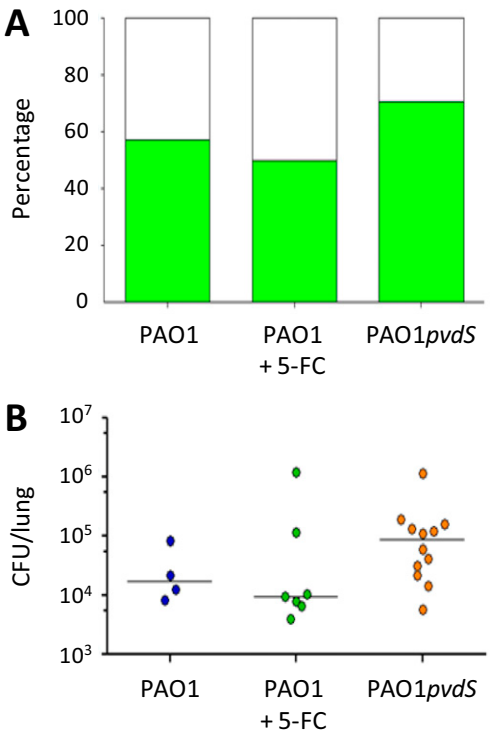

Fig. S3. 5-FC does not affect *P. aeruginosa* persistence in mouse lungs. (*A*) Percentage of mice showing $\geq 10^3$ viable *P. aeruginosa* cells per lung among mice surviving the pulmonary infection (corresponding to 100%) with *P. aeruginosa* PAO1 or the isogenic PAO1*pvdS* mutant treated or not treated with 5-FC (Fig. 4*A*). (*B*) Bacterial load [colony-forming unit (CFU) per lung] in the lungs of mice showing $\geq 10^3$ viable *P. aeruginosa* cells per lung. No statistically significant differences between groups ($0.16 \leq P \leq 1$) were observed (Fisher and unpaired two-tailed *t* tests for *A* and *B*, respectively).

Table S1. *P. aeruginosa* cystic fibrosis isolates used in this study

Strain*	Source
BT2	1
BT73	1
KK1	1
KK28	1
KK71	1
KK72	1
TR1	1
TR66	1
FM-01	2
FM-02	2
FM-04	2
FM-11	2
FM-12	2
FM-13	2
FM-14	2
FM-15	2
FM-17	2
FM-19	2
FM-20	2
FM-21	This work

*Numbers of FM isolates refer to the sputum samples and thus, patients from whom strains have been isolated (described in ref. 2). FM-21 was isolated from the sputum sample of a CF patient chronically infected by *P. aeruginosa* and hospitalized at the Policlinico Umberto I Hospital (Rome, Italy).

1. Bragonzi A, et al. (2009) *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am J Respir Crit Care Med* 180(2): 138–145.
2. Massai F, et al. (2011) A multitask biosensor for micro-volumetric detection of N-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal. *Biosens Bioelectron* 26(8):3444–3449.

Table S2. Bacterial strains and plasmids used in this study

	Genotype and/or relevant characteristics	Source
Strain		
P. aeruginosa		
PAO1	WT (prototroph), type I pyoverdine producer	ATCC
7NSK2	Type II pyoverdine producer (type IIa pyoverdine receptor)	1
ATCC27853	Type II pyoverdine producer (type IIb pyoverdine receptor)	1
LESB58	Type III pyoverdine producer	2
PAO1*pvdS*	PAO1 Δ*pvdS,* Gm^R	This work
PAO1*codA*	PAO1 Δ*codA*, in-frame deletion mutant in the *codA* (PA0437) gene	This work
PAO1*codB*	PAO1 Δ*codB*, in-frame deletion mutant in the *codB* (PA0438) gene	This work
PAO1*pvdA*	PAO1 Δ*pvdA*	3
K1660	K767 Δ*fpvA*	4
PAO1*fpvR*	PAO1 *fpvR*::Km^R	5
PAO1 A4	PAO1 carrying a *fur* mutation resulting in the H86→Y substitution in the Fur protein	6
Escherichia coli		
DH5αF′	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 [Φ*80*d*lacZ*ΔM15] Nal^R	7
S17-1 λ*pir*	*TpR SmR recA, thi, pro, hsdR*-M+RP4: 2-Tc:Mu: K_m Tn*7* λ*pir*	8
Plasmid		
pEX18Tc	pMB1 replicon, *oriT sacB lacZ*α, *Mob*$^+$ Tc^r, allelic exchange vector	9
pPS858	Ap^r; source of Gm^r-GFP cassette	9
pUCP18	*E. coli–Pseudomonas* shuttle vector; Ap/Cb^R	10
pDM4	Suicide vector; *sacBR*, *oriR6K*; Cm^R	11
Mini-CTX-*lux*	Promoter-probe vector containing the *luxCDABE* operon; Tc^R	12
Mini-CTX-P*pvdE*::*lux*	Plasmid to insert a P*pvdE*::*lux* fusion into the chromosome of *P. aeruginosa*; Tc^R	This work
pEXΔ*pvdS*	pEX18Tc derivative carrying the Gm^r-GFP cassette between flanking regions of the *pvdS* coding sequence	This work
pDM4Δ*codA*	pDM4 derivative carrying the flanking regions of the *codA* coding sequence	This work
pDM4Δ*codB*	pDM4 derivative carrying the flanking regions of the *codB* coding sequence	This work
pUCP*codA*	pUCP18 derivative carrying the entire *codA* coding sequence under the control of the P_{T5} *lacO* region	This work
pUCP*codB*	pUCP18 derivative carrying the entire *codB* coding sequence under the control of the P_{T5} *lacO* region	This work
pUCP*pvdS*	pUCP18 derivative carrying the entire *pvdS* coding sequence under the control of the P_{T5} *lacO* region; Ap^R	13
pMP220::P*pvdA*	pMP220 derivative carrying a P*pvdA*::*lacZ* transcriptional fusion; Tc^R	13
pMP190::P*pvdD*	pMP190 derivative carrying a P*pvdD*::*lacZ* transcriptional fusion; Cm^R	14
pMP190::P*pvdE*	pMP190 derivative carrying a P*pvdE*::*lacZ* transcriptional fusion; Cm^R	14
pPZ-*pvdS*	pPZTC derivative carrying a P*pvdS*::*lacZ* transcriptional fusion; Cb^R	15
pPZ-*toxA*	pPZ20 derivative carrying a P*toxA*::*lacZ* translational fusion; Cb^R	5
pPZ-*prpL*	pPZTC derivative carrying a P*prpL*::*lacZ* transcriptional fusion; Cb^R	5
pMP220::P*pchR*	pMP220 derivative carrying a P*pchR*::*lacZ* transcriptional fusion; Tc^R	This work
pMP220::P*feoA*	pMP220 derivative carrying a P*feoA*::*lacZ* transcriptional fusion; Tc^R	This work
pME6425	Translational *pchE′-′lacZ* fusion, Tc^R	16
pME3641	Plasmid carrying a translational P*proC*::*lacZ* fusion, Cb^R	17
Mini-CTX-*lux*::P*foxA*	Plasmid to insert a P*foxA*::*lux* fusion into the chromosome of *P. aeruginosa*; TcR	18

1. Bodilis J, et al. (2009) Distribution and evolution of ferripyoverdine receptors in *Pseudomonas aeruginosa*. *Environ Microbiol* 11(8):2123–2135.
2. Kukavica-Ibrulj I, et al. (2008) In vivo growth of *Pseudomonas aeruginosa* strains PAO1 and PA14 and the hypervirulent strain LESB58 in a rat model of chronic lung infection. *J Bacteriol* 190(8):2804–2813.
3. Imperi F, et al. (2008) Membrane-association determinants of the omega-amino acid monooxygenase PvdA, a pyoverdine biosynthetic enzyme from *Pseudomonas aeruginosa*. *Microbiology* 154(Pt 9):2804–2813.
4. Shen J, Meldrum A, Poole K (2002) FpvA receptor involvement in pyoverdine biosynthesis in *Pseudomonas aeruginosa*. *J Bacteriol* 184(12):3268–3275.
5. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonasaeruginosa*. *Proc Natl Acad Sci USA* 99(10): 7072–7077.
6. Barton HA, Johnson Z, Cox CD, Vasil AI, Vasil ML (1996) Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. *Mol Microbiol* 21(5):1001–1017.
7. Liss L (1987) New M13 host: DH5 F′ competent cells. *Focus* 9:13.
8. Simon R, Priefer U, Puhler A (1983) A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram-negative bacteria. *Biotechnology (NY)* 1:784–791.
9. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: Application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212(1):77–86.
10. Schweizer HP (1991) *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* 97(1):109–121.
11. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* 178(5):1310–1319.
12. Becher A, Schweizer HP (2000) Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal lacZ and lux gene fusions. *Biotechniques* 29(5): 948–950, 952.
13. Ambrosi C, Tiburzi F, Imperi F, Putignani L, Visca P (2005) Involvement of AlgQ in transcriptional regulation of pyoverdine genes in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 187(15):5097–5107.
14. Cunliffe HE, Merriman TR, Lamont IL (1995) Cloning and characterization of *pvdS*, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. *J Bacteriol* 177(10):2744–2750.
15. Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML (2002) GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: Identification of novel pyoverdine biosynthesis genes. *Mol Microbiol* 45(5):1277–1287.
16. Reimmann C, Serino L, Beyeler M, Haas D (1998) Dihydroaeruginoic acid synthetase and pyochelin synthetase, products of the *pchEF* genes, are induced by extracellular pyochelin in *Pseudomonas aeruginosa*. *Microbiology* 144(Pt 11):3135–3148.
17. Savioz A, Zimmermann A, Haas D (1993) *Pseudomonas aeruginosa* promoters which contain a conserved GG-N10-GC motif but appear to be RpoN-independent. *Mol Gen Genet* 238(1–2):74–80.
18. Mettrick KA, Lamont IL (2009) Different roles for anti-sigma factors in siderophore signalling pathways of *Pseudomonas aeruginosa*. *Mol Microbiol* 74(5):1257–1271.

Table S3. Primers used in this study

Primer name	Sequence*	Restriction site	Application
pvdS mut_UP_FW	5′-CGGAATTCCGTCTCCTGCTGCGC-3′	EcoRI	Generation of the PAO1*pvdS* deletion mutant
pvdS mut_UP_RV	5′-ACGCGTCGACCGACATGGAAATCACCTTG-3′	SalI	Generation of the PAO1*pvdS* deletion mutant
pvdS mut_DOWN_FW	5′-ACGCGTCGACTGACGGCGGCGAGCATTC-3′	SalI	Generation of the PAO1*pvdS* deletion mutant
pvdS mut_DOWN_RV	5′-CCCAAGCTTCCGTCCCCAGCCTC-3′	HindIII	Generation of the PAO1*pvdS* deletion mutant
codA mut_UP_FW	5′-GCTCTAGACCGCCGTGGTGGTGTGC-3′	XbaI	Generation of the PAO1*codA* in-frame deletion mutant
codA mut_UP_RV	5′-CGGAATTCGCGGCCGCGCAGGC-3′	EcoRI	Generation of the PAO1*codA* in-frame deletion mutant
codA mut_DOWN_FW	5′-CGGAATTCGACGCGGTGCGCCG-3′	EcoRI	Generation of the PAO1*codA* in-frame deletion mutant
codA mut_DOWN_RV	5′-GGCCTGCTCGAGGTCTGG-3′	XhoI	Generation of the PAO1*codA* in-frame deletion mutant
codB mut_UP_FW	5′-GCTCTAGATGGACAGCCATGGCTAC-3′	XbaI	Generation of the PAO1*codB* in-frame deletion mutant
codB mut_UP_RV	5′-CGGAATTCCGCCAGCGGGACCGG-3′	EcoRI	Generation of the PAO1*codB* in-frame deletion mutant
codB mut_DOWN_FW	5′-CGGAATTCGCTTGCGGCACCGCC-3′	EcoRI	Generation of the PAO1*codB* in-frame deletion mutant
codB mut_DOWN_RV	5′-GCGCACCTCGAGCATGGC-3′	XhoI	Generation of the PAO1*codB* in-frame deletion mutant
*codA*_FW	5′-CGGGATCCCCCACGGAGACTCGCG-3′	BamHI	Construction of *codA*-complementing plasmid
*codA*_RV	5′-CCCAAGCTTGTCAATCAGCATGGAGGAC-3′	HindIII	Construction of *codA*-complementing plasmid
*codB*_FW	5′-CGGGATCCAGAAGAGGTCCTCCATG-3′	BamHI	Construction of *codB*-complementing plasmid
*codB*_RV	5′-CCCAAGCTTCGCTAGAAGGCTCCGG-3′	HindIII	Construction of *codB*-complementing plasmid
P*pchR*_FW	5′-GGAAGATCTACCGTGTCGCCATGTG-3′	BglII	Construction of P*pchR*::*lacZ* transcriptional fusion
P*pchR*_RV	5′-GCGGAATTCGATGTGCGCGACGCC-3′	EcoRI	Construction of P*pchR*::*lacZ* transcriptional fusion
P*feoA*_FW	5′-CGGAATTCGACAACCGCCCGGCC-3′	EcoRI	Construction of P*feoA*::*lacZ* transcriptional fusion
P*feoA*_RV	5′-GCTCTAGATGCGGTAGGAACGGGAC-3′	XbaI	Construction of P*feoA*::*lacZ* transcriptional fusion

All PCRs were performed using the genomic DNA of the PAO1 strain as template.
*The restriction site used for cloning is underlined in the primer sequence.

Corrections

REVIEW

Correction for "A post-Kyoto partner: Considering the stratospheric ozone regime as a tool to manage nitrous oxide," by David Kanter, Denise L. Mauzerall, A. R. Ravishankara, John S. Daniel, Robert W. Portmann, Peter M. Grabiel, William R. Moomaw, and James N. Galloway, which appeared in issue 12, March 19, 2013, of *Proc Natl Acad Sci USA* (110:4451–4457; first published February 25, 2013; 10.1073/pnas.1222231110).

The authors note that on page 4454, left column, 2nd full paragraph, lines 7–9, "For example, oxidation catalysts are able to reduce N_2O emissions ~70% compared with models without the technology (22)" should instead appear as "For example, advanced three-way catalysts are able to reduce N_2O emissions ~65% compared with models without the technology (22)."

The authors also note that ref. 22 should appear as:

22. Eggleston HS, Buendia L, Miwa K, Ngara T, Tanabe K, eds (2006) *IPCC Guidelines for National Greenhouse Gas Inventories, Volume 2: Energy* (Institute for Global Environmental Strategies, Hayama, Japan), p 3.22.

www.pnas.org/cgi/doi/10.1073/pnas.1317243110

BIOPHYSICS AND COMPUTATIONAL BIOLOGY

Correction for "Charge interactions can dominate the dimensions of intrinsically disordered proteins," by Sonja Müller-Späth, Andrea Soranno, Verena Hirschfeld, Hagen Hofmann, Stefan Rüegger, Luc Reymond, Daniel Nettels, and Benjamin Schuler, which appeared in issue 33, August 17, 2010, of *Proc Natl Acad Sci USA* (107:14609–14614; first published July 16, 2010; 10.1073/pnas.1001743107).

The authors note that Eq. **5** appeared incorrectly. The corrected equation appears below.

$$R_g = N^{0.5}\frac{\alpha\, b}{\sqrt{6}}\left(1+\rho\frac{Ka}{1+Ka}\right)$$

www.pnas.org/cgi/doi/10.1073/pnas.1317338110

MICROBIOLOGY

Correction for "Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity," by Francesco Imperi, Francesco Massai, Marcella Facchini, Emanuela Frangipani, Daniela Visaggio, Livia Leoni, Alessandra Bragonzi, and Paolo Visca, which appeared in issue 18, April 30, 2013, of *Proc Natl Acad Sci USA* (110:7458–7463; first published April 8, 2013; 10.1073/pnas.1222706110).

The authors note that that the following statement should be added to the end of page 7461, right column, line 2:

"While exogenously provided 5-fluorouracil is toxic to *P. aeruginosa* (39), it has been found to inhibit several *P. aeruginosa* virulence-related traits at subinhibitory concentrations (40), though pyoverdine-dependent virulence gene expression was not previously shown as 5-fluorouracil target. Given that 5-fluorouracil affected *P. aeruginosa* growth, while flucytosine did not (see ref. 39 and this work), further studies are required to decipher the different specificities, impacts, and modes of action of flucytosine and 5-fluorouracil treatments on this bacterial pathogen."

Additionally, the authors note that they omitted references to articles by West et al. and Ueda et al. The complete references appear below.

39. West TP, Chu CP (1986) Utilization of pyrimidines and pyrimidine analogues by fluorescent pseudomonads. *Microbios* 47(192-193):149–157.
40. Ueda A, Attila C, Whiteley M, Wood TK (2009) Uracil influences quorum sensing and biofilm formation in Pseudomonas aeruginosa and fluorouracil is an antagonist. *Microb Biotechnol* 2(1):62–74.

www.pnas.org/cgi/doi/10.1073/pnas.1316459110

Chapter 3

The Gac/Rsm and cyclic-di-GMP signalling networks coordinately regulate iron uptake in *Pseudomonas aeruginosa*

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Chapter 3
The Gac/Rsm and cyclic-di-GMP signaling networks coordinately regulate iron uptake in *Pseudomonas aeruginosa*

During the colonization and infection of the human host the opportunistic pathogen *P. aeruginosa* produces an arsenal of virulence factors; some of them (i.e. pyoverdine, the protease PrpL and the exotoxin A) are regulated at transcriptional level by the alternative sigma factor PvdS. The prominent role of PvdS in the *P. aeruginosa* virulence, as demonstrated in the previous chapter, makes the knowledge of *pvdS* regulation important to better understand *P. aeruginosa* pathogenicity. The best characterized mechanisms which control the transcription and the activity of this alternative sigma factor are the master regulator of iron metabolism Fur and pyoverdine signaling, respectively (Chapter 1). Recently, new potential PvdS regulators have been identified, such as the master regulator of sulfur metabolism CysB (Imperi *et al*., 2010), the extracellular lipase LipA (Funken *et al*., 2011) and the Gac regulatory network (Brencic *et al*., 2009; Burrowes *et al*., 2006). Regarding the Gac/Rsm system, its effect on *pvdS* expression is still debated, since two independent transcriptomic analyses, carried out by two different research groups, provided opposite results (Brencic *et al*., 2009; Burrowes *et al*., 2006). The main aim of the present work was to clarify the role of the Gac system in *pvdS* expression, and to investigate whether this regulatory network also affects other iron uptake systems. This work represents the first clear evidence that the Gac system positively modulates the expression of iron uptake genes in *P. aeruginosa*. Our findings indicate that *P. aeruginosa* has evolved an impressive capacity to finally tune and integrate the expression of regulons implicated in virulence.

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The Gac/Rsm and cyclic-di-GMP signalling networks coordinately regulate iron uptake in *Pseudomonas aeruginosa*

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Summary

Pseudomonas aeruginosa **is a versatile bacterial pathogen capable of occupying diverse ecological niches. To cope with iron limitation, *P. aeruginosa* secretes two siderophores, pyoverdine and pyochelin, whose ability to deliver iron to the cell is crucial for biofilm formation and pathogenicity. In this study, we describe a link between iron uptake and the Gac/Rsm system, a conserved signal transducing pathway of *P. aeruginosa* that controls the production of extracellular products and virulence factors, as well as the switch from planktonic to biofilm lifestyle. We have observed that pyoverdine and pyochelin production in *P. aeruginosa* is strongly dependent on the activation state of the Gac/Rsm pathway, which controls siderophore regulatory and biosynthetic genes at the transcriptional level, in a manner that does not involve regulation of ferric uptake regulator (Fur) expression. Gac/Rsm-mediated regulation of iron uptake genes appears to be conserved in different *P. aeruginosa* strains. Further experiments led to propose that the Gac/Rsm system regulates siderophore production through modulation of the intracellular levels of the second messenger c-di-GMP, indicating that the c-di-GMP and the Gac/Rsm regulatory networks essential for biofilm formation can also coordinately control iron uptake in *P. aeruginosa*.**

Received 4 February, 2013; revised 20 May, 2013; accepted 25 May, 2013. *For correspondence. E-mail francesco.imperi@uniroma1.it; Tel. +39 06 49917576; Fax +39 06 49917594. †These authors contributed equally to this work.

Introduction

Bacteria adapt to changing environments by tightly regulating gene expression. Sensing environmental stimuli is primarily achieved via two-component systems, which enable bacteria to quickly induce the transcription of genes needed to increase their fitness in a defined niche (Mikkelsen *et al.*, 2011). The opportunistic human pathogen *Pseudomonas aeruginosa* colonizes a variety of different environments, from humans to water and soil, representing a true example of highly versatile adaptability, which is reflected by the abundance in its chromosome of genes involved in transcriptional regulation or environmental sensing (Stover *et al.*, 2000).

In *P. aeruginosa*, the Gac/Rsm signalling network plays a major role in the transition from the planktonic to the biofilm lifestyle and, in a clinical setting, from acute to chronic infection (Coggan and Wolfgang, 2012). This regulatory network is based on a two-component system consisting of a response regulator, GacA, which is activated through phosphorylation by the cognate inner membrane sensor kinase GacS. GacS activity is influenced by two additional inner membrane sensor kinases, LadS and RetS, that have indirect opposite effects (positive and negative, respectively) on the GacA activation status (Ventre *et al.*, 2006). Although the signals that activate the three sensor kinases have not yet been identified, it has been proposed that the Gac/Rsm system may sense environmental cues and/or signals produced by the cells themselves in a population density-dependent manner (Goodman *et al.*, 2004; Kay *et al.*, 2006). Once activated, GacA only promotes the transcription of two small non-coding RNAs (sRNAs), RsmY and RsmZ (Brencic *et al.*, 2009), which act by titrating out the RNA-binding protein RsmA (Kay *et al.*, 2006). RsmA, in turn, binds to specific motifs (usually ANGGA) in single-stranded loops of target messenger RNAs (mRNAs), and when these motifs overlap the ribosome binding site, this results in the repression of translation (Kay *et al.*, 2006). RsmA directly

inhibits some *P. aeruginosa* phenotypes involved in sessile growth and/or chronic infection, such as exopolysaccharide production and type VI secretion (Brencic and Lory, 2009; Irie *et al*., 2010). Interestingly, RsmA also has positive effects on gene expression in *P. aeruginosa*. Production of rhamnolipids and lipases, surface motility and expression of type III secretion system and type IV pilus genes are positively regulated by RsmA (Heurlier *et al*., 2004; Brencic and Lory, 2009). However, the mechanism(s) by which this positive control occurs remains unclear. Although it is plausible that RsmA indirectly regulates these functions by affecting the expression of specific regulators (Brencic and Lory, 2009), it has also been proposed that besides affecting mRNA translation, RsmA binding to some messengers may somehow increase their half-life, ultimately resulting in a positive effect on gene expression (Heurlier *et al*., 2004).

Iron availability is an environmental cue that has a strong impact on *P. aeruginosa* physiology. In environments where iron is scarce or where its bioavailability is poor, including the body fluids of infected mammals, *P. aeruginosa* dramatically modifies its gene expression profile activating a number of pathways involved not only in iron uptake but also in virulence (Ochsner *et al*., 2002; Banin *et al*., 2005; Visca *et al*., 2007). A central role in this response is played by the transcriptional repressor ferric uptake regulator (Fur), whose relevance in *P. aeruginosa* physiology is inferred by the evidence that *fur* is an essential gene in this bacterium (Vasil and Ochsner, 1999). Fur requires ferrous ion as a co-repressor for dimerization and binding to a specific operator site (the Fur box) within target promoters, shutting down the transcription of target genes in iron-replete cells. However, only a minority of iron-regulated genes have a Fur box in their promoter region (Ochsner *et al*., 2002), and indeed, it has been shown that Fur can affect numerous genes indirectly through several Fur-regulated transcriptional regulators and sRNAs (reviewed in Cornelis *et al*., 2009). Genes responsible for the biogenesis of the two *P. aeruginosa* siderophores, pyochelin and pyoverdine, for instance, are indirectly regulated by Fur through the AraC/XylS-type regulator PchR and the alternative sigma factor PvdS, respectively, the latter being also involved in the transcriptional control of two well-characterized virulence factors, i.e. exotoxin A and protease PrpL (Lamont *et al*., 2002; Imperi *et al*., 2013).

Two independent transcriptomic analyses of *P. aeruginosa rsmA* mutants suggested that the loss of RsmA alters the mRNA levels of many genes, including some involved in iron acquisition (Burrowes *et al*., 2006; Brencic and Lory, 2009). However, while the *rsmA* mutation was found to negatively affect siderophore gene expression in *P. aeruginosa* PAO1 (Burrowes *et al*., 2006), an opposite effect was observed in the strain PAK (Brencic and Lory, 2009). Although both microarray analyses were performed under non-inductive culture conditions, i.e. in an iron-replete complex medium, such analyses differ in several parameters other than the genetic background, including the growth phase at which these experiments were performed (exponential for PAO1 and stationary for PAK), which could also have affected the available iron content in the growth medium (Brencic and Lory, 2009). It is worth mentioning that a connection between the Gac/Rsm signalling network and iron homeostasis has also been suggested in other *Pseudomonas* species (Liao *et al*., 1997; Hassan *et al*., 2010; Kong *et al*., 2012). Overexpression of *P. aeruginosa* RsmA in *P. syringae* caused a decrease in pyoverdine production (Kong *et al*., 2012), and similarly, reduced pyoverdine levels were observed in a *P. marginalis gacA* mutant (Liao *et al*., 1997). On the other hand, a transcriptomic analysis of *P. fluorescens* Pf-5 showed that the transcript abundance of the alternative sigma factor gene *pvdS* was highly increased in a *gacA* mutant compared with the parental strain, suggesting a negative effect of the Gac system on iron uptake (Hassan *et al*., 2010).

Given the discordant literature on the relationship between the Gac/Rsm system and iron uptake, the aim of this work was to investigate in more detail the role played by the Gac/Rsm system in iron regulation using the opportunistic pathogen *P. aeruginosa* as the model organism and siderophore production as the read-out. By deleting and/or overexpressing specific components of the Gac/Rsm regulatory cascade, we demonstrate that this system positively controls siderophore production and expression of siderophore genes in different *P. aeruginosa* strains and irrespective of the iron availability in the growth medium. We also show that the Gac/Rsm system controls the intracellular levels of the second messenger cyclic-di-GMP and that the Gac/Rsm-mediated regulation of iron uptake genes likely occurs through a still-uncharacterized cyclic-di-GMP-dependent regulatory mechanism.

Results

The Gac/Rsm system controls pyoverdine production

In order to investigate the role of the Gac/Rsm system in iron uptake regulation, we first assessed the effect of the deletion of genes encoding key players of this regulatory network on the production of the siderophore pyoverdine in the type strain *P. aeruginosa* PAO1 under iron-depleted conditions. A deletion of *rsmA* resulted in an increase in pyoverdine levels in culture supernatants, particularly during early exponential phase (8 h), when they were threefold higher than those from the wild type (Fig. 1A). In contrast, the PAO1 Δ*rsmY* Δ*rsmZ* double mutant

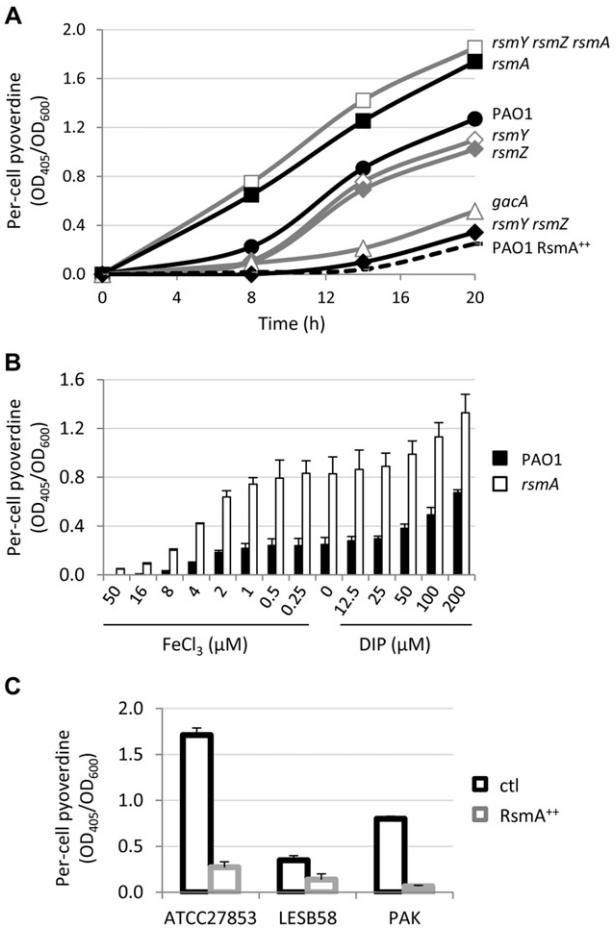

Fig. 1. The Gac/Rsm system affects pyoverdine production in *P. aeruginosa*.
A. Pyoverdine production (OD_{405}/OD_{600}) in *P. aeruginosa* PAO1, PAO1 overexpressing RsmA ($RsmA^{++}$) and selected isogenic mutants in the Gac/Rsm regulatory pathway (as indicated) grown in TSBD medium at 37°C. Values are the mean of at least three independent assays [standard deviation (SD) < 12% of the mean values].
B. Pyoverdine production (OD_{405}/OD_{600}) in *P. aeruginosa* PAO1 (black bars) and PAO1 Δ*rsmA* (white bars) grown for 8 h at 37°C in TSBD medium in the presence of increasing concentrations of $FeCl_3$ (0–50 μM) or 2,2-dipyridyl (DIP, 0–200 μM). Values represent the mean (± SD) of two independent assays performed in duplicate.
C. Pyoverdine production (OD_{405}/OD_{600}) in selected *P. aeruginosa* strains producing type II (ATCC27583), III (LESB58) or I (PAK) pyoverdine and containing a plasmid that overexpresses RsmA ($RsmA^{++}$) or the empty vector (ctl), grown in TSBD medium at 37°C for 14 (ATCC27583 and PAK) or 24 h (LESB58). Values represent the mean (± SD) of three assays.

produced very low levels of pyoverdine along the whole growth curve compared with the wild type (Fig. 1A). No significant growth differences were observed under iron-poor conditions between the wild type and mutant strains (Fig. S1). The PAO1 Δ*gacA* mutant and the Δ*rsmY* Δ*rsmZ* double mutant showed a similar behaviour, in line with the essential role of GacA in the transcription of the two small RNAs (Kay *et al*., 2006; Brencic *et al*., 2009). A triple Δ*rsmY* Δ*rsmZ* Δ*rsmA* deletion mutant displayed a pyoverdine production profile comparable with that of the single Δ*rsmA* mutant (Fig. 1A), suggesting that the pyoverdine-defective phenotype of the PAO1 Δ*rsmY* Δ*rsmZ* double mutant is directly associated to the increased availability of free RsmA protein in the absence of the negative control exerted by the two small RNAs. Accordingly, wild-type PAO1 overexpressing the *rsmA* gene produced very low levels of pyoverdine comparable with those observed in the PAO1 Δ*rsmY* Δ*rsmZ* mutant (Fig. 1A). Notably, single deletions of *rsmZ* or *rsmY* only slightly affect pyoverdine production (Fig. 1A), indicating that each sRNA is sufficient to counteract the negative effect of RsmA on pyoverdine production.

Variation of iron availability in the growth medium altered pyoverdine yields without affecting the RsmA-mediated regulation, as inferred by the systematically higher levels of pyoverdine in the supernatants of PAO1 Δ*rsmA* than the wild type after growth in the presence of increasing concentrations of $FeCl_3$ (increasing iron content) or the iron chelator 2,2′-dipyridyl (decreasing iron availability) (Fig. 1B). Moreover, overexpression of RsmA in *P. aeruginosa* prototypic strains producing different pyoverdine types (Bodilis *et al*., 2009) also resulted in a decrease in pyoverdine levels (Fig. 1C), suggesting that the negative regulation exerted by RsmA on pyoverdine production is conserved in *P. aeruginosa*.

As a whole, these experiments demonstrate that the Gac/Rsm system exerts a strong control over pyoverdine production in *P. aeruginosa*, which relies on the activity of the post-transcriptional regulator RsmA.

RsmA negatively affects pvdS transcription

To gain further insight into the mechanism of Gac/Rsm-dependent control of pyoverdine production, we compared the intracellular protein levels and promoter activities of selected genes involved in pyoverdine biogenesis between PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* by means of Western blotting and *lacZ* transcriptional fusion analyses. Intracellular levels of the biosynthetic enzyme PvdA and activity of the *pvdA* promoter showed a trend comparable with that observed for pyoverdine levels. With respect to the wild-type strain, both protein levels and promoter activities were markedly higher in the PAO1 Δ*rsmA* mutant during early exponential phase, while they were strongly reduced in the PAO1 Δ*rsmY* Δ*rsmZ* double mutant along the whole growth curve (Fig. 2A and B). The same activity profile was also observed in these mutants for the promoter of *pvdD*, another pyoverdine biosynthetic gene (data not shown). These results indicate that RsmA controls pyoverdine production by downregulating transcription of pyoverdine biosynthetic genes.

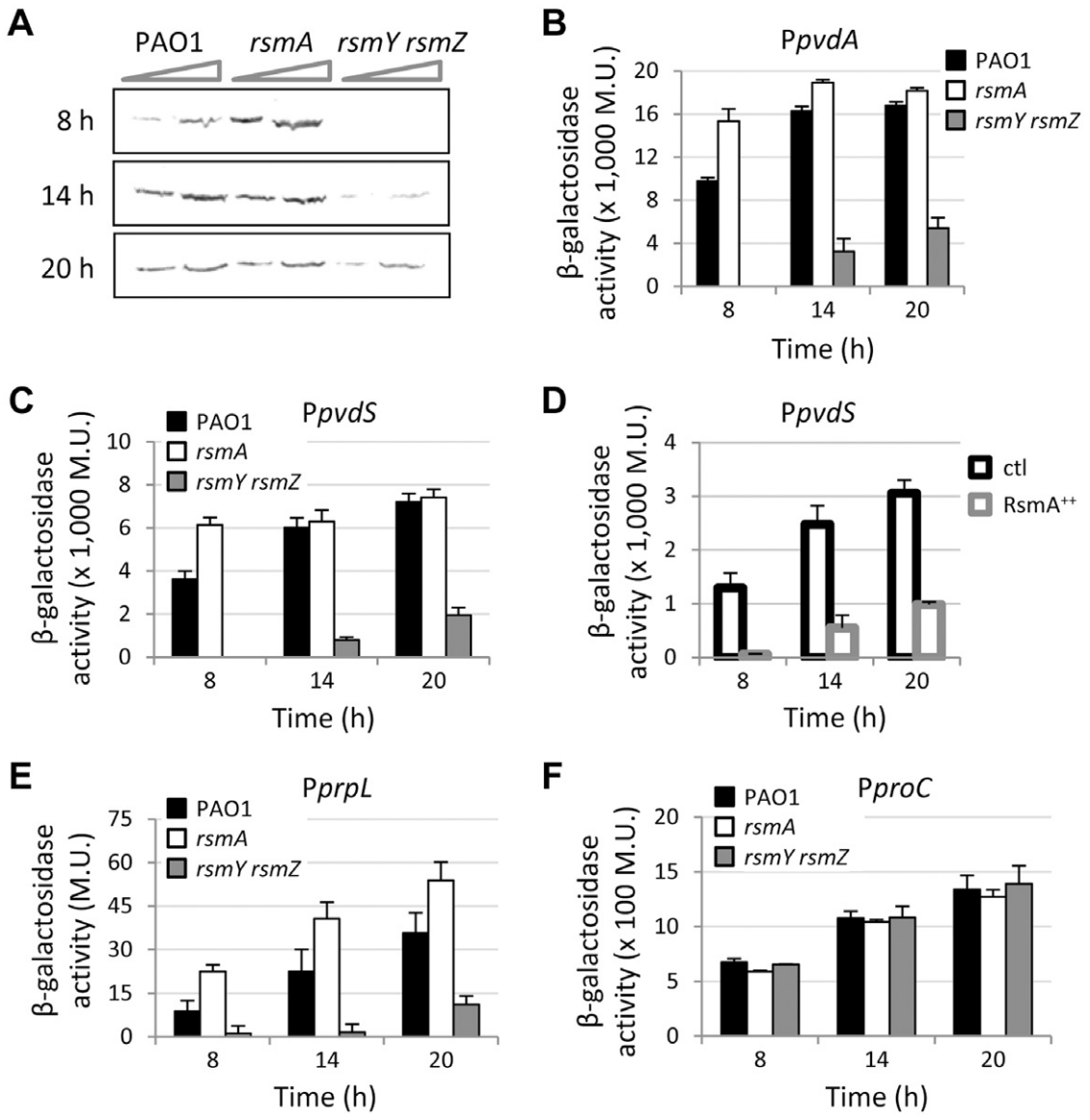

Fig. 2. The Gac/Rsm system controls pyoverdine production by regulating *pvdS* transcription.
A. Intracellular levels of PvdA in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* grown in TSBD medium at 37°C. PvdA levels were determined at different time points in growth by Western blot analysis of whole cell lysates (5–10 μg of proteins) with an anti-PvdA monoclonal antibody. The image is representative of two independent experiments showing similar results.
Activity of P*pvdA* (B), P*pvdS* (C) or P*prpL* (E) transcriptional fusions in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* (black, white and grey bars, respectively), cultured as in panel A.
D. Activity of the P*pvdS*::*lacZ* transcriptional fusion in *P. aeruginosa* PAO1 in the presence of a plasmid that overexpresses RsmA (RsmA^{++}) or the empty vector (ctl).
F. Activity of the P*proC'*-'*lacZ* translational fusion in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* (black, white and grey bars, respectively), cultured as in panel A. Values in panels B–F are expressed in Miller units (M.U.) and are the mean (± SD) of at least three independent assays.

Because transcription of pyoverdine genes is driven by the alternative sigma factor PvdS, we monitored the activity of the *pvdS* promoter in the above mutants using a P*pvdS*::*lacZ* transcriptional fusion. Activity of the *pvdS* promoter showed an opposite profile between the PAO1 Δ*rsmA* and Δ*rsmY* Δ*rsmZ* mutants, being higher at early stages of growth in the absence of RsmA and markedly reduced when the abundance of free RsmA is increased (PAO1 Δ*rsmY* Δ*rsmZ*) (Fig. 2C). Activity of the *pvdS* promoter was also negatively affected by the overexpression of RsmA in wild-type PAO1 (Fig. 2D). A comparable activity profile was also observed for a P*pvdS'*-'*lacZ* translational fusion (Fig. S2A), ruling out that the Gac/Rsm system also controls *pvdS* expression

at the post-transcriptional level. These results indicate that RsmA negatively affects pyoverdine production by reducing the transcription of *pvdS* and, as a result, of pyoverdine biosynthetic genes. As a further control, a transcriptional *lacZ* fusion to the promoter of *piv* (*prpL*), another PvdS-regulated gene encoding the PrpL protease IV, showed an expression profile similar to those observed for the promoters of the pyoverdine biosynthetic genes (Fig. 2E). Notably, the expression of the housekeeping gene *proC*, used as a control, was unaffected by the Gac/Rsm system (Fig. 2F; Pérez-Martínez and Haas, 2011), indicating that the observed regulation of the pyoverdine system mediated by RsmA cannot be ascribed to a generalized effect on gene expression or on the reporter system used.

RsmA also controls pyochelin production

In order to assess whether the Gac/Rsm system exerts a broader regulation on iron uptake in *P. aeruginosa*, we monitored the effects of altering the levels of free RsmA on the promoter activity of *pchR*, encoding an AraC/XylS-type regulator of pyochelin biosynthetic genes (Michel *et al*., 2005). As observed for pyoverdine genes (Fig. 2), under iron-depleted conditions the *pchR* promoter was also strongly repressed in the PAO1 Δ*rsmY* Δ*rsmZ* double mutant and in the RsmA-overexpressing PAO1 strain, while an increase in *pchR* promoter activity was observed in the Δ*rsmA* mutant compared with the wild type (Fig. 3A and B). As for *pvdS*, a P*pchR*'-'*lacZ* translational fusion showed an activity profile comparable with that of the transcriptional fusion (Fig. S2B), ruling out that the Gac/Rsm-mediated control on *pchR* expression also occurs at the post-transcriptional level. Consistent with the *pchR* gene expression analysis, relevant differences were observed in the pyochelin levels in culture supernatants between the wild type and the Δ*rsmA* and Δ*rsmY* Δ*rsmZ* mutants (Fig. 3C). Notably, the expression of another iron-regulated gene, i.e. *feoA*, which is involved in the uptake of ferrous iron (Cartron *et al*., 2006), was also found to be influenced by the activation state of the Gac/Rsm system (Fig. S3), suggesting that RsmA has a general effect on iron uptake regulation in *P. aeruginosa.*

RsmA does not control siderophore production through modulation of Fur expression

In order to investigate the mechanism by which RsmA exerts its control on target iron uptake genes, we first assessed whether RsmA directly affects the expression of the *pvdS* and *pchR* genes. To this aim, the transcriptional *lacZ* reporter fusions to *pvdS* and *pchR* promoters were introduced into *Escherichia coli* MC4100, together with a plasmid overexpressing RsmA or with the corresponding empty vector. Differently from what observed in *P. aeruginosa* (Figs 2D and 3B), the activity of the *pvdS* and *pchR* promoters under iron-depleted conditions was unaffected by RsmA in the heterologous host *E. coli* (Fig. 4A). As a positive control of RsmA activity in *E. coli*, we used a '*lacZ* translational fusion to PA4492, a *P. aeruginosa* gene known to be under the direct post-transcriptional control of RsmA (Brencic *et al*., 2009, Fig. S4). The reporter activity of this translational fusion was repressed in *E. coli* MC4100 cells carrying the plasmid overexpressing RsmA

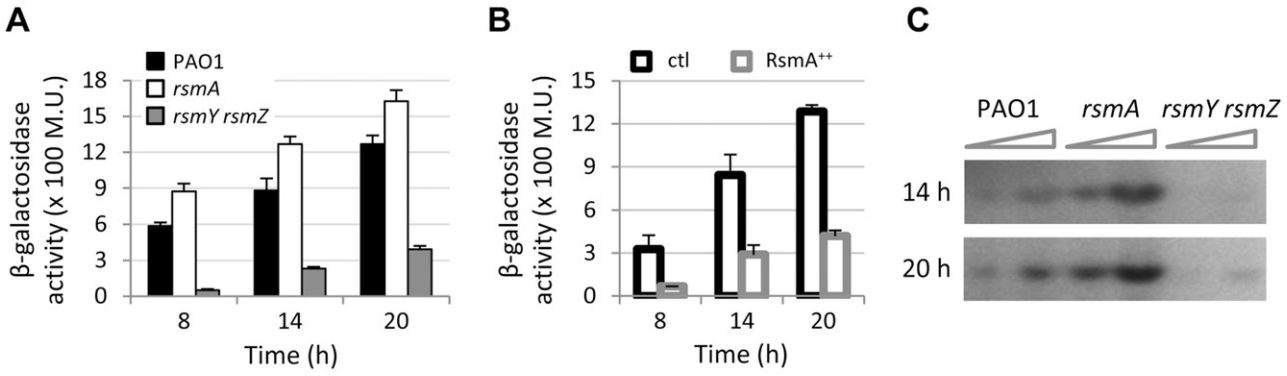

Fig. 3. The Gac/Rsm system regulates pyochelin production.
A. Activity of the P*pchR*::*lacZ* transcriptional fusion in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* (black, white, and grey bars, respectively) grown in TSBD medium at 37°C.
B. Activity of the P*pchR::lacZ* transcriptional fusion in *P. aeruginosa* PAO1 in the presence of a plasmid that overexpresses RsmA (RsmA^{++}) or the empty vector (ctl), cultured as in panel A. Values in panels A and B are expressed in Miller units (M.U.) and are the mean (± SD) of three independent assays.
C. Thin layer chromatography plate showing pyochelin levels in culture supernatants of *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* grown as described in panel A. Two different volumes (5 and 10 μl) of pyochelin extracted from 25 ml of culture supernatants and resuspended in 100 μl of methanol were loaded onto the plate. The image is representative of two independent experiments showing similar results.

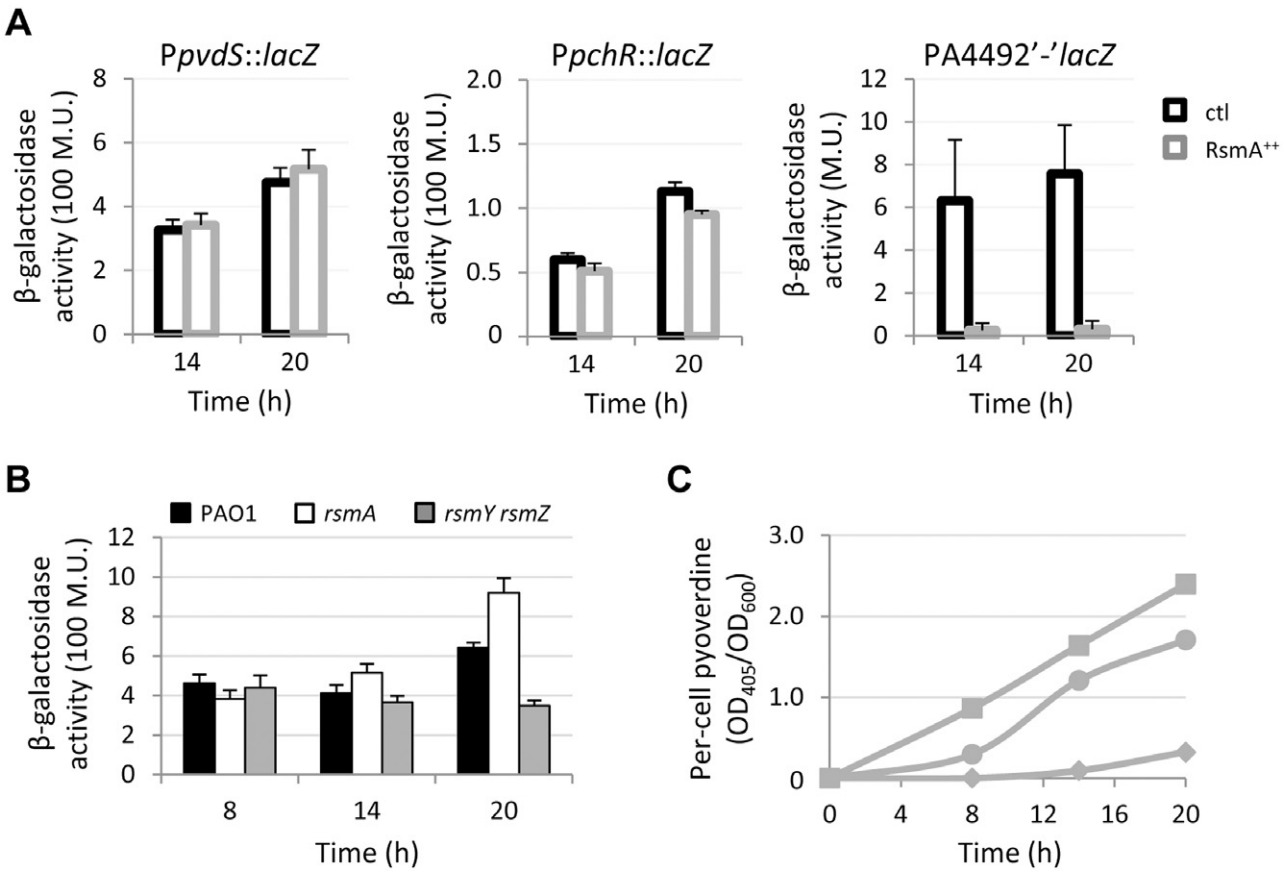

Fig. 4. RsmA-mediated regulation of iron uptake genes is indirect.
A. Activity of the P*pvdS*::*lacZ* and P*pchR*::*lacZ* transcriptional fusions and of the PA4492'-'*lacZ* translational fusion in *E. coli* MC4100 harbouring a plasmid that overexpresses RsmA (RsmA^{++}) or the empty vectors (ctl), grown in TSBD medium at 37°C.
B. Activity of a *fur*'-'*lacZ* translational fusion transcribed from the *fur* promoter (pME6014 *fur*'-'*lacZ*) in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* (black, white and grey bars, respectively), grown in TSBD medium at 37°C. Values in panels A and B are expressed in Miller units (M.U.) and are the mean (± SD) of at least three independent assays.
C. Pyoverdine production (OD_{405}/OD_{600}) in *P. aeruginosa* PAO1 ΔPA2384 (circles), PAO1 Δ*rsmA* ΔPA2384 (squares) and PAO1 Δ*rsmY* Δ*rsmZ* ΔPA2384 (diamonds) grown in TSBD medium at 37°C. Values are the mean of three independent assays (SD < 9% of the mean values).

but not in cells carrying the corresponding empty vector (Fig. 4A) confirming that *P. aeruginosa* RsmA retains its activity in *E. coli* cells. Taken together, these results ruled out a direct control of RsmA on the expression of the reporter fusions to target iron uptake genes, indicating that in *P. aeruginosa* RsmA acts on a regulatory factor that affects the promoter activity of both *pvdS* and *pchR* genes.

A possible effector of the RsmA-mediated regulation of iron uptake genes is Fur, which directly or indirectly controls almost all the iron-regulated genes in Gram-negative bacteria. Because Fur acts as a transcriptional repressor (Ochsner *et al.*, 1995) and taking into account the negative effect of RsmA on the promoter activity of siderophore genes (Figs 2 and 3), we first hypothesized that RsmA could downregulate these genes by positively affecting *fur* expression. Given that *fur* is an essential gene in *P. aeruginosa* (Vasil and Ochsner, 1999), we decided to verify such hypothesis by generating a translational reporter fusion between the entire *fur* gene, including its own promoter, and '*lacZ* (Table S1) in order to assess whether the Gac/Rsm system affects *fur* transcription, translation and/or mRNA stability. In our experimental setting, no differences were observed in the activity of the *fur'*-'*lacZ* reporter fusion between PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* during early stages of growth (8 and 14 h, Fig. 4B), i.e. when the Gac/Rsm system exerts its maximum effect on siderophore production (Figs 2 and 3). This evidence rules out that the observed regulation of iron uptake genes by RsmA occurs via modulation of Fur expression. Interestingly, during stationary phase, the activity of the *fur'*-'*lacZ* reporter fusion appeared ~40% higher in the PAO1 Δ*rsmA* and ~40% lower in the PAO1 Δ*rsmY* Δ*rsmZ* mutants compared with the wild type (Fig. 4B). This result suggests a negative effect of RsmA on *fur* expression, the contrary of what would be expected

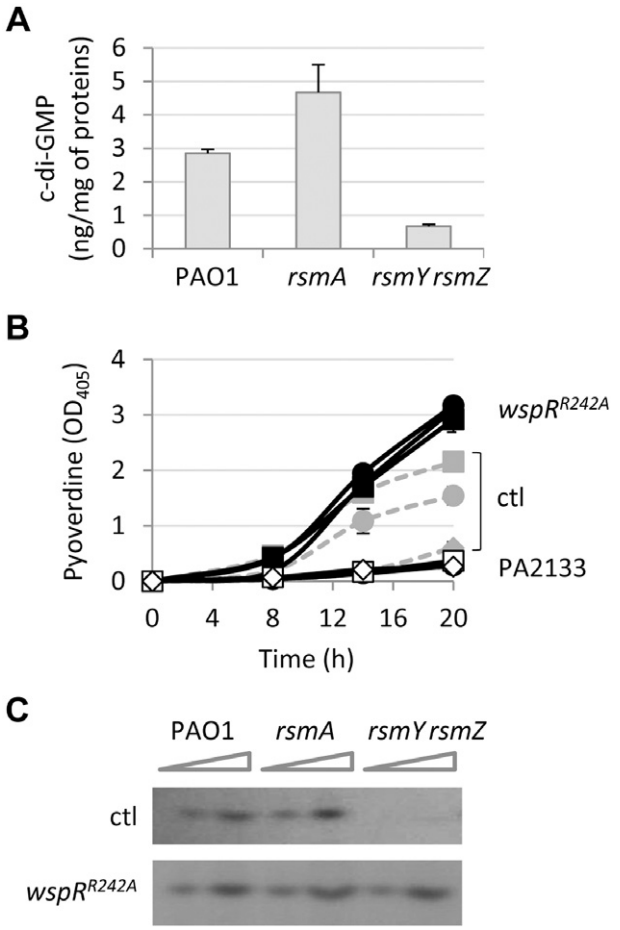

Fig. 5. The Gac/Rsm system controls siderophore production via c-di-GMP.
A. Relative intracellular levels of c-di-GMP (ng mg^{-1} of cellular proteins) in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* cells grown for 14 h in TSBD medium at 37°C. Results are the mean (± SD) of three independent assays.
B. Pyoverdine levels (OD_{405}) in culture supernatants of *P. aeruginosa* PAO1 (circles), PAO1 Δ*rsmA* (squares) and PAO1 Δ*rsmY* Δ*rsmZ* (diamonds) harbouring the empty vector pBBR1MCS-4 (ctl, dotted grey lines) or the same plasmid that constitutively expresses either the diguanylate cyclase $WspR^{R242A}$ (*wspR*^{R242A}, solid black lines, filled symbols) or the phosphodiesterase PA2133 (PA2133, solid black lines, empty symbols), grown in TSBD at 37°C. Results are the mean of at least three independent assays.
C. TLC plate showing pyochelin levels in culture supernatants of *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* harbouring a plasmid that expresses the constitutively active diguanylate cyclase $WspR^{R242A}$ (*wspR*^{R242A}) or the corresponding empty plasmid (ctl), cultured as in panel A. Two different volumes (5 and 10 μl) of pyochelin extracted from 25 ml of culture supernatants and resuspended in 100 μl of methanol were loaded onto the plate. The image is representative of two independent experiments showing similar results.

for an involvement of Fur in the RsmA-mediated repression of iron uptake genes. Therefore, although interesting *per se*, this finding was not investigated further in this work.

Besides Fur, a microarray analysis proposed the protein encoded by PA2384 as a possible additional regulator involved in the transcriptional control of both *pvdS* and *pchR* genes in *P. aeruginosa* PAO1 (Zheng *et al.*, 2007). We thus tested whether this protein was a potential effector of RsmA-mediated control on the siderophore genes by deleting the PA2384 gene in the wild-type, Δ*rsmA* and Δ*rsmY* Δ*rsmZ* backgrounds, and monitoring pyoverdine production in the resulting mutant strains. As shown in Fig. 4C, the deletion of the PA2384 gene had no effect on the ability of RsmA to affect pyoverdine production, thus ruling out the involvement of PA2384 in the Gac/Rsm-mediated control of iron uptake genes.

RsmA controls siderophore production through modulation of cyclic-di-GMP levels

Recently, two independent works showed that the Gac/Rsm system is interconnected with the cyclic-di-GMP (c-di-GMP) signalling network in *P. aeruginosa* (Moscoso *et al.*, 2011; Irie *et al.*, 2012) in line with a previous observation that the overexpression of a c-di-GMP phosphodiesterase (PDE) can suppress the swarming-negative phenotype of a *P. aeruginosa rsmA* mutant (Ryan *et al.*, 2006). Therefore, we investigated whether c-di-GMP could play a role in the Gac/Rsm-mediated control of iron uptake genes. We preliminarily investigated the effect of *rsmA* and *rsmY rsmZ* deletions on c-di-GMP levels in *P. aeruginosa* cells grown under iron depletion. As shown in Fig. 5A, c-di-GMP levels were higher in the PAO1 Δ*rsmA* mutant than in the wild type, while they were strongly reduced in PAO1 Δ*rsmY* Δ*rsmZ*, indicating that the Gac/Rsm pathway plays an important role in the control of c-di-GMP homeostasis under iron-depleted conditions. This result prompted us to assess the effect of c-di-GMP on siderophore production. To this aim, *P. aeruginosa* wild-type and mutant strains were transformed with a plasmid encoding the constitutively activated diguanylate cyclase (DGC) $WspR^{R242A}$, which has previously been used to artificially increase c-di-GMP levels in *P. aeruginosa* (Moscoso *et al.*, 2011). Overproduction of c-di-GMP led to a large increase of pyoverdine production in the PAO1 Δ*rsmY* Δ*rsmZ* mutant and, to a lesser extent, in the wild-type and PAO1 Δ*rsmA* strains (Fig. 5B). Notably, in the presence of $WspR^{R242A}$, the wild-type strain and the Δ*rsmA* and Δ*rsmY* Δ*rsmZ* mutants showed an identical pyoverdine production profile (Fig. 5B). In agreement with the earlier data demonstrating that the Gac/Rsm system affects pyoverdine production by controlling *pvdS* transcription (Fig. 2), the WspR-mediated increase in c-di-GMP levels was found to restore the activity of the P*pvdS*::*lacZ* transcriptional fusion in the Δ*rsmY* Δ*rsmZ* double mutant to levels comparable with those of the wild type and Δ*rsmA* mutant

strains (Fig. S5). Notably, a similar trend was also observed in the case of pyochelin production (Fig. 5C), highlighting the relevant role of c-di-GMP in the regulation of siderophore biosynthesis. This is further supported by the finding that the overexpression of a c-di-GMP degrading enzyme (the PDE PA2133; Moscoso *et al*., 2011) almost abolished pyoverdine production in all strains tested, irrespective of the activation state of the Gac/Rsm signalling system (Fig. 5B).

Discussion

The Gac/Rsm signalling cascade plays a key role in the control of *P. aeruginosa* lifestyle and pathogenicity. Several *P. aeruginosa* virulence-related traits are directly or indirectly controlled by the Gac/Rsm system through the post-transcriptional regulator RsmA (Heurlier *et al*., 2004; Kay *et al*., 2006), and a functional Gac/Rsm system is required for *P. aeruginosa* pathogenicity in different animal models (Rahme *et al*., 1995; Coleman *et al*., 2003; Mulcahy *et al*., 2008), raising the interest on this system as a potential drug target (Pérez-Martínez and Haas, 2011; Yamazaki *et al*., 2012).

In this work, we further characterized the regulatory network of the Gac/Rsm system in *P. aeruginosa* by describing a new link between Gac/Rsm, c-di-GMP and iron uptake. Although RsmA has previously been suggested to affect the expression of *P. aeruginosa* iron uptake genes by two different microarray analyses (Burrowes *et al*., 2006; Brencic and Lory, 2009), the actual regulatory activity of RsmA on iron uptake genes was still unclear. By using two complementary *P. aeruginosa* PAO1 mutant strains mirroring opposite activation states of the Gac/Rsm system, i.e. a Δ*rsmA* mutant and a Δ*rsmY* Δ*rsmZ* double mutant, we showed that RsmA inhibits the production of the siderophores pyoverdine and pyochelin by reducing the activity of the promoters of the two transcriptional regulators PvdS and PchR (Figs 1–3). The negative effect of RsmA on siderophore production occurs in a broad iron concentration range and has been observed in different *P. aeruginosa* strains (Fig. 1), suggesting that Gac/Rsm-mediated regulation on iron uptake genes likely involves an iron-independent regulative pathway conserved in *P. aeruginosa*.

Attempts to characterize the molecular mechanism of Gac/Rsm-mediated control of siderophore genes revealed that RsmA has indirect effects on *pvdS* and *pchR* transcription and that these effects do not rely on RsmA-mediated regulation of Fur expression (Fig. 4B). Moreover, because Fur structure and function are highly conserved in Gram-negative bacteria (Escolar *et al*., 1999), the inability of RsmA to repress *pvdS* and *pchR* transcription in *E. coli* (Fig. 4A) also rules out a direct involvement of RsmA in modulating Fur activity. However, because we found that, besides the siderophore genes, also another Fur-dependent gene involved in ferrous iron uptake is under the control of the Gac/Rsm system (Fig. S3), it cannot be excluded that RsmA might indirectly influence Fur activity through a still-uncharacterized *P. aeruginosa* regulatory circuit. Although not related to the observed effect of the Gac/Rsm system on iron uptake genes, RsmA was also found to slightly affect Fur expression at late stages of growth (Fig. 4B). In *E. coli*, Fur expression is controlled at the transcriptional level by several regulators, including Fur itself, the catabolite-activator protein (CAP) (de Lorenzo *et al*., 1988) and the oxidative stress regulators OxyR and SoxS (Zheng *et al*., 1999), and at the translational level by the sRNA RhyB (Vecerek *et al*., 2007). Fur expression and activity have been succinctly investigated in *P. aeruginosa*; further studies are thus required to verify whether any of the many proteins and/or cellular functions directly or indirectly affected by RsmA (Burrowes *et al*., 2006; Kay *et al*., 2006; Brencic and Lory, 2009) could somehow influence Fur expression and/or activity in this bacterium.

Although the specific intermediary regulatory factor(s) by which RsmA regulates *pvdS* and *pchR* promoters remains unknown, c-di-GMP appeared to play a role in Gac/Rsm-mediated control on iron uptake gene expression. c-di-GMP levels were found to be higher in a Δ*rsmA* mutant and strongly reduced in a Δ*rsmY* Δ*rsmZ* double mutant (Fig. 5A) in line with the increased c-di-GMP content previously reported in a *P. aeruginosa retS* mutant (Moscoso *et al*., 2011) and, very recently, in an *rsmA* mutant (Irie *et al*., 2012). Moreover, artificially induced dysregulation of c-di-GMP homeostasis by overexpression of a constitutively active DGC or of a c-di-GMP PDE abrogates the Gac/Rsm control of siderophore production (Fig. 5). Finally, while the two sRNAs RsmY and RsmZ have been previously found to be essential for the c-di-GMP-dependent switch between type III and type VI secretion systems in *P. aeruginosa* (Moscoso *et al*., 2011), c-di-GMP does not require a functional Gac/Rsm system to positively regulate iron uptake, as demonstrated by the fact that c-di-GMP overproduction efficiently promotes siderophore synthesis in an *rsmY rsmZ* defective background (Fig. 5). Taken together, our results indicates that the Gac/Rsm system regulates iron uptake genes by modulating the intracellular level of c-di-GMP, which in turn promotes siderophore production (Fig. 6).

c-di-GMP is a nearly ubiquitous intracellular signalling molecule in bacteria. It acts as a secondary messenger that controls fundamental bacterial behaviours such as motility, sessility and virulence. c-di-GMP is synthesized from GTP by DGCs and broken down by specific PDEs (Hengge, 2009; Schirmer and Jenal, 2009). The *P. aeruginosa* genome encodes more than 40 predicted

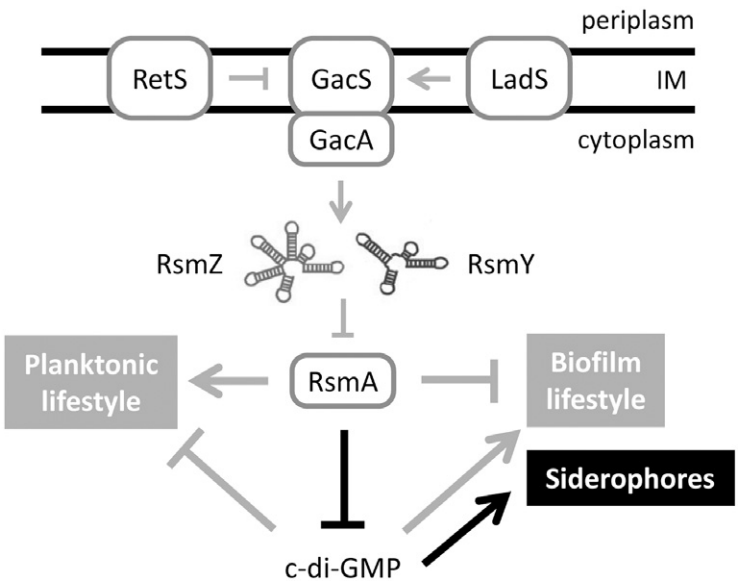

Fig. 6. Model depicting the link between the Gac/Rsm system, c-di-GMP and siderophore production. Grey lines indicate the existence of an already described regulation, and black lines highlight the connections described in this work. T-shaped lines represent negative control; arrows represent positive control.

proteins with potential DGC, PDE or DGC-PDE domains that could participate in c-di-GMP homeostasis (Kulasakara *et al*., 2006; Ryan *et al*., 2009). Although the involvement of c-di-GMP in iron uptake has not been previously investigated, there are some literature reports that indirectly support our findings. Several siderophore genes were shown to be upregulated by microarray analysis in a *P. aeruginosa wspF* mutant overproducing c-di-GMP (Hickman *et al*., 2005). Moreover, a *P. aeruginosa* mutant in the transmembrane signalling system YfiBNR, characterized by high intracellular c-di-GMP levels and a small colony variant phenotype, also showed enhanced pyoverdine and pyochelin production (Malone *et al*., 2010). Concomitant increase in both c-di-GMP and pyoverdine levels was also observed in a *P. aeruginosa* strain lacking PA4781, encoding a HD-GYP domain protein with predicted PDE activity (Ryan *et al*., 2009). As a whole, these results are in line with the proposed role of c-di-GMP as an activator of siderophore production in *P. aeruginosa*; further studies are however needed to unravel the specific mode of action and/or effector(s) involved in c-di-GMP-mediated control of iron uptake genes.

Besides the known functions directly regulated by RsmA at the post-transcriptional level, such as the regulation of genes involved in exopolysaccharide production and type VI secretion (Brencic and Lory, 2009; Irie *et al*., 2010), RsmA also indirectly affects, by still unknown mechanism(s), a number of *P. aeruginosa* phenotypes, including quorum sensing, protein secretion, cytotoxicity and motility (Mikkelsen *et al*., 2011). Given the observed role of the Gac/Rsm system in c-di-GMP homeostasis (Fig. 5) and considering that RsmA and c-di-GMP display partially overlapping regulons in *P. aeruginosa* (Coggan and Wolfgang, 2012), it is tempting to speculate that c-di-GMP could be the mediator of Gac/Rsm-dependent regulation not only of iron uptake genes but also of other genes indirectly affected by RsmA.

Our study shows that the Gac/Rsm system and c-di-GMP act in a concerted way to regulate iron uptake in *P. aeruginosa* (Fig. 6). Both c-di-GMP and Gac/Rsm signalling networks control the *P. aeruginosa* switch between planktonic and biofilm lifestyles, as well as between acute virulence and chronic persistence during infection (Ventre *et al*., 2006; Hengge, 2009; Mikkelsen *et al*., 2011; Coggan and Wolfgang, 2012). In particular, high c-di-GMP levels and activation of the two-component system GacS/GacA reduce virulence and motility while inducing biofilm formation (Coggan and Wolfgang, 2012). Our results demonstrate that they also act in concert to promote siderophore production (Figs 1, 3 and 5).

What could be the biological significance of coupling biofilm development to enhanced siderophore production? Several lines of evidence indicate the importance of siderophores in *P. aeruginosa* biofilms. Siderophore genes are expressed by *P. aeruginosa* biofilm-forming cells (Banin *et al*., 2005; Yang *et al*., 2009), and siderophore-mediated iron uptake is essential for biofilm formation under iron depletion (Banin *et al*., 2005; Kaneko *et al*., 2007). Moreover, *P. aeruginosa* cells living in biofilms are more susceptible to iron limitation than planktonic cells (Patriquin *et al*., 2008), suggesting that biofilm-forming cells either require more iron for their metabolism or are disadvantaged in iron acquisition compared with planktonic cells. Linking siderophore gene expression to regulatory mechanisms crucial for switching from motile to biofilm lifestyles would therefore represent a strategy to guarantee the iron uptake potential required during biofilm

development. Siderophores were also found to be important for biofilm formation in other bacteria (Ojha and Hatfull, 2007; May and Okabe, 2011), and Csr/Rsm and c-di-GMP are key components of widespread bacterial signalling networks promoting the biofilm lifestyle (Römling, 2012; Romeo *et al*., 2013). Whether coordinated expression of siderophore and biofilm-related genes might represent a common regulatory strategy in biofilm-forming bacteria is an interesting question that deserves future research.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. Bacteria were routinely grown in LB medium (Sambrook *et al*., 1989). The iron-depleted complex medium trypticase soy broth dialysate (TSBD) was used as iron-poor medium (Imperi *et al*., 2010) to which $FeCl_3$ or 2,2-dipyridyl were added at the indicated concentrations when required. Unless otherwise stated, bacteria were grown at 37°C in microtitre plates under static conditions. Antibiotics were added at the following concentrations for work with *E. coli*, with the concentrations used for *P. aeruginosa* shown in brackets: ampicillin, 100 μg ml^{-1}; carbenicillin (250 μg ml^{-1}); chloramphenicol 30 μg ml^{-1} (350 μg ml^{-1}); gentamicin, 15 μg ml (200 μg ml^{-1}); nalidixic acid, 20 μg ml^{-1}; tetracycline 12 μg ml^{-1} (100 μg ml^{-1}).

Deletion mutants and reporter plasmids

Recombinant DNA was manipulated as described elsewhere (Sambrook *et al*., 1989). All the primers and restriction sites used for PCR and cloning are listed in Table S2. Deletion mutants in *rsmA*, *rsmY*, *rsmZ*, *gacA* and PA2384 were generated using the suicide plasmids pZH13, pME3087Δ*rsmY*, pME3332, pME6111 and pME9643, respectively (Table S1). Gene replacements in *P. aeruginosa* were verified by PCR and DNA sequencing. Reporter plasmids pMP220::P*pvdS*, pMP220::P*pvdA*, pMP190::P*pvdD*, pMP220::P*feoA*, pPZ-*prpL*, pME3641 have been previously described (Table S1). The P*pchR-lacZ* transcriptional fusion was generated by amplifying by PCR and cloning a 573-bp DNA fragment encompassing the entire *pchR* promoter into the promoter probe plasmid pMP220 (Spaink *et al*., 1987). The PA4492'-'*lacZ* translational fusion was generated by amplifying by PCR and cloning a 511-bp DNA fragment encompassing the promoter and the start codon of PA4492 into the plasmid pME6014 (Schnider-Keel *et al*., 2000) in frame with the truncated '*lacZ* gene. The pME6014::*fur*'-'*lacZ* plasmid encoding a Fur-'LacZ fusion protein expressed from the *fur* promoter was generated by amplifying by PCR and cloning into the plasmid pME6014 a 500-bp DNA fragment encompassing the promoter and the whole coding sequence of *fur* minus the last two codons fused in frame with the truncated '*lacZ* gene. All constructs were verified by DNA sequencing. Plasmid pME 3849 was used for RsmA overexpression experiments using the empty plasmid pME6001 as negative control (Table S1).

β-galactosidase assays

The β-galactosidase activity from *P. aeruginosa* cells carrying the different reporter plasmids (Table S1) was determined spectrophotometrically using *o*-nitrophenyl-β-D-galactopyranoside as the substrate, normalized to the OD_{600} of the bacterial culture and expressed in Miller units (Miller, 1972).

Western blot analysis

Appropriate volumes of bacterial cultures were centrifuged, and pellets were suspended in SDS-PAGE loading buffer (0.25 M Tris-HCl, pH 6.8; 2% SDS; 10% β-mercaptoethanol; 20% glycerol) for SDS-PAGE analysis of whole-cell extracts. Pellets from identical culture volumes were also collected to determine the cellular protein concentration for each sample by using the DC protein assay kit (Bio-Rad, Milan, Italy) and bovine serum albumin as the standard. Volumes of SDS-PAGE samples corresponding to 5 or 10 μg of proteins were loaded onto the gel. Proteins resolved by SDS-PAGE were electrotransferred onto a nitrocellulose filter (Hybond-C extra, Amersham, Milan, Italy) and probed for PvdA using a mouse monoclonal anti-PvdA antibody (Putignani *et al*., 2004). Filters were developed with 5-bromo-4-chloro-3-indoyl-phosphate and nitro blue tetrazolium chloride reagents for colorimetric alkaline phosphatase detection (Promega, Milan, Italy).

Pyoverdine, pyochelin and c-di-GMP quantifications

Pyoverdine quantities were measured as the OD_{405} of culture supernatants appropriately diluted in 0.1 M Tris-HCl (pH 8) (Imperi *et al*., 2009). Pyochelin was isolated by ethyl acetate extraction of acidified culture supernatants (Zhang *et al*., 2005). After evaporation, the dry residue was suspended in 100 μl of methanol. Pyochelin extracts (5–10 μl) were applied to a Silica Gel ($60F_{254}$) thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany) using acetone : methanol : 0.2 M acetic acid (5:2:1) as the mobile phase (Zhang *et al*., 2005). Pyochelin on TLC plates was qualitatively characterized by iron-binding capacity when sprayed with 0.1 M $FeCl_3$ in 0.1 M HCl resulting in red-brown spots (Cox and Graham, 1979). Intracellular levels of c-di-GMP were determined by liquid chromatography coupled with tandem mass spectrometry, as described (Spangler *et al*., 2010), and normalized to the corresponding cellular protein content determined using the DC protein assay kit (Bio-Rad) and bovine serum albumin as the standard.

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References

Banin, E., Vasil, M.L., and Greenberg, E.P. (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A* **102:** 11076–11081.

Bodilis, J., Ghysels, B., Osayande, J., Matthijs, S., Pirnay, J.P., Denayer, S., *et al*. (2009) Distribution and evolution of ferripyoverdine receptors in *Pseudomonas aeruginosa*. *Environ Microbiol* **11:** 2123–2135.

Brencic, A., and Lory, S. (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* **72:** 612–632.

Brencic, A., McFarland, K.A., McManus, H.R., Castang, S., Mogno, I., Dove, S.L., and Lory, S. (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol* **73:** 434–445.

Burrowes, E., Baysse, C., Adams, C., and O'Gara, F. (2006) Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. *Microbiology* **152:** 405–418.

Cartron, M.L., Maddocks, S., Gillingham, P., Craven, C.J., and Andrews, S.C. (2006) Feo – transport of ferrous iron into bacteria. *Biometals* **19:** 143–157.

Coggan, K.A., and Wolfgang, M.C. (2012) Global regulatory pathways and cross-talk control *Pseudomonas aeruginosa* environmental lifestyle and virulence phenotype. *Curr Issues Mol Biol* **14:** 47–70.

Coleman, F.T., Mueschenborn, S., Meluleni, G., Ray, C., Carey, V.J., Vargas, S.O., *et al*. (2003) Hypersusceptibility of cystic fibrosis mice to chronic *Pseudomonas aeruginosa* oropharyngeal colonization and lung infection. *Proc Natl Acad Sci U S A* **100:** 1949–1954.

Cornelis, P., Matthijs, S., and Van Oeffelen, L. (2009) Iron uptake regulation in *Pseudomonas aeruginosa*. *Biometals* **22:** 15–22.

Cox, C.D., and Graham, R. (1979) Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. *J Bacteriol* **137:** 357–364.

De Lorenzo, V., Herrero, M., Giovannini, F., and Neilands, J.B. (1988) Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. *Eur J Biochem* **173:** 537–546.

Escolar, L., Pérez-Martín, J., and de Lorenzo, V. (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein. *J Bacteriol* **181:** 6223–6229.

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

Hassan, K.A., Johnson, A., Shaffer, B.T., Ren, Q., Kidarsa, T.A., Elbourne, L.D., *et al*. (2010) Inactivation of the GacA response regulator in *Pseudomonas fluorescens* Pf-5 has far-reaching transcriptomic consequences. *Environ Microbiol* **12:** 899–915.

Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7:** 263–273.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., *et al*. (2004) Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186:** 2936–2945.

Hickman, J.W., Tifrea, D.F., and Harwood, C.S. (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci U S A* **102:** 14422–14427.

Imperi, F., Tiburzi, F., and Visca, P. (2009) Molecular basis of pyoverdine siderophore recycling in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **106:** 20440–20445.

Imperi, F., Tiburzi, F., Fimia, G.M., and Visca, P. (2010) Transcriptional control of the *pvdS* iron starvation sigma factor gene by the master regulator of sulfur metabolism CysB in *Pseudomonas aeruginosa*. *Environ Microbiol* **12:** 1630–1642.

Imperi, F., Massai, F., Facchini, M., Frangipani, E., Visaggio, D., Leoni, L., *et al*. (2013) Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity. *Proc Natl Acad Sci U S A* **110:** 7458–7463.

Irie, Y., Starkey, M., Edwards, A.N., Wozniak, D.J., Romeo, T., and Parsek, M.R. (2010) *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol Microbiol* **78:** 158–172.

Irie, Y., Borlee, B.R., O'Connor, J.R., Hill, P.J., Harwood, C.S., Wozniak, D.J., and Parsek, M.R. (2012) Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **109:** 20632–20636.

Kaneko, Y., Thoendel, M., Olakanmi, O., Britigan, B.E., and Singh, P.K. (2007) The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. *J Clin Invest* **117:** 877–888.

Kay, E., Humair, B., Dénervaud, V., Riedel, K., Spahr, S., Eberl, L., *et al*. (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* **188:** 6026–6033.

Kong, H.S., Roberts, D.P., Patterson, C.D., Kuehne, S.A., Heeb, S., Lakshman, D.K., and Lydon, J. (2012) Effect of overexpressing rsmA from *Pseudomonas aeruginosa* on virulence of select phytotoxin-producing strains of *P. syringae*. *Phytopathology* **102:** 575–587.

Kulasakara, H., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., *et al*. (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′-5′)-cyclic-GMP in virulence. *Proc Natl Acad Sci U S A* **103:** 2839–2844.

Lamont, I.L., Beare, P.A., Ochsner, U., Vasil, A.I., and Vasil, M.L. (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **99:** 7072–7077.

Liao, C.H., McCallus, D.E., Fett, W.F., and Kang, Y. (1997) Identification of gene loci controlling pectate lyase production and soft-rot pathogenicity in *Pseudomonas marginalis*. *Can J Microbiol* **43:** 425–431.

Malone, J.G., Jaeger, T., Spangler, C., Ritz, D., Spang, A., Arrieumerlou, C., *et al*. (2010) YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. *PLoS Pathog* **6:** e1000804.

May, T., and Okabe, S. (2011) Enterobactin is required for biofilm development in reduced-genome *Escherichia coli*. *Environ Microbiol* **13:** 3149–3162.

Michel, L., González, N., Jagdeep, S., Nguyen-Ngoc, T., and Reimmann, C. (2005) PchR-box recognition by the AraC-type regulator PchR of *Pseudomonas aeruginosa* requires the siderophore pyochelin as an effector. *Mol Microbiol* **58:** 495–509.

Mikkelsen, H., Sivaneson, M., and Filloux, A. (2011) Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ Microbiol* **13:** 1666–1681.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory, pp. 252–255.

Moscoso, J.A., Mikkelsen, H., Heeb, S., Williams, P., and Filloux, A. (2011) The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretion via c-di-GMP signalling. *Environ Microbiol* **13:** 3128–3138.

Mulcahy, H., O'Callaghan, J., O'Grady, E.P., Maciá, M.D., Borrell, N., Gómez, C., *et al*. (2008) *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation. *Infect Immun* **76:** 632–638.

Ochsner, U.A., Vasil, A.I., and Vasil, M.L. (1995) Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. *J Bacteriol* **177:** 7194–7201.

Ochsner, U.A., Wilderman, P.J., Vasil, A.I., and Vasil, M.L. (2002) GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. *Mol Microbiol* **45:** 1277–1287.

Ojha, A., and Hatfull, G.F. (2007) The role of iron in *Mycobacterium smegmatis* biofilm formation: the exochelin siderophore is essential in limiting iron conditions for biofilm formation but not for planktonic growth. *Mol Microbiol* **66:** 468–483.

Patriquin, G.M., Banin, E., Gilmour, C., Tuchman, R., Greenberg, E.P., and Poole, K. (2008) Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. *J Bacteriol* **190:** 662–671.

Pérez-Martínez, I., and Haas, D. (2011) Azithromycin inhibits expression of the GacA-dependent small RNAs RsmY and RsmZ in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **55:** 3399–3405.

Putignani, L., Ambrosi, C., Ascenzi, P., and Visca, P. (2004) Expression of L-ornithine N^{δ}-oxygenase (PvdA) in fluorescent *Pseudomonas* species: an immunochemical and *in silico* study. *Biochem Biophys Res Commun* **313:** 245–257.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., and Ausubel, F.M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268:** 1899–1902.

Romeo, T., Vakulskas, C.A., and Babitzke, P. (2013) Post-transcriptional regulation on a global scale: form and function of Csr/Rsm systems. *Environ Microbiol* **15:** 313–324.

Römling, U. (2012) Cyclic di-GMP, an established secondary messenger still speeding up. *Environ Microbiol* **14:** 1817–1829.

Ryan, R.P., Fouhy, Y., Lucey, J.F., Crossman, L.C., Spiro, S., He, Y.W., *et al*. (2006) Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc Natl Acad Sci U S A* **103:** 6712–6717.

Ryan, R.P., Lucey, J., O'Donovan, K., McCarthy, Y., Yang, L., Tolker-Nielsen, T., and Dow, J.M. (2009) HD-GYP domain proteins regulate biofilm formation and virulence in *Pseudomonas aeruginosa*. *Environ Microbiol* **11:** 1126–1136.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York, NY, USA: Cold Spring Harbor Laboratory.

Schirmer, T., and Jenal, U. (2009) Structural and mechanistic determinants of c-di-GMP signalling. *Nat Rev Microbiol* **7:** 724–735.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., *et al*. (2000) Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J Bacteriol* **182:** 1215–1225.

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E., and Lugtenberg, B.J.J. (1987) Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol Biol* **9:** 27–39.

Spangler, C., Böhm, A., Jenal, U., Seifert, R., and Kaever, V. (2010) A liquid chromatography-coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine monophosphate. *J Microbiol Methods* **81:** 226–231.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., *et al*. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406:** 959–964.

Vasil, M.L., and Ochsner, U.A. (1999) The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* **34:** 399–413.

Vecerek, B., Moll, I., and Bläsi, U. (2007) Control of Fur synthesis by the non-coding RNA RyhB and iron-responsive decoding. *EMBO J* **26:** 965–975.

Ventre, I., Goodman, A.L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., *et al*. (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci USA* **103:** 171–176.

Visca, P., Imperi, F., and Lamont, I.L. (2007) Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol* **15:** 22–30.

Yamazaki, A., Li, J., Zeng, Q., Khokhani, D., Hutchins, W.C., Yost, A.C., *et al*. (2012) Derivatives of plant phenolic compound affect the type III secretion system of *Pseudomonas aeruginosa* via a GacS-GacA two-component signal transduction system. *Antimicrob Agents Chemother* **56:** 36–43.

Yang, L., Nilsson, M., Gjermansen, M., Givskov, M., and Tolker-Nielsen, T. (2009) Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginosa* biofilm formation. *Mol Microbiol* **74:** 1380–1392.

Zhang, S., Chen, Y., Potvin, E., Sanschagrin, F., Levesque, R.C., McCormack, F.X., and Lau, G.W. (2005) Comparative signature-tagged mutagenesis identifies *Pseudomonas* factors conferring resistance to the pulmonary collectin SP-A. *PLoS Pathog* **1:** 259–268.

Zheng, M., Doan, B., Schneider, T.D., and Storz, G. (1999) OxyR and SoxRS regulation of *fur*. *J Bacteriol* **181:** 4639–4643.

Zheng, P., Sun, J., Geffers, R., and Zeng, A.P. (2007) Functional characterization of the gene PA2384 in large-scale gene regulation in response to iron starvation in *Pseudomonas aeruginosa*. *J Biotechnol* **132:** 342–352.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Growth (OD_{600}) of *P. aeruginosa* PAO1 (circles), PAO1 Δ*rsmA* (squares) and PAO1 Δ*rsmY* Δ*rsmZ* (diamonds) at 37°C in TSBD medium. Values represent the mean (± standard deviation) of at least three independent assays.

Fig. S2. Activity of (A) P*pvdS*'-'*lacZ* and (B) P*pchR*'-'*lacZ* translational fusions in *P. aeruginosa* PAO1 (black bars), PAO1 Δ*rsmA* (white bars) and PAO1 Δ*rsmY* Δ*rsmZ* (grey bars) grown in TSBD medium at 37°C. Values are the mean (± standard deviation) of three independent assays.

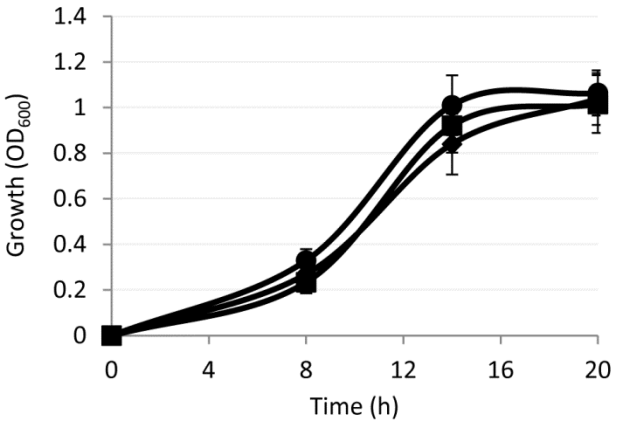
Fig. S3. Activity of the P*feoA*::*lacZ* translational fusions in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* grown for 14 h in TSBD medium at 37°C. Values are the mean (± standard deviation) of three independent experiments performed in duplicate.

Fig. S4. Activity of the PA4492'-'*lacZ* translational fusions in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* grown for 8 h in TSBD medium at 37°C. Values are the mean (± standard deviation) of three independent assays.

Fig. S5. Activity of the P*pvdS*::*lacZ* translational fusions in *P. aeruginosa* PAO1 (black bars), PAO1 Δ*rsmA* (white bars) and PAO1 Δ*rsmY* Δ*rsmZ* (grey bars) harbouring a plasmid that constitutively expresses the diguanylate cyclase $WspR^{R242A}$ (*wspR*^{R242A}) or the empty plasmid (ctl), grown for 14 h in TSBD at 37°C. Results are the mean (± standard deviation) of four independent assays.

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Primers used in this study[1].



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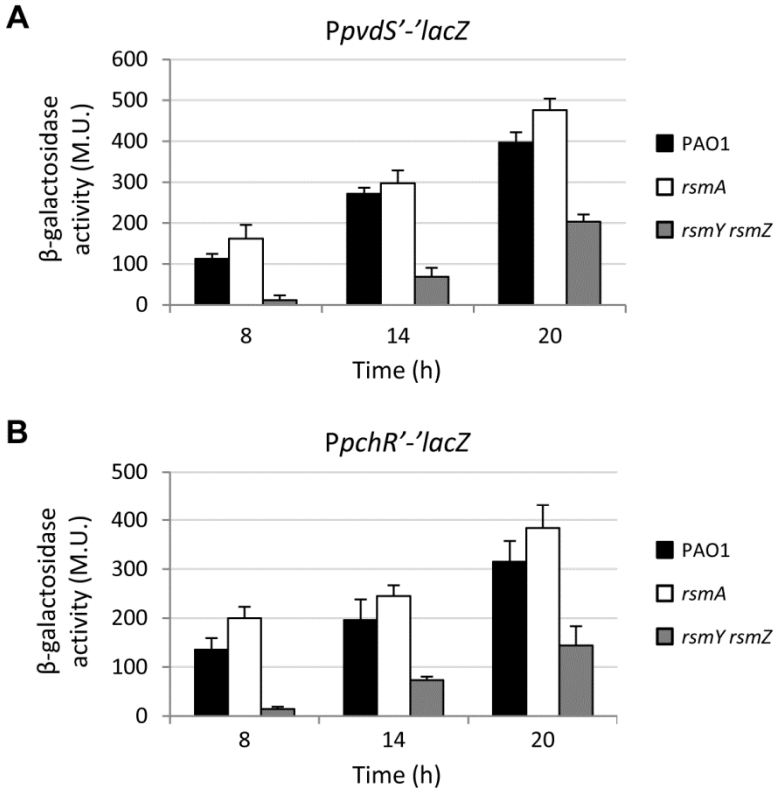

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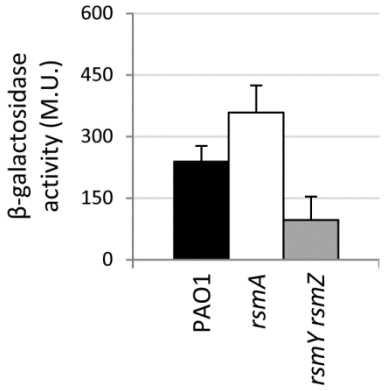
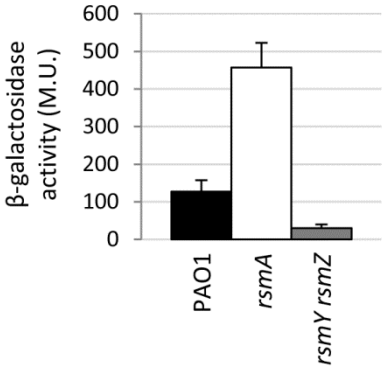


Figure S3. Activity of the P*feoA*::*lacZ* transcriptional fusion in *P. aeruginosa* PAO1, PAO1 Δ*rsmA* and PAO1 Δ*rsmY* Δ*rsmZ* grown for 14 h in TSBD medium at 37 °C. Values are the mean (± SD) of three independent experiments performed in duplicate.



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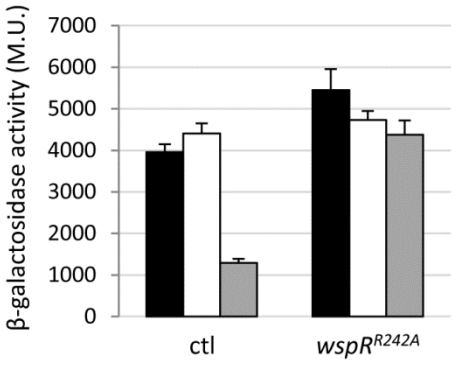

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Table S1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristics	Reference or source
P. aeruginosa		
PAO1 (ATCC15692)	Prototroph	American type culture collection
PAO1 Δ*rsmA*	PAO1 deleted of the *rsmA* coding sequence	This study
PAO1 Δ*gacA*	PAO1 deleted of the *gacA* coding sequence	This study
PAO1 Δ*rsmY*	PAO1 deleted of *rsmY*	This study
PAO1 Δ*rsmZ*	PAO1 deleted of *rsmZ*	This study
PAO1 Δ*rsmY* Δ*rsmZ*	PAO1 Δ*rsmY* deleted of the *rsmZ* coding sequence	This study
PAO1 Δ*rsmY* Δ*rsmZ* Δ*rsmA*	PAO1 Δ*rsmY rsmZ* deleted of the *rsmA* coding sequence	This study
PAO1 Δ2384	PAO1 deleted of the PA2384 coding sequence	This study
PAO1 Δ*rsmA* Δ2384	PAO1 Δ*rsmA* deleted of the PA2384 coding sequence	This study
PAO1 Δ*rsmY* Δ*rsmZ* Δ2384	PAO1 Δ*rsmY* Δ*rsmZ* deleted of the PA2384 coding sequence	This study
E. coli		
S17.1λ*pir*	*thi pro hsdR hsdM*$^+$ *recA RP4-2-Tc::Mu-Km::Tn7 λpir, Gm*^{R}	Simon *et al.*, 1983
DH5αF′	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169[ϕ*80 dlacZ*Δ*M15*], Nal^R	Liss, 1987
MC4100	*araD139 rpsL150 relA1 flbB5301 deoC1 pstF25 rbsR* Δ(*lacZYA-argF*)*U169* Str^r	Casadaban and Cohen, 1980
Plasmid		
pBluescript-II KS+	Cloning vector; ColE1 replicon; Ap^R	Stratagene
pBBR1MCS-4	Broad-host range cloning vector; Ap^R	Kovach *et al.*, 1995
pMP220	Broad-host-range, low-copy-number promoter-probe vector, IncP replicon, *lacZ* Tc^r Tra$^-$	Spaink *et al.*, 1987
pME6014	Cloning vector for translational *lacZ* fusions; Tc^R	Schnider-Keel *et al.*, 2000
pME3641	Translational P*proC*′-′*lacZ* fusion	Savioz *et al.*, 1993
pME6001	Cloning vector derived from pBRR1MCS, Gm^R	Blumer *et al*., 1999
pME3849	pME6001 containing the *rsmA* gene under the control of the P_{lac} promoter	Pessi *et al.*, 2001
pME6111	*gacA* gene-exchange construct; pME3088 suicide vector carrying a 4.8 kb fragment containing *gacA*::ΩSm/Sp; Tc^R, Sm/Sp^R	Reimmann *et al.*, 1997
pPZ-*prpL*	pPZTC derivative carrying a P*prpL-lacZ* transcriptional fusion; Cb^R. *prpL = piv*	Lamont *et al.*, 2002
pZH13	Suicide plasmid for *rsmA* deletion; Cm^R	Pessi *et al.*, 2001
pME3087Δ*rsmY*	Suicide plasmid for *rsmY* deletion; Tc^R	Kay *et al.*, 2006
pME3332	Suicide plasmid for *rsmZ* deletion; Tc^R	Heurlier *et al*., 2004
pME9643	Suicide plasmid for PA2384 deletion; Tc^R	C. Reimmann, unpublished
pMP220::P*pvdA*	Promoter of *pvdA* directionally cloned into pMP220	Leoni *et al.*, 1996

pMP220::P*pvdS*	Promoter of *pvdS* directionally cloned into pMP220	Ambrosi *et al.*, 2002
pMP220::P*pchR*	Promoter of *pchR* directionally cloned into pMP220	This study
pMP190::P*pvdD*	Promoter of *pvdD* directionally cloned into pMP190	Cunliffe *et al.*, 1995
pMP220::P*feoA*	Promoter of *feoA* directionally cloned into pMP220	Imperi *et al.*, 2013
pME9301	Translational P*pvdS*`-`*lacZ* fusion	Frangipani *et al.*, 2008
pME7226	Translational P*pchR*`-`*lacZ* fusion	Michel *et al.*, 2005
pME6014 PA4492'-`*lacZ*	Promoter of PA4492 cloned into pME6014 in frame with *lacZ*	This study
pME6014 *fur'*-`*lacZ*	*fur* gene (including promoter and coding sequence) cloned into pME6014 in frame with *lacZ*	This study
pBBR1MCS-4-PA2133	PA2133 cloned into pBBR1MCS-4	Moscoso *et al.*, 2011
pBBR1MCS-4-*wspR*^{R242A}	R252A-*wspR* cloned into pBBR1MCS-4	Moscoso *et al.*, 2011

Additional references (not included in the main text):

Ambrosi, C., Leoni, L., and Visca, P. (2002) Different responses of pyoverdine genes to autoinduction in *Pseudomonas aeruginosa* and the group *Pseudomonas fluorescens-Pseudomonas putida*. Appl Environ Microbiol 68: 4122-4126.

Blumer, C., Heeb, S., Pessi, G., and Haas, D. (1999) Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc Natl Acad Sci U S A 96: 14073-14078.

Casabadan, M.J., and Cohen, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J Mol Biol 138: 179-207.

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

Frangipani, E., Slaveykova, V.I., Reimmann, C., and Haas, D. (2008) Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation. J Bacteriol 190: 6706-6717.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M. I., and Peterson, K.M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 175–176.

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

Liss, L. (1987). New M13 host: DH5 F' competent cells. Focus 9:13.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M.T., Cámara, M., et al., (2001) The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. J Bacteriol 183: 6676-6683.

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

Savioz, A., Zimmermann, A., and Haas, D. (1993) *Pseudomonas aeruginosa* promoters which contain a conserved GG-N10-GC motif but appear to be RpoN-independent. Mol Gen Genet 238: 74-80.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology 1: 784-790.

Table S2. Primers used in this study[1]

Primer name	Sequence[2]	Restriction site	Application
P*pchR*_FW	5′-GGAAGATCTACCGTGTCGCCATGTG-3′	BglII	Construction of P*pchR*::*lacZ* transcriptional fusion
P*pchR*_RV	5′-GCGGAATTCGATGTGCGCGACGCC-3′	EcoRI	Construction of P*pchR*::*lacZ* transcriptional fusion
*fur-lacZ*_FW	5′-CGCGAATTCATCGGTGAGCGACTTCC-3′	EcoRI	Construction of *fur′-'lacZ* translational fusion
*fur-lacZ*_RV	5′-GCGGGATCCTCTTCTTGCGCACGTAGAG-3′	BamHI	Construction of *fur′-'lacZ* translational fusion
PA4492_FW	5′-GCGGAATTCTGCTGGAGCTGCACGC-3′	EcoRI	Construction of PA4492′-*'lacZ* translational fusion
PA4492_RV	5′-CCGGGATCCTCGAACCGAAGATACGCATG-3′	BamHI	Construction of PA4492′-*'lacZ* translational fusion

[1] All PCRs were performed using the genomic DNA of the PAO1 strain as template.
[2] Restriction site used for cloning is underlined in the primer sequence.

Chapter 4

Exopolysaccharide-mediated cell aggregation promotes pyoverdine dependent iron uptake and virulence in *Pseudomonas aeruginosa*

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Chapter 4
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Among the virulence factors produced by *P. aeruginosa*, pyoverdine is one of the most fascinating, this is not only because of its for its dual personality (as iron scavenging molecule and as virulence-inducing signal molecule) but also because it seems to play a key role in both acute and chronic infections (Chapter 1). During chronic infections, *P. aeruginosa* generally grows as biofilm, and pyoverdine is essential for biofilm formation under iron-depleted conditions (Banin *et al*., 2005). The switch between the planktonic and the biofilm lifestyle is mainly regulated by the Gac regulatory system and the signaling molecule c-di-GMP. This two regulatory networks control the formation of biofilm matrix by modulating the expression of the genes responsible for the biosynthesis of the aggregative exopolysaccharides Pel and Psl (Hickman *et al*., 2005; Brencic and Lory, 2009). In our previous work (Chapter 2), we demonstrated that the Gac system and c-di-GMP also coordinately promote pyoverdine production, although the underlying mechanism(s) remains unclear (Frangipani *et al*., 2014). In order to gain insight into the regulation of pyoverdine production mediated by these regulatory networks, we focused our attention on the overlap between the Gac and c-di-GMP regulons, and this led us to investigate the possible involvement of the exopolysaccharides Pel and Psl. By generating single and double deletion mutants in the *pel* and *psl* operons, we demonstrated that Gac and c-di-GMP require at least one of the two exopolysaccharides to exert their control on pyoverdine biosynthesis. In the *pelpsl* double mutant the production of pyoverdine, PrpL and exotoxin A was almost abolished, indicating that exopolysaccharides have a strong impact on pyoverdine-dependent virulence gene expression. The molecular mechanism by which Pel and Psl control pyoverdine production remains unclear, but the experimental evidence obtained so far leads us to propose that cell aggregation mediated, by Pel and/or Psl, rather than the exopolysaccharides by themselves, is the cue which promotes siderophore and virulence gene expression in *P. aeruginosa*. This work has been submitted to Environmental Microbiology and is currently under revision.

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Exopolysaccharide-mediated cell aggregation promotes pyoverdine-dependent iron uptake and virulence in *Pseudomonas aeruginosa*

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Keywords:	bacteria, environmental signal/stress responses, gene expression/regulation, metals, microbial behaviour/signalling, microbial genetics

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Running title: Planktonic aggregation boosts *P. aeruginosa* virulence

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Summary

In *Pseudomonas aeruginosa* the Gac signaling system and the second messenger cyclic-di-GMP participate in the control of the switch between planktonic and biofilm lifestyles, by regulating the production of the two exopolysaccharides Pel and Psl. The Gac and cyclic-di-GMP regulatory networks also coordinately promote the expression of the pyoverdine siderophore, although the underlying mechanism remains unknown. By coupling reverse genetics with gene expression and phenotypic assays, we demonstrate that the extracellular polysaccharides Pel and Psl are essential for Gac- and cyclic-di-GMP-mediated activation of pyoverdine production. An exopolysaccharide-null *pel psl* mutant produces very low levels of pyoverdine and shows a marked reduction in exotoxin A and PrpL protease expression. Exopolysaccharides-dependent control of pyoverdine gene expression is independent of both Fur and pyoverdine signaling regulatory pathways. While the exopolysaccharide-proficient parent strain forms multicellular planktonic aggregates in liquid cultures, the exopolysaccharide-null mutant mainly grows as dispersed cells. Notably, artificially-induced cell aggregation is able to restore pyoverdine-dependent gene expression in the exopolysaccharide-null mutant. This study demonstrates that cell aggregation is an important cue triggering the expression of pyoverdine-related genes in *P. aeruginosa*, highlighting a novel link between virulence gene expression, cell-cell interaction and the multicellular community lifestyle.

Introduction

Pseudomonas aeruginosa is a metabolically versatile Gram-negative bacterium and an opportunistic pathogen in cystic fibrosis (CF) and otherwise critical patients, causing both chronic and acute infections. The ability of *P. aeruginosa* to rapidly adapt to diverse ecological niches and to switch from acute to chronic infections is related to the tightly regulated expression of specific sub-sets of genes in response to environmental cues (Stover *et al.*, 2000). Characteristic traits of *P. aeruginosa* chronic infection are the microcolony and biofilm mode of growth. Microcolonies are small aggregates of cells that open the way to the communal organization of a biofilm. Biofilms are surface-associated communities of bacteria encased in a self-generated polymeric matrix. Extracellular polysaccharides represent a key component of the biofilm matrix, and are involved in surface attachment and cell-cell interaction (Ma *et al.*, 2009). Bacteria growing as biofilms show enhanced resistance to antimicrobials and to the host immune response, and this makes biofilm-associated infections difficult to eradicate (Parsek and Singh, 2003).

P. aeruginosa has been considered a model organism for studies on biofilm formation and resistance. It produces three main exopolysaccharides, namely alginate, Pel and Psl (Colvin *et al.*, 2012). Alginate was the first exopolysaccharide described in *P. aeruginosa*, and confers the typical mucoid phenotype to producing strains (Sherbrock-Cox *et al.*, 1984). Alginate plays a crucial role in CF lung colonization, while Psl and Pel are normally produced by non mucoid strains, and failure to produce Pel and/or Psl exopolysaccharides impairs biofilm formation *in vitro* (Colvin *et al.*, 2012). The exopolysaccharide Psl, encoded by the polysaccharides synthesis locus (PA2231-PA2245 in the reference strain PAO1), consists of repeating pentamers of D-mannose, L-rhamnose and D-glucose (Ma *et al.*, 2007). The helical distribution of Psl on the individual cell surface promotes cell-cell and cell-surface interactions in microcolonies (Zhao *et al.*, 2013), although it also plays an essential role in the maintenance of the mature biofilm structure (Jackson *et al.*, 2004; Ma *et al.*, 2009). The exopolysaccharide Pel is encoded by a seven-genes operon (PA3064-PA3058 in PAO1), and was identified by a transposon mutagenesis screening for the loss of

surface pellicle formation. Pel structure has not yet been determined, although carbohydrate analysis suggested a glucose-rich composition (Friedman and Kolter, 2004).

The production of the Pel and Psl exopolysaccharides is directly or indirectly controlled by many regulatory networks at the level of transcription, translation and sugar chain biosynthesis (Goodman *et al.*, 2004; Ventre *et al.*, 2006; Sakuragi *et al.*, 2007; Gilbert *et al.*, 2009; Lee *et al.*, 2007). The best characterized regulatory networks involved in the regulation of *pel* and *psl* gene expression and, consequently, in the switch from the planktonic to the biofilm lifestyle are the Gac system and the signaling molecule cyclic-di-GMP (c-di-GMP). The Gac system relies on a two component system consisting of the sensor kinase GacS that, in response to a still unknown signal, activates the transcriptional regulator GacA, which in turn promotes the transcription of two small noncoding RNAs (sRNAs), RsmZ and RsmY (Brencic *et al.*, 2009). These sRNAs bind to and sequester the mRNA binding protein RsmA, thereby inhibiting its activity as translational repressor. When the Gac system is inactive, RsmA binds to specific motifs (usually ANGGA) in the 5' untranslated region of target genes and prevents translation (Hebb *et al.*, 2006). While *psl* gene expression is directly regulated by RsmA at the translational level (Irie *et al.*, 2010), there are no evidence of the direct effect of RsmA on the *pel* genes, although transcriptomic analysis showed that the Gac network also affects transcription of the *pel* operon (Brencic and Lory, 2009). The Gac system has recently been shown to play a key role also in the modulation of the intracellular levels of the signaling molecule c-di-GMP, which induces biofilm formation by promoting exopolysaccharides production (Moscoso *et al.*, 2011; Irie *et al.*, 2012; Frangipani *et al.*, 2014; Moscoso *et al.*, 2014). This second messenger exerts its regulatory activity on Pel production both at the transcriptional and post-transcriptional level. In fact, c-di-GMP inhibits the activity of FleQ, a transcriptional repressor of the *pel* operon (Hickman and Harwood, 2008), and activates the inner membrane protein PelD, which is involved in the generation of the polysaccharide chain (Lee *et al.*, 2007). Although the molecular mechanism has not been elucidated yet, high intracellular levels of c-di-GMP also increase the expression of *psl* genes (Hickman *et al.*, 2005).

Exopolysaccharides production and biofilm formation are not the only common targets of Gac and c-di-GMP signaling, as these systems also act in a concerted way to control iron uptake. In particular, an active state of the Gac system and high intracellular levels of c-di-GMP promote the expression of genes involved in pyoverdine production (Frangipani *et al.*, 2014). Pyoverdine is a green fluorescent siderophore which plays a prominent role in *P. aeruginosa* pathogenicity (Visca *et al.*, 2007). Pyoverdine acts not only as a high-affinity iron scavenger, providing *P. aeruginosa* cells with the required amount of iron for biofilm formation under iron-depleted conditions (Banin *et al.*, 2005) and growth in human serum (Ankenbauer *et al.*, 1985), but also serves as a signal molecule to promote expression of important *P. aeruginosa* virulence factors (Lamont *et al.*, 2002). Pyoverdine stimulates virulence *via* a cell-surface signaling cascade which involves the other membrane ferri-pyoverdine receptor FpvA, the cytoplasmic membrane-spanning antisigma factor FpvR, and the extracytoplasmic function (ECF) sigma factor PvdS. The binding of ferri-pyoverdine to FpvA transmits a signal to the antisigma factor FpvR, ultimately resulting in the release of PvdS from FpvR in a state that is competent for interaction with the RNA polymerase core (Lamont *et al.*, 2002; Tiburzi *et al.*, 2008). PvdS drives transcription of almost thirty *P. aeruginosa* genes, including those involved in pyoverdine biosynthesis and transport, the gene for the extracellular protease PrpL and, indirectly, the exotoxin A gene *toxA* (Ochsner *et al.*, 2002; Llamas *et al.*, 2014). The dual function of pyoverdine (an iron-uptake molecule and a virulence-inducing signal) makes it an essential element for *P. aeruginosa* virulence, as demonstrated in different mouse models of infection (Meyer *et al.*, 1996; Takase *et al.*, 2000; Imperi *et al.*, 2013). As for any iron-uptake system, pyoverdine production needs to be promptly shut down when intracellular iron levels are sufficiently high. This iron-mediated control occurs through the ferric uptake regulator Fur, an iron-sensing transcriptional repressor which binds to its co-repressor Fe^{2+} and inhibits transcription of the sigma factor gene *pvdS* (Ochsner *et al.*, 2002).

In the present study we addressed the mechanism by which Gac and c-di-GMP control pyoverdine biosynthesis. By coupling reverse genetics experiments with gene expression and phenotypic assays, we demonstrate that the extracellular polysaccharides Pel Psl are essential for Gac and c-

di-GMP-mediated regulation of pyoverdine production, which indeed results almost abrogated in a *pel psl* double mutant. We also show that artificially-induced cell aggregation restores pyoverdine-dependent gene expression in the exopolysaccharide-null mutant. Our findings suggest that aggregation of P*. aeruginosa* cells, rather than polysaccharide production *per se*, is an important cue triggering production of pyoverdine and pyoverdine-controlled virulence factors in *P. aeruginosa*.

Results

Exopolysaccharide production is essential for Gac- and c-di-GMP-mediated regulation of pyoverdine production. We recently demonstrated that both the Gac signaling system and the second messenger c-di-GMP positively control the production of the *P. aeruginosa* siderophore pyoverdine (Frangipani *et al.*, 2014). The Gac and c-di-GMP regulons are quite large, and include genes involved in several functions, including biofilm formation, virulence, protein transport and secondary metabolism (Hickman *et al.*, 2005; Burrowes *et al.*, 2006; Brencic and Lory, 2009). However, some overlap between Gac and c-di-GMP regulons exists, with the best characterized example being the regulation of biosynthetic genes for the exopolysaccharides Pel and Psl, which are involved in *P. aeruginosa* attachment and biofilm formation (Mann and Wozniak, 2012; Colvin *et al.*, 2012).

Thus, we attempted to verify whether the Pel and Psl exopolysaccharides are involved in the Gac and c-di-GMP mediated control of pyoverdine production. To this aim, we generated single and double deletion mutants in *pel* and *psl* genes in *P. aeruginosa* PAO1 (wild type) and in isogenic mutants in which the Gac signaling is inactive (*rsmY rsmZ* mutant) or constitutively active (*rsmA* mutant) (Table S1). As previously reported (Frangipani *et al.*, 2014), pyoverdine production was higher in the *rsmA* mutant and strongly reduced in the *rsmY rsmZ* mutant compared with the wild type (Fig. 1A). This pyoverdine production profile was maintained in strains lacking either Pel or Psl, while pyoverdine production was drastically reduced in the *pel psl* double mutant irrespective of the activation state of the Gac system (Fig. 1A). This response appeared to be independent of the previously-reported effect of the Gac system on c-di-GMP production, since mass spectrometry analysis revealed that the Gac system is still able to control intracellular c-di-GMP levels in a Pel/Psl deficient background (Fig. 1B). Accordingly, the overexpression of a constitutively activated diguanylate cyclase ($WspR^{R242A}$; Moscoso *et al.*, 2011) was able to promote pyoverdine production in *pel* or *psl* single mutants, but not in the *pel psl* double mutant (Fig. 1C), although it resulted in almost 100-fold increase in c-di-GMP intracellular levels in both wild type and Pel/Psl deficient cells (Fig. 1D). Thus, both the Gac system and the c-di-GMP second

messenger require at least one of two exopolysaccharides Pel and Psl to exert their control on pyoverdine biosynthesis, suggesting that these exopolysaccharides, or an exopolysaccharide-dependent phenotype, play a role in pyoverdine production by *P. aeruginosa*.

Exopolysaccharide-defective *P. aeruginosa* cells are impaired in the production of pyoverdine and pyoverdine-regulated virulence factors. To investigate the effect of Pel and Psl deficiency on pyoverdine biosynthesis, we compared pyoverdine production between wild type PAO1 and the *pel psl* double mutant. While the growth of the *pel psl* mutant was slightly impaired with respect to the wild type, the relative pyoverdine production by the mutant was almost abolished along the whole growth curve (Fig. 2A). This well correlates with the expression of the pyoverdine biosynthetic gene *pvdD*, since both the activity of a P*pvdD*::*lacZ* transcriptional fusion (Fig. 2B) and the levels of *pvdD* mRNA (Fig. 2C) were greatly reduced in the *pel psl* mutant, relative to the wild type. Accordingly, the expression of the pyoverdine-dependent virulence genes *toxA* and *prpL*, as well as the levels of the corresponding virulence factors in culture supernatants, was strongly reduced in the *pel psl* double mutant (Figs. 2C-E). Therefore, lack of exopolysaccharides causes a significant reduction in the production of the siderophore pyoverdine and of pyoverdine-dependent virulence factors by *P. aeruginosa*.

Exopolysaccharides affect *pvdS* gene expression and pyoverdine production independently of Fur and pyoverdine signaling. Expression of pyoverdine synthesis genes and pyoverdine-dependent virulence genes is under the primary control of the ECF sigma factor PvdS. Thus, we investigated the link between production of exopolysaccharides and PvdS expression. Compared with the wild type, the activity of a P*pvdS*::*lacZ* transcriptional fusion was significantly lower in the *pel psl* mutant (Fig. 3A). Comparable results were obtained with a P*pvdS*'-'*lacZ* translational fusion (Fig. 3A), indicating that exopolysaccharides affects *pvdS* expression at the transcriptional level. No differences were observed between the *pel psl* mutant and wild type in the expression of the housekeeping gene *proC* (Fig. 3A; Pérez-Martínez and Haas,

2011). Therefore, exopolysaccharides regulation *pvdS* is a specific effect which cannot be ascribed to a generalized effect on gene expression or on the reporter used. By comparison with reporter gene assays, however, real-time PCR experiments showed a less pronounced effect of exopolysaccharide deficiency on *pvdS* mRNA levels, which were reduced in the *pel psl* mutant less than two-fold compared to the wild type strain (Fig. 3B). Such a moderate decrease in the *pvdS* mRNA levels poorly correlates with the strong reduction in pyoverdine production observed in the exopolysaccharide mutant (Fig. 2A), and suggests that additional steps of pyoverdine production could also be affected by the lack of Pel and Psl exopolysaccharides. To definitely assess the contribution of *pvdS* expression to the exopolysaccharides-mediated control of pyoverdine biosynthesis, we generated arabinose-dependent *pvdS* conditional mutants in wild type and Pel/Psl defective backgrounds (Table S1). As expected, pyoverdine production in the *pvdS* conditional mutant was proportional to the amount of arabinose added to the growth medium, with 0.5% arabinose being sufficient to restore pyoverdine production to wild-type levels (Fig. S1). Such arabinose levels increased pyoverdine production in the *pel psl pvdS* conditional mutant without restoring the wild type levels (Fig. 3C). A similar trend was observed for the activity of the P*pvdD*::*lacZ* transcriptional fusion (Fig. 3D), and the production of the PvdS-dependent virulence factor PrpL (Fig. 3E). Taken together, these observations suggest that exopolysaccharides control the production of pyoverdine and pyoverdine-related virulence factors not only via *pvdS,* but also via an additional regulatory mechanism that plausibly occurs downstream of *pvdS* expression.

In a previous work, it was demonstrated that the Gac system does not regulate *pvdS* transcription by affecting the expression of the master regulator of iron uptake Fur (Frangipani *et al.*, 2014). However, we cannot rule out that regulation of Fur activity, rather than expression, could be involved in the observed effect of exopolysaccharides on *pvdS* gene expression. Fur is considered an essential protein in *P. aeruginosa* (Barton *et al.*, 1996; Cornelis *et al.*, 2009), and *fur* null mutants have never been obtained in this species. Thus, to verify the possible involvement of Fur in exopolysaccharide-mediated control of *pvdS* transcription, we performed conditional mutagenesis of the *fur* gene in PAO1 and *pel psl*. For this purpose, a *fur* conditional mutant was

generated by replacing the native *fur* gene with an arabinose-inducible allele (Table S1). The PAO1 *fur* conditional mutant grew poorly in the iron-poor TSBD medium, and even less in TSBD supplemented with 50 µM free iron, but growth was restored to wild type levels upon arabinose induction of *fur* gene expression (Fig. S2). Compared with the wild type, pyoverdine levels and *pvdS* gene expression were significantly higher in the *fur* conditional mutant grown without arabinose, and were not shut down by the addition of iron to the growth medium (Fig. S2). This evidence confirmed the suitability of our conditional mutagenesis strategy to obtain Fur-depleted *P. aeruginosa* cells. Notably, the differences in pyoverdine production and activity of the P*pvdS*::*lacZ* transcriptional fusion between *fur* and *pel psl fur* conditional mutants in the absence of arabinose were comparable to those observed for the wild type and the *pel psl* mutant (Fig. 4A-B), clearly indicating that exopolysaccharides control *pvdS* gene expression in a way that is independent of Fur.

Since exopolysaccharides affect the expression of pyoverdine-related genes in part by mechanisms that appear independent of *pvdS* expression (Fig. 3), we hypothesized that the absence of extracellular polysaccharides in the *pel psl* mutant negatively affects the pyoverdine surface signaling cascade (Lamont *et al.*, 2002; Llamas *et al.*, 2014), ultimately resulting in lower PvdS activity and, consequently, reduced expression of PvdS-dependent genes. To test this hypothesis, we deleted the *fpvR* gene in the wild type and Pel/Psl-negative backgrounds, in order to obtain strains in which PvdS activity cannot be influenced by pyoverdine signaling through the anti-sigma factor FpvR (Lamont *et al.*, 2002; Llamas *et al.*, 2014). However, deletion of *fpvR* did not restore pyoverdine production in the *pel psl* mutant (Fig. 4C), ruling out any involvement of FpvR and, thus, of the pyoverdine signaling cascade on the exopolysaccharides-mediated control of PvdS-dependent gene expression.

Artificially-induced cell aggregation restores production of virulence factors in exopolysaccharide-defective cells. Irrespective of the regulatory mechanisms involved, our findings argue for a prominent role of extracellular polysaccharides in triggering the expression of

pyoverdine and PvdS-dependent virulence genes. As shown in Figure 1, each single exopolysaccharide is able to promote pyoverdine production, suggesting that a phenotype that depends on the presence of exopolysaccharide(s), rather than a specific exopolysaccharide molecule, could be important for pyoverdine-related gene expression. Extracellular polysaccharides have a role in cell attachment to biotic and abiotic surfaces and in biofilm formation, and indeed the PAO1 *pel psl* mutant was unable to adhere and form biofilms on plastic tubes (Fig. 5A) (Colvin *et al.*, 2012). Moreover, exopolysaccharides have also been proposed to mediate planktonic aggregation (Klebensberger *et al.*, 2007), as confirmed by our observation that Pel/Psl-deficient cells prevalently grow as dispersed cells, while Pel/Psl-proficient wild-type cells grow as large aggregates including hundreds of cells (Fig. 5B). These bacterial aggregates are quite loose, and can be dispersed by vigorous pipetting or vortexing (data not shown).

We thus hypothesized that cell-to-cell contacts, instead of exopolysaccharides by themselves, could represent the signal triggering PvdS-dependent gene expression. This hypothesis was first tested by growing wild-type and *pel psl* mutant cells as colonies on the surface of TSBD medium solidified with different gelling substances, such as polyacrylamide and the polysaccharides agar and phytagel, and qualitatively assessing pyoverdine production by comparing fluorescence emission under UV light (Visca *et al.*, 2007). While the fluorescence of the *pel psl* mutant grown in liquid medium was much lower than that of the wild type and comparable to that of the pyoverdine-deficient mutant PAO1 *pvdA* (Imperi *et al.*, 2008), the exopolysaccharide mutant showed fluorescence levels that were indistinguishable from those of the wild type, and much higher than those of the *pvdA* mutant, during colony growth on solid surfaces (Fig. 6A). Although this assay clearly showed that growth on solid surfaces, where cells can interact with each other irrespective of aggregative polysaccharides, enhanced pyoverdine production in the *pel psl* mutant, the qualitative nature of the assay did not allow a quantitative comparison of pyoverdine levels between strains. To overcome this limitation, strains were cultured in TSBD medium supplemented with different sub-gelling concentrations of agar (0.0125-0.2%, Fig. 6B) or phytagel (0.08-0.125%, Fig. S3), thus allowing to quantitatively assess pyoverdine levels in

culture supernatants. Pyoverdine levels in the supernatants of *pel psl* mutant cultures were increased by sub-gelling concentrations of agar in a concentration-dependent manner, while agar had minor or no effects on pyoverdine production by the wild-type strain (Fig. 6B). Substantially similar results were obtained with phytagel (Fig. S3). At the microscopic level, the presence of sub-gelling concentrations of agar in the growth medium forced the *pel psl* mutant, as well as the wild type, to grow as large clusters of thousands of cells (Fig. 6C), consistent with the conclusion that cell aggregation stimulates pyoverdine production.

Notably, pyoverdine production by the *pel psl* mutant in the presence of agar was abrogated by agar degradation with β-agarase I, which however had no effect on pyoverdine production by wild type PAO1 (Fig. 6B). This result confirms that the effect of agar on the *pel psl* mutant has to be ascribed to the polymeric nature of the gelling agent rather than to any specific constituent of the polymer or to a general increase in the osmolarity of the growth medium. The latter point was further corroborated by the observation that, differently from agar and phytagel, high concentrations of sucrose (up to 10%) did not stimulate pyoverdine production by the exopolysaccharides-null mutant (Fig. S3). To also rule out any involvement of the increased viscosity of the growth medium due to the presence of agar, we verified that the addition of glycerol (up to 5%) did not promote pyoverdine production in both wild type and *pel psl* cultures (Fig. S3). Coherent with the observed effects on pyoverdine production, addition of 0.2% agar stimulated *pvdS* and *pvdD* expression (Fig. 6D), as well as PrpL and ToxA production by the *pel psl* mutant (Figs. 6E-F), while it had no effect on the expression of the housekeeping gene *proC* (Fig. 6D).

It appears therefore that artificially-induced cell aggregation, obtained by growing cells either as colonies on solid surface or in liquid cultures in the presence of aggregating agents such as agar or phytagel, is able to restore pyoverdine-dependent phenotypes in the exopolysaccharide-deficient mutant *pel psl*, suggesting that cell-to-cell contacts and/or growth as aggregates by itself are cues that stimulate pyoverdine production and virulence in *P. aeruginosa*.

Unspecific physical contacts do not promote pyoverdine production. In order to verify whether the effect of aggregation on pyoverdine production was due to specific cell-to-cell interactions or to an increase in unspecific surface contacts during growth as cellular aggregates, the *pel psl* mutant was cultured in the presence of 5×10^7 or 5×10^8 3-µm size polystyrene beads/ml (Polysciences), under vigorous shaking in order to maintain beads in suspension. The *pel psl* mutant is not able to attach to polystyrene (Fig. 5A); thus, the presence of such beads should only increase the number of contacts between planktonic cells and the inert plastic material. As shown in Figure 7, polystyrene beads had no effect on pyoverdine production by the *pel psl* mutant or the wild type used as control, strongly suggesting that cell aggregation, rather than unspecific physical contacts, represents the signal which triggers pyoverdine gene expression in *P. aeruginosa*.

Discussion

In the last decades, the old vision of bacteria as strictly unicellular organisms living in a planktonic single-cell status was swept away by the finding that bacterial cells in natural, industrial and many clinical settings predominantly exist as biofilms, *i.e.* structured microbial communities attached to a surface and encased in an extracellular matrix (Mann and Wozniak, 2012). But also during planktonic growth in liquid cultures bacteria can assemble into aggregates of densely packed cells, and it is believed that planktonic aggregation can play a role in resistance to stresses and antibiotics (Schleheck *et al.*, 2009; Blom *et al.*, 2010; Haaber *et al.*, 2012), as well as in microbe-host cell interaction (Lepanto *et al.*, 2011). Although hundreds of studies have investigated the physiology of biofilm-living bacterial cells, very little is known about the effects of aggregate growth on planktonic cells.

Here we provide evidence that growth as planktonic aggregates promotes production of three major virulence factors in the opportunistic pathogen *P. aeruginosa*, namely pyoverdine, extracellular protease PrpL and exotoxin A. Indeed, we observed that an exopolysaccharide-null mutant unable to aggregate in liquid cultures is also defective in the expression of virulence factor genes, and that this effect can be rescued by artificially-induced cell aggregation (Figs. 6 and S3). Exopolysaccharide-mediated aggregation appeared to control pyoverdine and virulence gene expression at two different levels. We noticed that exopolysaccharides enhance transcription of the *pvdS* regulatory gene (Fig. 3). However, expression of PvdS from an arabinose-dependent regulatory region, which abolishes any transcriptional effect on *pvdS* promoter activity or post-transcriptional control involving the 5' untranslated region, only partially restored expression of pyoverdine and virulence genes (Fig. 3). This indicates that a mechanism downstream of *pvdS* transcription and translation is mainly responsible for the exopolysaccharide-induced increase of PvdS-dependent gene expression. At present, the existence of a still-unknown regulator with a regulon that partially overlaps with that of PvdS cannot be ruled out. However, it is also possible that exopolysaccharides can indirectly affect the activity of the PvdS protein. While *pvdS* gene expression is modulated by several environmental signals and regulatory pathways in addition to

the well-characterized Fur regulation, pyoverdine signaling is the only system known to affect PvdS activity (reviewed in Llamas *et al.*, 2014). Since pyoverdine signaling relies on a cascade involving different components residing in the cell envelope, we excluded that the absence of exopolysaccharides and the consequent inability to form cell aggregates could influence some cell envelope properties that are important for pyoverdine signal transduction. In fact, inactivation of the *fpvR* antisigma gene, which results in a constitutively active state of the PvdS sigma factor (Lamont *et al.*, 2002; Tiburzi *et al.*, 2008), had no effect on pyoverdine production by the *pel psl* mutant (Fig. 4), clearly ruling out any involvement of the pyoverdine signaling cascade in exopolysaccharide-mediated activation of PvdS-dependent genes.

Another issue that deserves further investigation is how cell-to-cell contacts and/or cell aggregation are perceived by the bacterial cell and integrated into a regulatory cascade ultimately leading to the activation of virulence gene expression. At least two hypotheses can be made. First, a sensory machinery could transduce a contact signal deriving from the cell envelope into a cytoplasmic response. Our experiments lead to exclude the involvement of physical changes in the cell envelope due to unspecific contacts with abiotic surfaces (Fig. 7), suggesting that specific cell-to-cell interactions may be implicated. Such a kind of cell contact-sensitive signaling systems are involved in the regulation of surface motility in *Myxococcus xanthus* (Mauriello *et al.*, 2009), carbon source utilization in *Pseudomonas putida* (Joshi *et al.*, 2009), and contact-dependent secretion systems in many bacteria (reviewed in Hayes *et al.*, 2010). Also the Wsp system of *P. aeruginosa*, a chemotaxis-like signal transduction complex which promotes c-di-GMP production by activating the diguanylate cyclase WspR, has been found to be stimulated by growth on surfaces (Güvener and Harwood, 2007). Since we have ruled out c-di-GMP as the effector of exopolysaccharide-mediated increase of pyoverdine-dependent gene expression (Fig. 1), the involvement of the Wsp system can be reasonably excluded. However, other contact-dependent systems could exist in *P. aeruginosa*. For instance, the *P. aeruginosa* PAO1 genome is predicted to encode 26 methyl accepting chemotaxis proteins (Whitchurch *et al.*, 2004), 13 cell-surface signaling systems (Llamas *et al.*, 2014), up to 50 canonical two-component systems and more

than 10 orphan sensor kinases (Gooderham and Hancock, 2009), many of them being involved in virulence gene regulation. Very recently, it has been reported that surface-attached *P. aeruginosa* PA14 cells are more virulent than planktonic ones against the amoeba *Dictyostelium* discoideum, and that the pilus-biogenesis factor PilY1 could be involved in surface contact-dependent activation of virulence (Siryaporn *et al.*, 2014). Although our experimental evidence suggests that cell aggregation rather than surface contact is the signal which triggers pyoverdine gene expression (Figs 6 and 7), we cannot rule out, at present, the involvement of PilY1 (or other mechanosensitive factors) in the exopolysaccharides-mediated activation of virulence during planktonic growth.

An alternative hypothesis is that growth as aggregates determines changes in the cell surrounding environment that could influence virulence gene expression. Studies on the physiology of planktonic aggregates are still at an early stage, but a recent paper reported that localized oxygen depletion occurs in aggregates of *P. aeruginosa* cells grown in a gelatin-based microtrap (Wessel *et al.*, 2014). Notably, oxygen is a well known inducer of *pvdS* gene expression (Ochsner *et al.*, 1996; Llamas *et al.*, 2014); thus oxygen limitation can hardly explain the observed activation of PvdS-dependent genes in bacterial aggregates. It has also been reported that pyoverdine concentration is heterogeneous in *P. aeruginosa* microcolonies on solid surface, with a maximum at the colony center (Jolou *et al.*, 2013). Although it can be speculated that this heterogeneity could influence the efficiency of pyoverdine-dependent signaling and/or iron uptake in cell aggregates, we have demonstrated that aggregation-mediated increase of PvdS-dependent gene expression is independent of pyoverdine signaling and the iron sensor Fur (Fig. 4). However, other chemical and/or physiological changes occurring in densely packed bacterial cells could be responsible for the observed activation of virulence genes in *P. aeruginosa* planktonic aggregates.

In the whole, our study highlights a link between planktonic aggregation of *P. aeruginosa* cells and enhanced expression of secreted virulence factors. Such a kind of cell contact- or aggregation-dependent activation of virulence could represent a further strategy to modulate bacterial pathogenicity in response to population density, additional or complementary to chemical

signaling via quorum sensing. Cellular aggregation also represents the first committed step of biofilm formation. Although some recent transcriptomics and proteomics studies highlighted an overall attenuation of virulence gene expression in mature *P. aeruginosa* biofilms (Li *et al.*, 2014; Park *et al.*, 2014), which include many slowly growing or quiescent cells, our finding indirectly suggests that the virulence potential of *P. aeruginosa* may be actually increased during the first stages of biofilm formation, when siderophores, extracellular enzymes and toxins would provide cells with essential nutrients for the energy-demanding biofilm development process. This hypothesis is in line with the evidence that the gene expression profile of *P. aeruginosa* developing biofilms is more similar to that of exponential phase cultures rather than mature biofilms (Waite *et al.*, 2006). Finally, since planktonic aggregation seems to be widespread among bacteria (Schleheck *et al.*, 2009; Blom *et al.*, 2010; Haaber *et al.*, 2012), it could be verified whether the observed correlation between cell aggregation and virulence represents a conserved strategy in bacterial pathogens.

Experimental procedures

Bacterial strains, growth conditions and plasmids

Bacterial strains and plasmids used in this study are listed in Table S1. Bacteria were routinely grown in LB medium (Sambrook *et al.*, 1989). The iron-depleted complex medium TSBD was used as iron-poor medium (Ohman *et al.*, 1980), to which $FeCl_3$ was added at the indicated concentrations to increase iron availability, when required. The assays were performed on bacteria grown at 37°C in 96-well microtiter plates (250 µl of medium in each well) under static conditions. Antibiotics were added at the following concentrations for work with *E. coli*, with the concentrations used for *P. aeruginosa* shown in brackets: ampicillin, 100 µg/ml; carbenicillin (250 µg/ml); chloramphenicol 30 µg/ml (350 µg/ml); gentamicin, 15 µg/ml (200 µg/ml); nalidixic acid, 20 µg/ml; tetracycline 12 µg/ml (100 µg/ml). When specified, TSBD medium was supplemented with L-arabinose, agar, phytagel, sucrose or glycerol (Sigma-Aldrich) at the indicated final concentrations. Polystyrene beads (Polybead® Microspheres 3.00 µm, Polysciences) were washed several times with sterile water and then resuspended in TSBD at the desired concentrations.

Generation of deletion and conditional mutants

Recombinant DNA was manipulated as described elsewhere (Sambrook *et al.,* 1989). All the primers and restriction sites used for PCR and cloning are listed in Table S2. Deletion mutants in *pelABCD*, *pslABCD* or *fpvR* were generated using specific derivatives of the pDM4 suicide plasmid (Table S1). The *fur* conditional mutant was generated using a recently-described strategy (Lo Sciuto *et al.*, 2014), based on the mini-CTX1-mediated insertion of the *fur* coding sequence under the control of an arabinose-dependent promoter into a neutral site of the *P. aeruginosa* genome, followed by the in-frame deletion of the endogenous *fur* gene using the pDM4Δ*fur* suicide plasmid (Table S1), under permissive conditions (*i.e.* growth in the presence of arabinose). The same strategy was employed to obtain the *pvdS* conditional mutant, with the only exception that a previously-generated suicide plasmid (pEXΔ*pvdS*; Table S1) was used for *pvdS* deletion. Gene replacements and insertions were verified by PCR and DNA sequencing.

425

426 **Growth and pyoverdine measurements**

427 Growth was measured as the OD_{600} of appropriate dilutions of bacterial cultures in sterile growth
428 medium. Pyoverdine production was measured as the OD_{405} of culture supernatants appropriately
429 diluted in 0.1 M Tris-HCl (pH 8), and normalized to the OD_{600} of the corresponding cultures
430 (Imperi *et al.,* 2009). When indicated, pyoverdine production was normalized to the number of
431 colony forming units/ml.

432

433 **β-galactosidase and c-di-GMP assays**

434 The β-galactosidase activity from *P. aeruginosa* cells carrying the different reporter plasmids
435 (Table S1) was determined spectrophotometrically using *o*-nitrophenyl-β-D-galactopyranoside as
436 the substrate, normalized to the OD_{600} of the bacterial culture and expressed in Miller units (Miller,
437 1972). Intracellular levels of c-di-GMP were determined by liquid chromatography coupled with
438 tandem mass spectrometry as described (Spangler *et al.,* 2010), and normalized to the
439 corresponding cellular protein content determined using the DC protein assay kit (Bio-Rad) and
440 bovine serum albumin as the standard.

441

442 **Western blot analysis of ToxA and PrpL enzymatic assay**

443 For detection of ToxA, 100-µl aliquots of culture supernatants were supplemented with 20 µl of 6×
444 SDS-PAGE loading dye [375 mM Tris.HCl (pH 6.8), 9% SDS, 50% Glycerol, 0.03% Bromophenol
445 blue]. In order to normalize the amount of secreted proteins to bacterial growth, the volume of
446 supernatants loaded into SDS-PAGE gels was calculated according to the formula: loading volume
447 (µl) = $10/OD_{600}$ of the corresponding bacterial culture. Proteins resolved by SDS-PAGE were
448 electrotransferred onto a nitrocellulose filter (Hybond-C extra, Amersham), and probed for ToxA
449 using a rabbit polyclonal anti-ToxA antibody (Sigma-Aldrich). Filters were developed with 5-
450 bromo-4-chloro-3-indoyl-phosphate and nitro blue tetrazolium chloride reagents for colorimetric
451 alkaline phosphatase detection (Promega).

PrpL activity was determined by means of a specific enzyme assay based on the use of the chromogenic substrate Chromozym PL (tosyl-Gly-Pro-Lys-p-nitroanilide; Sigma-Aldrich) (Imperi *et al.*, 2010). Briefly, 10 µl of culture supernatants were mixed with 10 µl of 2 mg/ml Chromozym PL and 180 µl of phosphate buffer (pH 7.0) in 96-well microtiter plates. The OD_{410} was read at 2 min intervals for 30 min in a Victor^{3}V plate reader (Perkin-Elmer), and the change in optical density (ΔOD_{410}) per minute was determined. PrpL activity units were determined as: (ΔOD_{410} × total assay volume/ml)/(sample volume × E410 × light path); where total assay volume was 200 µl, sample volume was 10 µl, the extinction coefficient (E) of the product (p-nitroaniline) at 410 nm was 9.75, and the light path was 0.35 cm. PrpL activity was then normalized to the OD_{600} of the corresponding bacterial culture.

Real-time PCR

Total RNA was purified by using RNeasy minicolumns (Qiagen), including the on-column DNase I digestion described by the manufacturer. Eluted RNA samples were treated with 6 units of Turbo DNase (Ambion) for 1 h at 37°C in the presence of 1 µl of RNase inhibitor (Qiagen). DNase I was removed by using the RNeasy MinElute cleanup kit (Qiagen) according to manufacturer's instructions. cDNA was reverse transcribed from 0.5 µg of total RNA with PrimeScript RT reagent Kit (TaKaRa). cDNA was then used as the template for quantitative real-time PCR in a 7300 Real-Time PCR System (Applied Biosystems) using SYBR green with the ROX detection system (Bio-Rad). The primers used for real-time PCR are listed in Table S2. At least three wells were run for each cDNA sample. The threshold cycle value (Ct) of each gene was used to calculate relative gene expression with respect to the housekeeping gene *rpsL* by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Confocal microscopy

Thirty µl of *P. aeruginosa* cultures in TSBD were spotted on a glass slide that was freshly coated with 0.5% agarose in water, and covered with a cover slip. Images were acquired with

a Leica TCS SP5 inverted confocal microscope equipped with a HCX PLAPO lamba blue 40X/1.25 OIL objective (Zeiss). Images were recorded using specific sets for GFP (excitation at 488 nm, emission window from 500 to 600 nm).

Biofilm assay

Visualization of biofilm formation was carried out in 12 ml polystyrene tubes. Bacteria were inoculated in 1 ml of TSBD medium to an OD_{600} of 0.02. After 14-h incubation at 37°C under static conditions, planktonic cells were discarded and the tubes were washed with water three times. Attached cells were stained with 0.1% crystal violet and tubes were washed with water to remove unbound dye.

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References

Ankenbauer, R., Sriyosachati, S., and Cox, C.D. (1985) Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infect Immun. 49: 132-140.

Banin, E., Vasil, M.L., and Greenberg, E. P. (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci U S A 102: 11076-11081.

Barton, H.A., Johnson, Z., Cox, C.D., Vasil, A.I., and Vasil, M.L. (1996) Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. Mol Microbiol 21: 1001-1017.

Blom, J.F., Horňák, K., Simek, K., and Pernthaler, J. (2010) Aggregate formation in a freshwater bacterial strain induced by growth state and conspecific chemical cues. Environ Microbiol 12: 2486-2495.

Brencic, A., and Lory, S. (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. Mol Microbiol 72: 612-632.

Brencic, A., McFarland, K.A., McManus, H.R., Castang, S., Mogno, I., Dove, S.L., and Lory, S. (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. Mol Microbiol 73: 434-445.

Burrowes, E., Baysse, C., Adams, C., and O'Gara, F. (2006) Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. Microbiology 152: 405-418.

Colvin, K.M., Irie, Y., Tart, C. S., Urbano, R., Whitney, J. C., Ryder, C., *et al.,* (2012) The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environ Microbiol 14: 1913-1928.

Cornelis, P., Matthijs, S., and Van Oeffelen, L. (2009) Iron uptake regulation in *Pseudomonas aeruginosa*. Biometals 22: 15-22.

Frangipani, E., Visaggio, D., Heeb, S., Kaever, V., Cámara, M., Visca, P., and Imperi, F. (2014) The Gac/Rsm and cyclic-di-GMP signalling networks coordinately regulate iron uptake in *Pseudomonas aeruginosa*. Environ Microbiol 16: 676-688.

Friedman, L., and Kolter, R. (2004) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol Microbiol 51: 675-690.

Gilbert, K.B., Kim, T.H., Gupta, R., Greenberg, E.P., and Schuster, M. (2009) Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR. Mol Microbiol 73: 1072-1085.

Gooderham, W.J., and Hancock, R.E. (2009) Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. FEMS Microbiol Rev 33: 279-294.

548 Goodman, A.L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R.S., and Lory, S. (2004) A signaling
549 network reciprocally regulates genes associated with acute infection and chronic persistence in
550 *Pseudomonas aeruginosa*. Dev Cell 7: 745-754.
551
552 Güvener, Z.T., and Harwood, C.S. (2007) Subcellular location characteristics of the *Pseudomonas*
553 *aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on
554 surfaces. Mol Microbiol 66: 1459-1473.
555
556 Haaber, J., Cohn, M.T., Frees, D., Andersen, T.J., and Ingmer, H. (2012) Planktonic aggregates of
557 *Staphylococcus aureus* protect against common antibiotics. PLoS One 7: e41075.
558
559 Hayes, C.S., Aoki, S.K., and Low, D.A. (2010) Bacterial contact-dependent delivery systems. Annu
560 Rev Genet 44: 71-90.
561
562 Heeb, S., Kuehne, S.A., Bycroft, M., Crivii, S., Allen, M.D., Haas, D., *et al.,* (2006) Functional
563 analysis of the post-transcriptional regulator RsmA reveals a novel RNA-binding site. J Mol Biol
564 355: 1026-1036.
565
566 Hickman, J.W., and Harwood, C.S. (2008) Identification of FleQ from *Pseudomonas aeruginosa* as
567 a c-di-GMP-responsive transcription factor. Mol Microbiol 69: 376-389.
568
569 Hickman, J.W., Tifrea, D.F., and Harwood, C.S. (2005) A chemosensory system that regulates
570 biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A 102:
571 14422-14427.
572

573 Imperi, F., Massai, F., Facchini, M., Frangipani, E., Visaggio, D., Leoni, L., *et al.,* 2013.
574 Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa*
575 pathogenicity. Proc Natl Acad Sci U S A 110: 7458-7463.
576
577 Imperi, F., Putignani, L., Tiburzi, F., Ambrosi, C., Cipollone, R., Ascenzi, P., and Visca, P. (2008)
578 Membrane-association determinants of the omega-amino acid monooxygenase PvdA, a pyoverdine
579 biosynthetic enzyme from *Pseudomonas aeruginosa*. Microbiology 154: 2804-2813.
580
581 Imperi, F., Tiburzi, F., and Visca, P. (2009) Molecular basis of pyoverdine siderophore recycling in
582 *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 106: 20440-20445.
583
584 Imperi, F., Tiburzi, F., Fimia, G.M., and Visca, P. (2010) Transcriptional control of the *pvdS* iron
585 starvation sigma factor gene by the master regulator of sulfur metabolism CysB in *Pseudomonas*
586 *aeruginosa*. Environ Microbiol 12: 1630-1642.
587
588 Irie, Y., Borlee, B.R., O'Connor, J.R., Hill, P.J., Harwood, C.S., Wozniak, D.J., and Parsek, M.R.
589 (2012) Self-produced exopolysaccharide is a signal that stimulates biofilm formation in
590 *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 109: 20632-20636.
591
592 Irie, Y., Starkey, M., Edwards, A.N., Wozniak, D.J., Romeo, T., and Parsek, M.R. (2010)
593 *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS
594 and post-transcriptionally by RsmA. Mol Microbiol 78: 158-172.
595
596 Jackson, K.D., Starkey, M., Kremer, S., Parsek, M.R., and Wozniak, D.J. (2004) Identification of
597 *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa*
598 PAO1 biofilm formation. J Bacteriol 186: 4466-4475.
599

Joshi, H., Dave, R., and Venugopalan, V.P. (2009) Competition triggers plasmid-mediated enhancement of substrate utilisation in *Pseudomonas putida*. PLoS One 4: e6065.

Julou, T., Mora, T., Guillon, L., Croquette, V., Schalk, I.J., Bensimon, D., and Desprat, N. (2013) Cell-cell contacts confine public goods diffusion inside *Pseudomonas aeruginosa* clonal microcolonies. Proc Natl Acad Sci U S A 110: 12577-12582.

Klebensberger, J., Lautenschlager, K., Bressler, D., Wingender, J., and Philipp, B. (2007) Detergent-induced cell aggregation in subpopulations of *Pseudomonas aeruginosa* as a preadaptive survival strategy. Environ Microbiol 9: 2247-2259.

Lamont, I.L., Beare, P.A., Ochsner, U., Vasil, A.I., and Vasil, M.L. (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 99: 7072-7077.

Lee, V.T., Matewish, J.M., Kessler, J.L., Hyodo, M., Hayakawa, Y., and Lory, S.A. (2007) cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Mol Microbiol 65: 1474-1484.

Lepanto, P., Bryant, D.M., Rossello, J., Datta, A., Mostov, K.E., and Kierbel, A. (2011) *Pseudomonas aeruginosa* interacts with epithelial cells rapidly forming aggregates that are internalized by a Lyn-dependent mechanism. Cell Microbiol 13: 1212-1222.

Li, Y., Petrova, O.E., Su, S., Lau, G.W., Panmanee, W., Na, R., *et al.,* (2014) BdlA, DipA and induced dispersion contribute to acute virulence and chronic persistence of *Pseudomonas aeruginosa*. PLoS Pathog 10: e1004168.

626 Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time
627 quantitative PCR and the 2(-Delta Delta C(T)). Method Methods 5: 402-408.
628
629 Llamas, M.A., Imperi, F., Visca, P., and Lamont, I.L. (2014) Cell-surface signaling in
630 *Pseudomonas*: stress responses, iron transport, and pathogenicity. FEMS Microbiol Rev 38: 569-
631 597.
632
633 Lo Sciuto, A., Fernández-Piñar, R., Bertuccini, L., Iosi, F., Superti, F., and Imperi, F. (2014) The
634 periplasmic protein TolB as a potential drug target in *Pseudomonas aeruginosa*. PLoS One 9:
635 e103784.
636
637 Ma, L., Conover, M., Lu, H., Parsek, M.R., Bayles, K., and Wozniak, D.J. (2009) Assembly and
638 development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathog 5: e1000354.
639
640 Ma, L., Lu, H., Sprinkle, A., Parsek, M.R., and Wozniak, D. J. (2007) *Pseudomonas aeruginosa* Psl
641 is agalactose- and mannose-rich exopolysaccharide. J Bacteriol 189: 8353-8356.
642
643 Mann, E.E., and Wozniak, D.J. (2012) *Pseudomonas* biofilm matrix composition and niche biology.
644 FEMS Microbiol Rev 36: 893-916.
645
646 Mauriello, E.M., Astling, D.P., Sliusarenko, O., and Zusman, D.R. (2009) Localization of a bacterial
647 cytoplasmic receptor is dynamic and changes with cell-cell contacts. Proc Natl Acad Sci U S A 106:
648 4852-4857.
649
650 Meyer, J.M., Neely, A., Stintzi, A., Georges, C., and Holder, I.A. (1996) Pyoverdin is essential for
651 virulence of *Pseudomonas aeruginosa*. Infect Immun 64: 518-523.
652

Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, pp. 252–255.

Moscoso, J.A., Jaeger, T., Valentini, M., Hui, K., Jenal, U., and Filloux, A. (2014) The diguanylate cyclase SadC is a central player of the Gac/Rsm-mediated biofilm formation in *Pseudomonas aeruginosa*. J Bacteriol 196: 4081-4088.

Moscoso, J.A., Mikkelsen, H., Heeb, S., Williams, P., and Filloux, A. (2011) The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretion via c-di-GMP signalling. Environ Microbiol 13: 3128-3138.

Ochsner, U.A., Johnson, Z., Lamont, I.L., Cunliffe, H.E., and Vasil, M.L. (1996) Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated *pvdS* gene encoding an alternative sigma factor. Mol Microbiol 21: 1019-1028.

Ochsner, U.A., Wilderman, P.J., Vasil, A.I., and Vasil, M.L. (2002) GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. Mol Microbiol 45: 1277-1287.

Ohman, D.E., Sadoff, J.C., and Iglewski, B.H. (1980) Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect Immun 28: 899-908.

Park, A.J., Murphy, K., Krieger, J.R., Brewer, D., Taylor, P., Habash, M., and Khursigara, C.M. (2014) A temporal examination of the planktonic and biofilm proteome of whole cell *Pseudomonas aeruginosa* PAO1 using quantitative mass spectrometry. Mol Cell Proteomics 13: 1095-1105.

679 Parsek, M.R., and Singh, P.K. (2003) Bacterial biofilms: an emerging link to disease pathogenesis.
680 Annu Rev Microbiol 57: 677-701.
681
682 Pérez-Martínez, I., and Haas, D. (2011) Azithromycin inhibits expression of the GacA-dependent
683 small RNAs RsmY and RsmZ in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 55: 3399-
684 405.
685
686 Sakuragi, Y., and Kolter, R. (2007) Quorum-sensing regulation of the biofilm matrix genes (*pel*) of
687 *Pseudomonas aeruginosa*. J Bacteriol 189: 5383-5386.
688
689 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd
690 edn. New York, USA: Cold Spring Harbor Laboratory.
691
692 Schleheck, D., Barraud, N., Klebensberger, J., Webb, J.S., McDougald, D., Rice, S.A., and
693 Kjelleberg, S. (2009) *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid
694 batch cultures and disperses upon starvation. PLoS One 4: e5513.
695
696 Sherbrock-Cox, V., Russell, N.J., and Gacesa, P. (1984) The purification and chemical
697 characterisation of the alginate present in extracellular material produced by mucoid strains of
698 *Pseudomonas aeruginosa*. Carbohydr Res. 135: 147-154.
699
700 Siryaporn, A., Kuchma, S.L., O'Toole, G.A., and Gitai, Z. (2014) Surface attachment induces
701 *Pseudomonas aeruginosa* virulence. Proc Natl Acad Sci U S A In press.
702
703 Spangler, C., Böhm, A., Jenal, U., Seifert, R., and Kaever, V. (2010) A liquid chromatography-
704 coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine
705 monophosphate. J Microbiol Methods 81: 226-231.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J. *et al.,* (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406: 959-964.

Takase, H., Nitanai, H., Hoshino, K., and Otani, T. (2000) Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. Infect Immun 68: 1834-1839.

Tiburzi, F., Imperi, F., and Visca, P. (2008) Intracellular levels and activity of PvdS, the major iron starvation sigma factor of *Pseudomonas aeruginosa*. Mol Microbiol 67: 213-227.

Ventre, I., Goodman, A.L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., *et al.,* 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. Proc Natl Acad Sci U S A 103: 171-176.

Visca, P., Imperi, F., and Lamont, I.L. (2007) Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol 15: 22-30.

Waite, R.D., Paccanaro, A., Papakonstantinopoulou, A., Hurst, J.M., Saqi, M., Littler, E., and Curtis, M.A. (2006) Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. BMC Genomics 7: 162.

Wessel, A.K., Arshad, T.A., Fitzpatrick, M., Connell, J.L., Bonnecaze, R.T., Shear, J.B., and Whiteley, M. (2014) Oxygen limitation within a bacterial aggregate. MBio 5: e00992.

731 Whitchurch, C.B., Leech, A.J., Young, M.D., Kennedy, D., Sargent, J.L., Bertrand, J.J. *et al.,*
732 (2004) Characterization of a complex chemosensory signal transduction system which controls
733 twitching motility in *Pseudomonas aeruginosa*. Mol Microbiol 52: 873-893.
734
735 Zhao, K., Tseng, B.S., Beckerman, B., Jin, F., Gibiansky, M.L., Harrison, J.J., *et al.,* (2013) Psl
736 trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. Nature
737 497: 388-391.
738

Legends to figures

Fig. 1. Exopolysaccharides are crucial for the Gac- and c-di-GMP-mediated control on pyoverdine production. (A) Pyoverdine production by *P. aeruginosa* PAO1, *pel*, *psl* and *pel psl* mutants, and their derivatives deleted in the *rsmA* or in the *rsmY* and *rsmZ* genes. (B) Intracellular levels of c-di-GMP (relative to wild type PAO1) in *P. aeruginosa* PAO1, the *pel psl* mutant, and their derivatives deleted in the *rsmA* or the *rsmY* and *rsmZ* genes. (C) Pyoverdine production by *P. aeruginosa* PAO1, *pel*, *psl* and *pel psl* mutants harboring the empty vector pBBR1MCS-4 or its derivative constitutively expressing the diguanylate cyclase $WspR^{R242A}$. (D) Intracellular levels of c-di-GMP (relative to PAO1 pBBR1MCS-4) in *P. aeruginosa* PAO1 and the *pel psl* mutant harboring pBBR1MCS-4 or its derivative constitutively expressing the diguanylate cyclase $WspR^{R242A}$. Bacteria were grown in TSBD at 37°C under static conditions for 14 h. Values are the mean (± SD) of at least three independent assays.

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782 Fig. 5. Role of exopolysaccharides on attachment and aggregation of *P. aeruginosa* cells. (A)
783 Biofilm formation on polystyrene tubes by *P. aeruginosa* PAO1 and the *pel psl* mutant after 14 h of
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786 static conditions for 14 h. The images are representative of several micrographs from five
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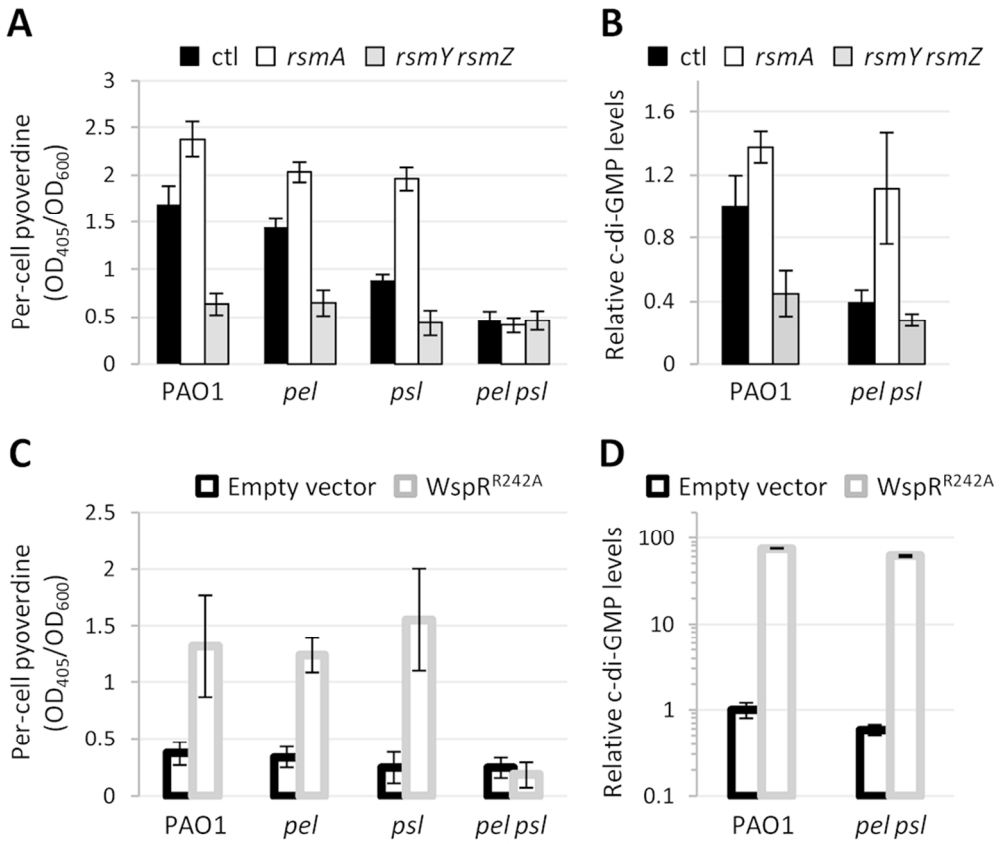
789 Fig. 6. Cell aggregation is involved in exopolysaccharide-mediated control of pyoverdine-
790 dependent virulence factors. (A) Fluorescent phenotype upon UV light exposure of *P. aeruginosa*
791 PAO1, the *pel psl* mutant and the *pvdA* mutant (used as pyoverdine-deficient negative control)

grown in liquid TSBD medium (Control) or on TSBD solidified with 1.5% agar, 1% phytagel or 10% acrylamide. (B) Pyoverdine production by *P. aeruginosa* PAO1 and the *pel psl* mutant grown in TSBD supplemented with increasing concentrations of agar (0-0.2%) and/or β-agarase I (3.3 units/ml). (C) Confocal microscopy images of *P. aeruginosa* PAO1 and *pel psl* cells harboring the GFP-expressing vector pMMG cultured in TSBD supplemented or not with 0.2% agar. Images are representative of several micrographs from at least three independent experiments. Bar: 50 µm. (D) Activity of the P*pvdS::lacZ*, P*pvdD::lacZ* and P*proC'-'lacZ* reporter fusions, (E) PrpL enzymatic activity and (F) ToxA levels in culture supernatants from *P. aeruginosa* PAO1 and *pel psl* cultures in TSBD supplemented or not with 0.2% agar. Bacteria were cultured in TSBD at 37°C under static conditions for 14 h. Values in panels B, D and E are the mean (± SD) of at least three independent assays. Images in panels A and F are representative of two independent experiments giving similar results.

Fig. 7. Pyoverdine production is not stimulated by unspecific physical contacts. Pyoverdine production by *P. aeruginosa* PAO1 and *pel psl* in the presence or in the absence of 3-µm size polystyrene beads (5×10^7 or 5×10^8 beads/ml), normalized to the number of colony forming units/ml and expressed as percentage with respect to the untreated wild type. Bacteria were grown in TSBD at 37°C for 14 h under vigorous shaking (220 rpm). Values are the mean (± SD) of three independent assays.



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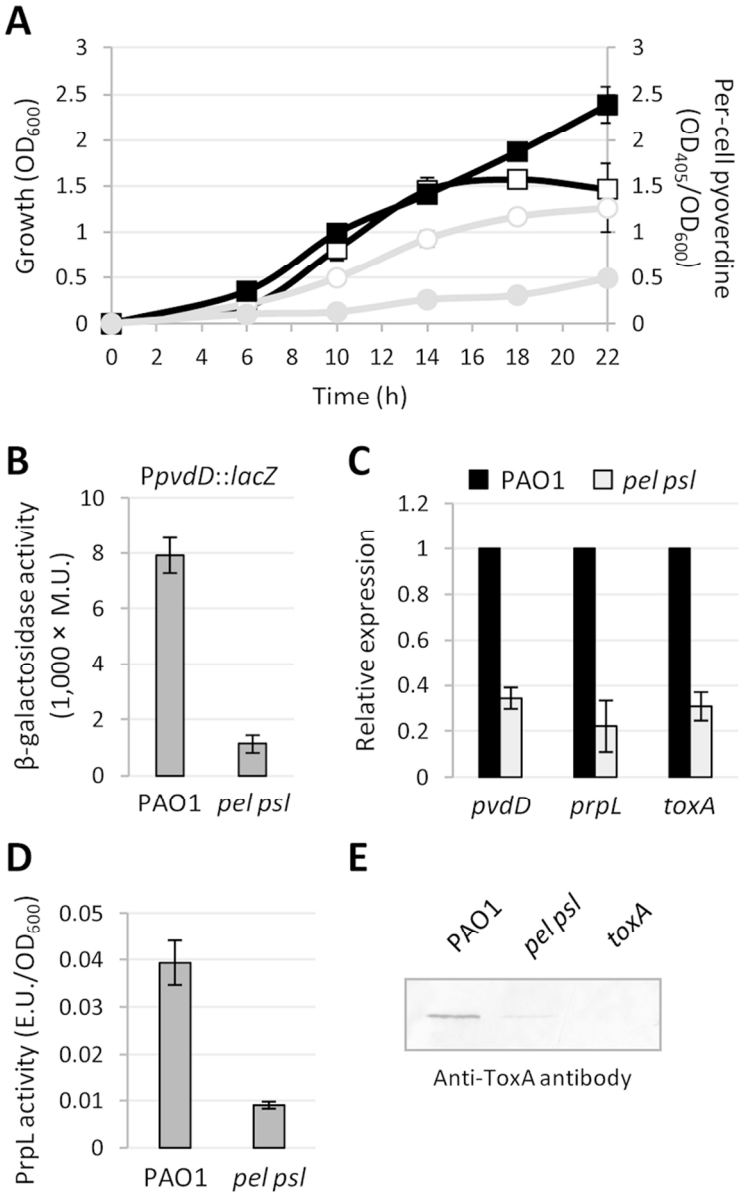

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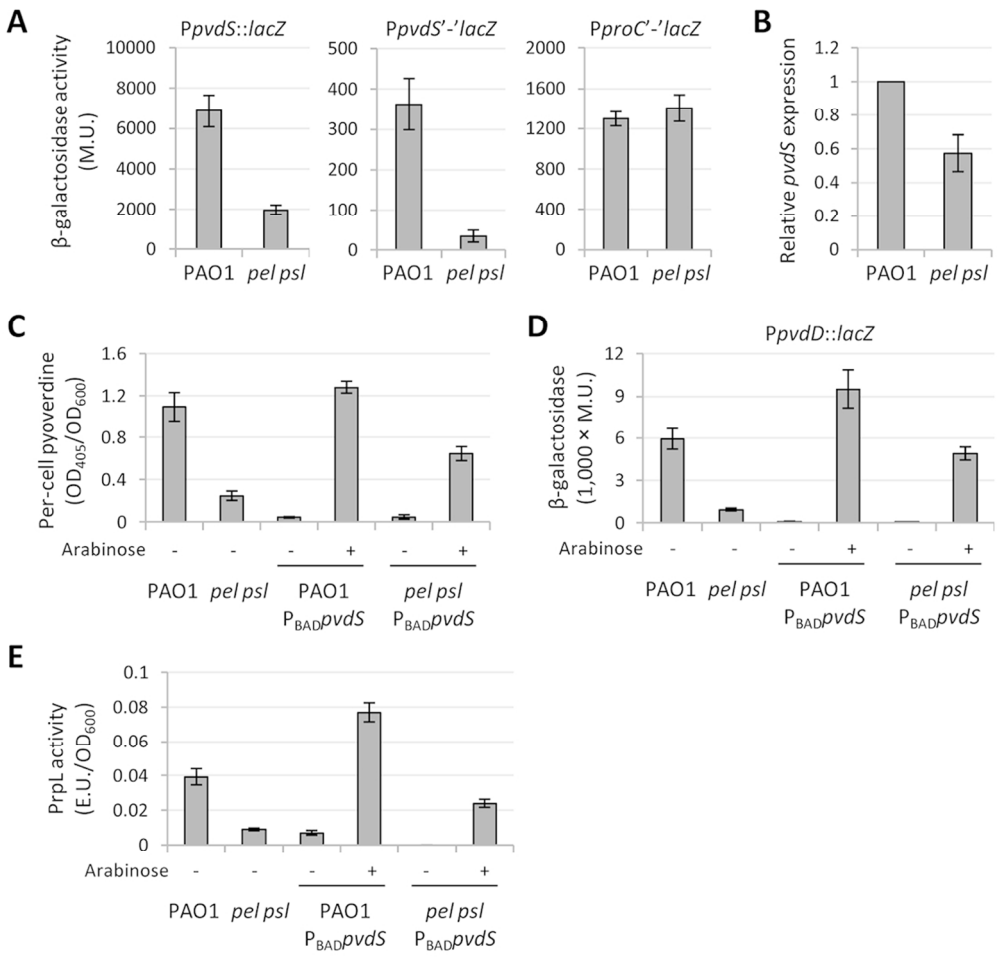

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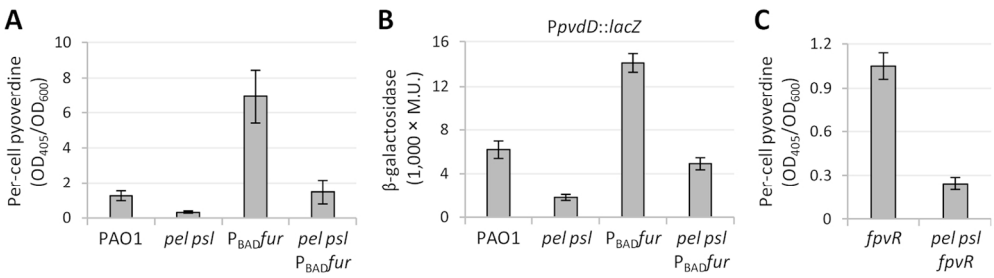

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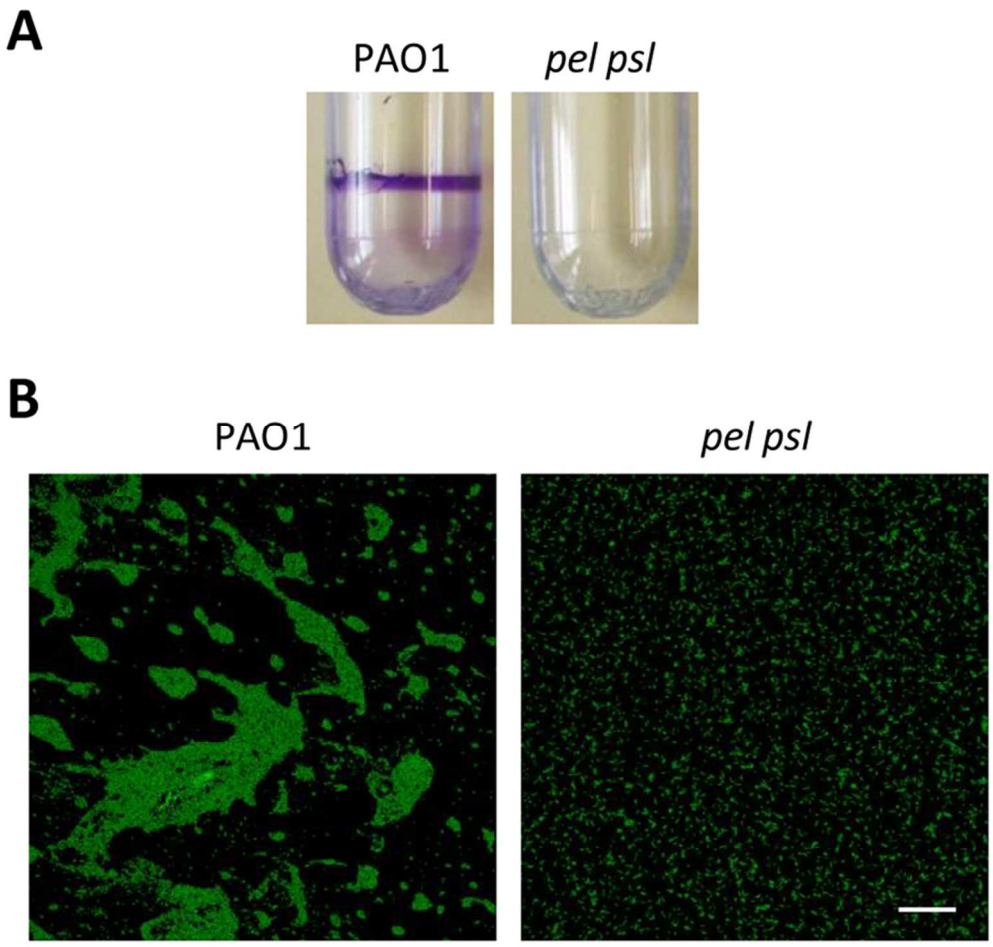

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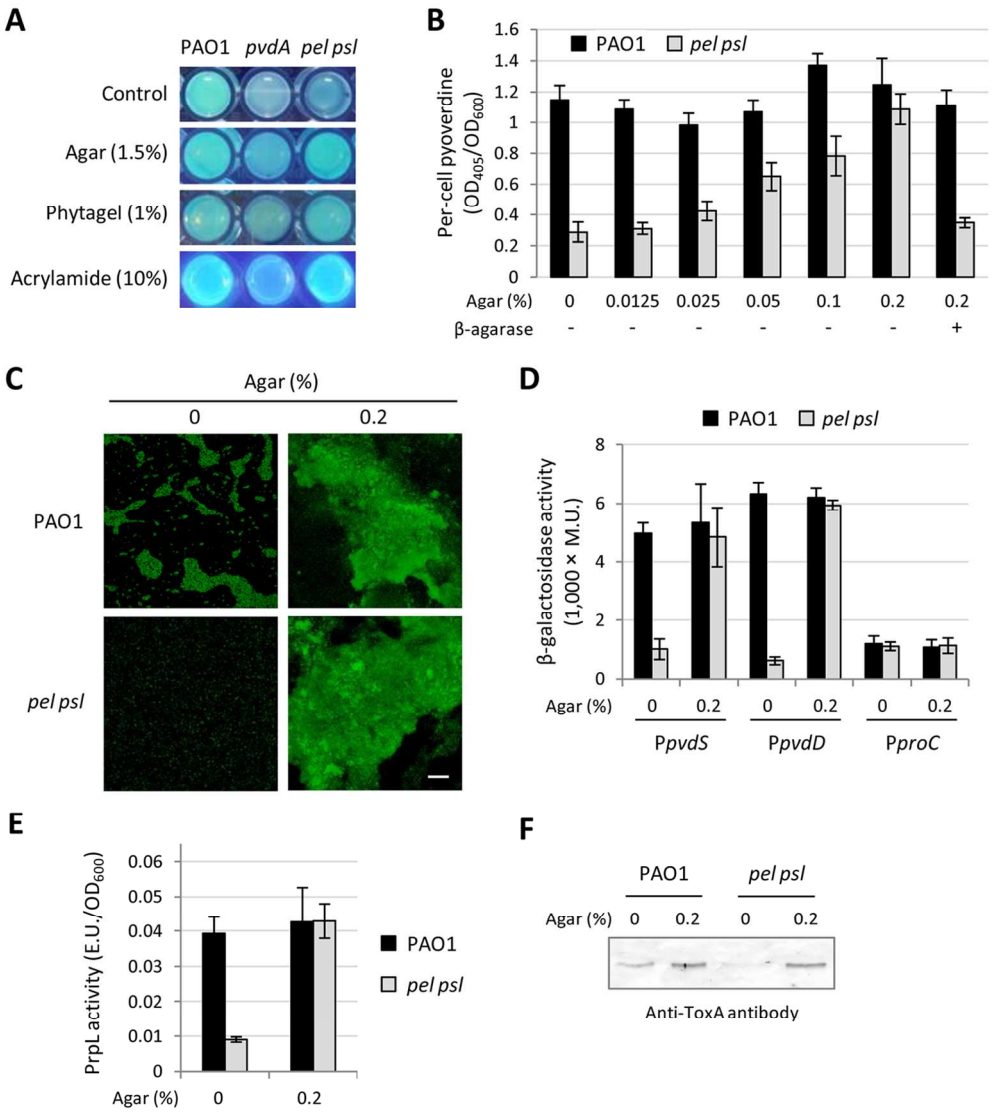

Fig. 6. Cell aggregation is involved in exopolysaccharide-mediated control of pyoverdine-dependent virulence factors. (A) Fluorescent phenotype upon UV light exposure of *P. aeruginosa* PAO1, the *pel psl* mutant and the *pvdA* mutant (used as pyoverdine-deficient negative control) grown in liquid TSBD medium (Control) or on TSBD solidified with 1.5% agar, 1% phytagel or 10% acrylamide. (B) Pyoverdine production by *P. aeruginosa* PAO1 and the *pel psl* mutant grown in TSBD supplemented with increasing concentrations of agar (0-0.2%) and/or β-agarase I (3.3 units/ml). (C) Confocal microscopy images of *P. aeruginosa* PAO1 and *pel psl* cells harboring the GFP-expressing vector pMMG cultured in TSBD supplemented or not with 0.2% agar. Images are representative of several micrographs from at least three independent experiments. Bar: 50 µm. (D) Activity of the P*pvdS*::*lacZ*, P*pvdD*::*lacZ* and P*proC*'-'*lacZ* reporter fusions, (E) PrpL enzymatic activity and (F) ToxA levels in culture supernatants from *P. aeruginosa* PAO1 and *pel psl* cultures in TSBD supplemented or not with 0.2% agar. Bacteria were cultured in TSBD at 37°C under static conditions for 14 h. Values in panels B, D and E are the mean (± SD) of at least three independent assays. Images in panels A and F are representative of two independent experiments giving similar results.
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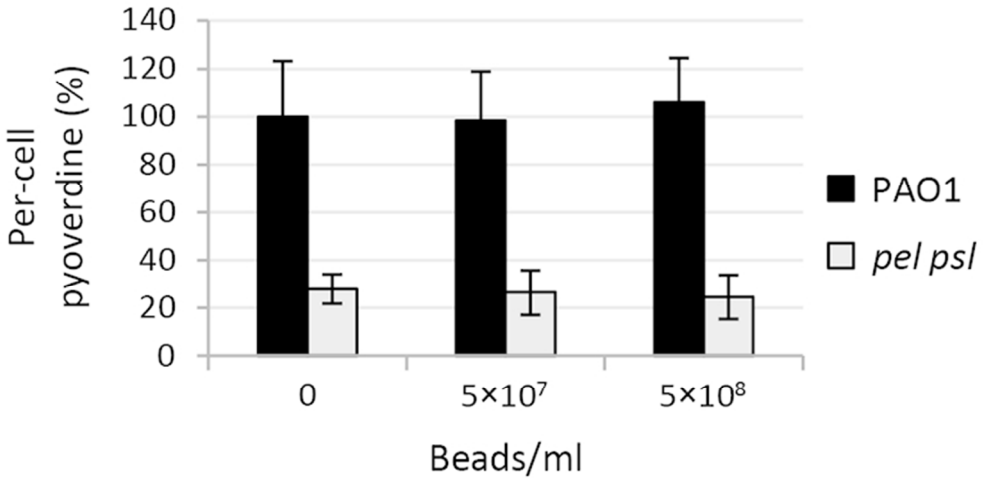

Fig. 7. Pyoverdine production is not stimulated by unspecific physical contacts. Pyoverdine production by *P. aeruginosa* PAO1 and *pel psl* in the presence or in the absence of 3-µm size polystyrene beads (5×10^7 or 5×10^8 beads/ml), normalized to the number of colony forming units/ml and expressed as percentage with respect to the untreated wild type. Bacteria were grown in TSBD at 37°C for 14 h under vigorous shaking (220 rpm). Values are the mean (± SD) of three independent assays.
66x33mm (300 x 300 DPI)

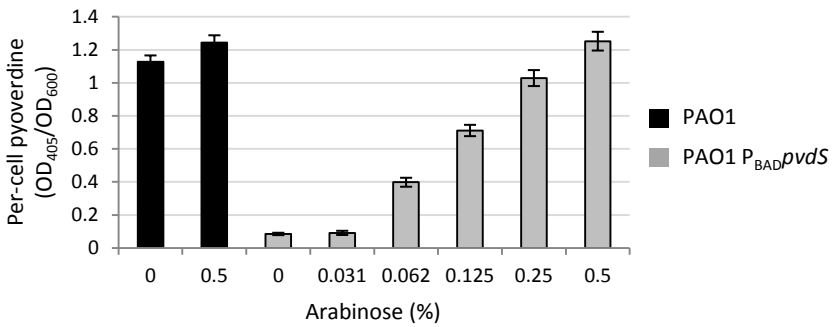

Fig. S1. Pyoverdine production by *P. aeruginosa* PAO1 and the *pvdS* conditional mutant PAO1 P_{BAD}*pvdS* grown in TSBD at 37°C under static conditions for 14 h in the presence of increasing concentrations of arabinose. Values are the mean (± SD) of two independent assays.

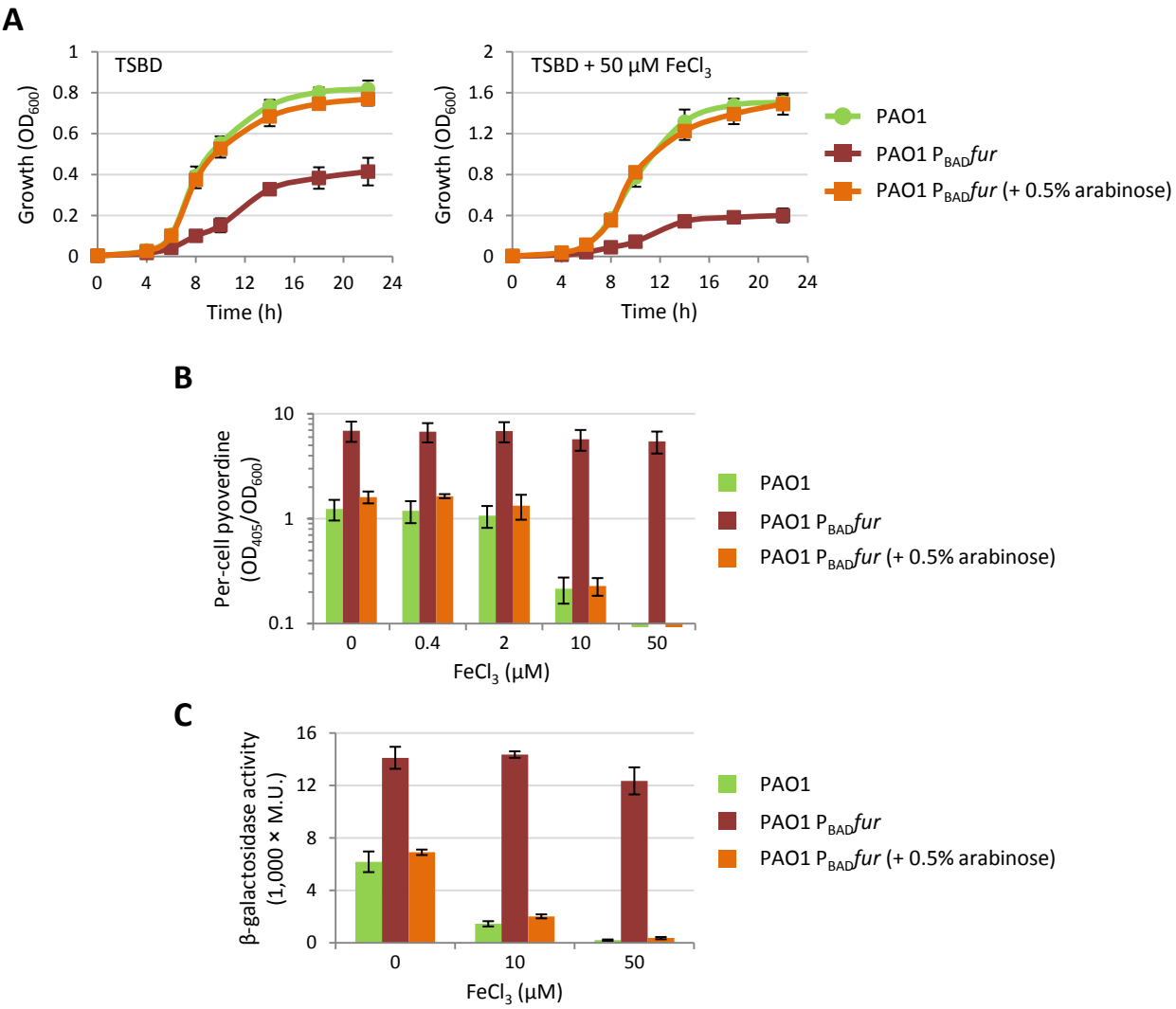

Fig. S2. Validation of the *fur* conditional mutant. (A) Growth of *P. aeruginosa* PAO1 and the *fur* conditional mutant (PAO1 P_{BAD}*fur*) in TSBD, supplemented or not with 50 µM $FeCl_3$ and/or 0.5% arabinose, at 37°C in microtiter plates under static conditions, measured in Victor²V plate reader (Perkin-Elmer). (B) Pyoverdine production and (C) activity of the P*pvdS*::*lacZ* transcriptional fusion in the *P. aeruginosa* PAO1 and the *fur* conditional mutant (PAO1 P_{BAD}*fur*) grown in TSBD, supplemented or not with 50 µM $FeCl_3$ and/or 0.5% arabinose, at 37°C in microtiter plates under static conditions for 14 h. Values are the mean (± SD) of at least two independent assays.

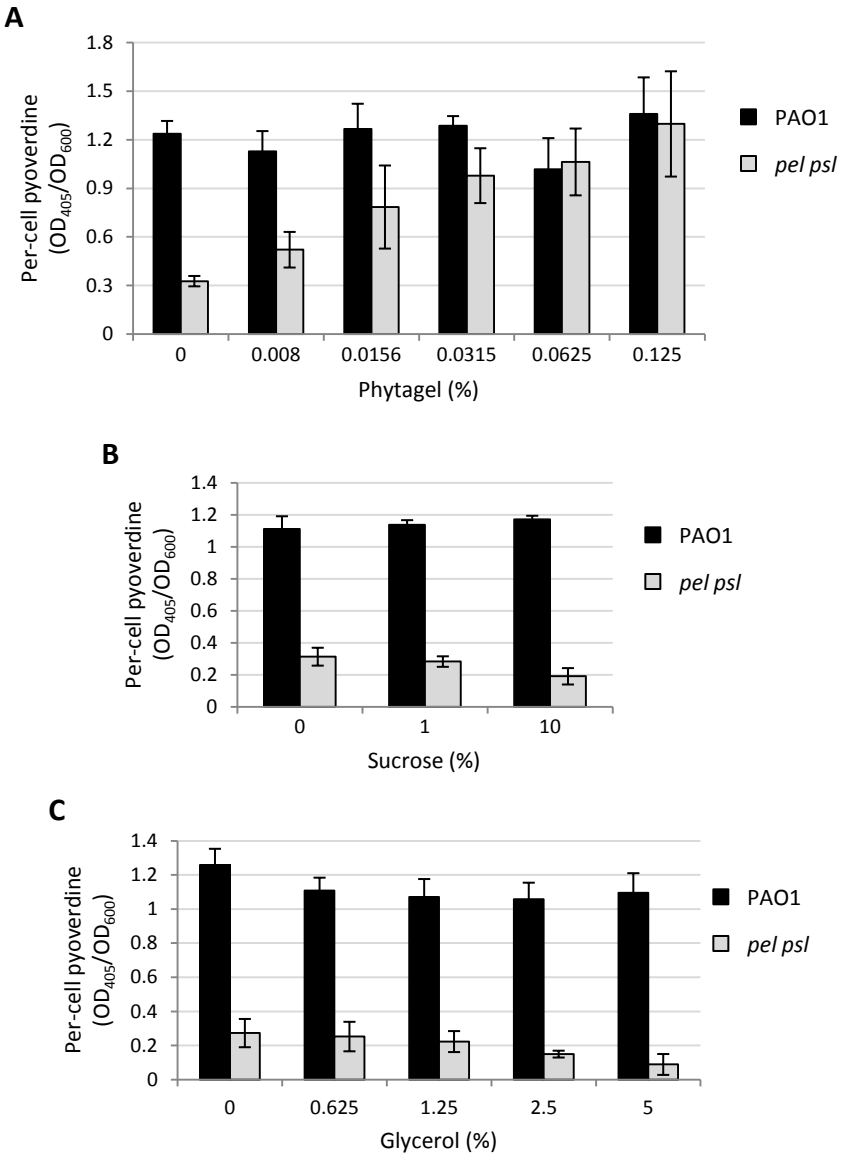

Fig. S3. Effect of phytagel, sucrose and glycerol on pyoverdine production. Pyoverdine production by *P. aeruginosa* PAO1 and the *pel psl* mutant grown in TSBD at 37°C in microtiter plates under static conditions for 14 h, in the presence of increasing concentrations of (A) phytagel (0-0.125%), (B) sucrose (0-10%) or (C) glycerol (0-5%). Values are the mean (± SD) of at least three independent assays.

Table S1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype and/or relevant characteristics	Reference or source
P. aeruginosa		
PAO1 (ATCC15692)	Prototroph	American type culture collection
PAO1 *rsmA*	PAO1 deleted of the *rsmA* coding sequence	Frangipani *et al.*, 2014
PAO1 *rsmY rsmZ*	PAO1 deleted of the *rsmY* and *rsmZ* genes	Frangipani *et al.*, 2014
PAO1 *pel*	PAO1 deleted of the *pelABCD* genes	This work
PAO1 *psl*	PAO1 deleted of the *pslABCD* genes	This work
PAO1 *pel psl*	PAO1 *pel* deleted of the *pslABCD* genes	This work
PAO1 *rsmA pel*	PAO1 *rsmA* deleted of the *pelABCD* genes	This work
PAO1 *rsmA psl*	PAO1 *rsmA* deleted of the *pslABCD* genes	This work
PAO1 *rsmA pel psl*	PAO1 *rsmA pel* deleted of the *pslABCD* genes	This work
PAO1 *rsmY rsmZ pel*	PAO1 *rsmY rsmZ* deleted of the *pelABCD* genes	This work
PAO1 *rsmY rsmZ psl*	PAO1 *rsmY rsmZ* deleted of *pslABCD* genes	This work
PAO1 *rsmY rsmZ pel psl*	PAO1 *rsmY rsmZ pel* deleted of *pslABCD* genes	This work
PAO1 *fpvR*	PAO1 deleted of the *fpvR* coding sequence	This work
PAO1 *pel psl fpvR*	PAO1 *pel psl* deleted of *fpvR* coding sequence	This work
PAO1 *pvdS*	PAO1 deleted of the *pvdS* coding sequence	Imperi *et al.*, 2013
PAO1 *pvdS araC*P_{BAD}*pvdS*	PAO1 *pvdS* carrying an arabinose-dependent copy of *pvdS*	This work
PAO1 *pel psl pvdS araC*P_{BAD}*pvdS*	PAO1 *pel psl* deleted of the *pvdS* coding sequence and carrying an arabinose-dependent copy of *pvdS*	This work
PAO1 *fur araC*P_{BAD}*fur*	PAO1 deleted of the *fur* coding sequence and carrying an arabinose-dependent copy of *fur*	This work
PAO1 *pel psl fur araC*P_{BAD}*fur*	PAO1 *fur araC*-P_{BAD}*fur* deleted of *pslABCD* and *pelABCD* genes	This work
PAO1 *pvdA*	PAO1 deleted of the *pvdA* coding sequence	Imperi *et al.*, 2008
PAO1 *toxA*	PA01 mutant with a transposon insertion in *toxA* (Mutant ID 40695), Tc^R	Jacobs *et al.*, 2003
E. coli		
S17.1λ*pir*	*thi pro hsdR hsdM*$^+$ *recA RP4-2-Tc::Mu-Km::Tn7 λpir, Gm*^{R}	Simon *et al.*, 1983
DH5αF'	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169[ϕ*80 dlacZ*Δ*M15*], Nal^R	Liss, 1987
Plasmid		
pBluescript-II KS+	Cloning vector; ColE1 replicon; Ap^R	Stratagene
pDM4	Suicide vector; *sacBR*, *oriR6K*; Cm^R	Milton *et al.*, 1996
pDM4Δ*pelABCD*	pDM4 derivative for *pelABCD* deletion	Leoni L., unpublished
pDM4Δ*pslABCD*	pDM4 derivative for *pslABCD* deletion	Leoni L., unpublished
pDM4Δ*fpvR*	pDM4 derivative for *fpvR* in-frame deletion	Visca P., unpublished
pDM4Δ*fur*	pDM4 derivative for *fur* in-frame deletion	This work
pEXΔ*pvdS*	pEX18Tc derivative for *pvdS* deletion, Gm^R, Tc^R	Imperi *et al.*, 2013

mini-CTX1	Self-proficient integration vector with *tet, Ω-FRT-attP*-MCS, *ori, int,* and *oriT*; Tc^R	Hoang *et al.*, 2000
mini-CTX1-*araC*P_{BAD}*tolB*	mini-CTX1 derivative carrying *araC*P_{BAD}*tolB*	Lo Sciuto *et al.*, 2014
mini-CTX1-*araC*P_{BAD}*fur*	mini-CTX1-*araC*P_{BAD}*tolB* derivative in which *tolB* has been replaced with *fur* by HindIII/EcoRI digestion	This work
mini-CTX1-*araC*P_{BAD}*pvdS*	mini-CTX1-*araC*P_{BAD}*tolB* derivative in which *tolB* has been replaced with *pvdS* by HindIII/EcoRI digestion	This work
pBBR1MCS-4	Broad-host range cloning vector; Ap^R	Kovach *et al.*, 1995
pBBR1MCS-4-*wspR*^{R242A}	R252A-*wspR* cloned into pBBR1MCS-4	Moscoso *et al.*, 2011
pME3641	Plasmid carrying a translational P*proC*::*lacZ* fusion, Cb^R	Savioz *et al.*, 1993
pMP220::P*pvdS*	Plasmid carrying a transcriptional P*pvdS*::*lacZ* fusion, Tc^R	Ambrosi *et al.*, 2002
pMP190::P*pvdD*	Plasmid carrying a transcriptional P*pvdD*::*lacZ* fusion, Cm^R	Cunliffe *et al.*, 1995
pME9301	Plasmid carrying a translational P*pvdS*`-`*lacZ* fusion, Tc^R	Frangipani *et al.*, 2008
pMMG	pME6032 derivative constitutively expressing the green fluorescent protein (GFP), Tc^R	Popat *et al.*, 2012

Additional references (not included in the main text):

Ambrosi, C., Leoni, L., and Visca, P. (2002) Different responses of pyoverdine genes to autoinduction in *Pseudomonas aeruginosa* and the group *Pseudomonas fluorescens-Pseudomonas putida*. Appl Environ Microbiol 68: 4122-4126.

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

Frangipani, E., Slaveykova, V.I., Reimmann, C., and Haas, D. (2008) Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation. J Bacteriol 190: 6706-6717.

Hoang, T.T., Kutchma, A.J., Becher, A., and Schweizer, H.P. (2000) Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid 43: 59-72.

Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., *et al.*, (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 100: 14339-14344.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M.I., and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 175–176.

Liss, L. (1987) New M13 host: DH5 F′ competent cells. Focus 9: 13.

Milton, D.L., O'Toole, R., Horstedt, P., and Wolf-Watz, H. (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. J Bacteriol 178: 1310-1319.

Popat, R., Crusz, S.A., Messina, M., Williams, P., West, S.A., and Diggle, S.P. (2012) Quorum-sensing and cheating in bacterial biofilms. Proc Biol Sci 279: 4765-4771.

Savioz, A., Zimmermann, A., and Haas, D. (1993) *Pseudomonas aeruginosa* promoters which contain a conserved GG-N10-GC motif but appear to be RpoN-independent. Mol Gen Genet 238: 74-80.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology 1: 784-790.

Table S2. Primers used in this study[1]

Primer name	Sequence (5'-3')[2]	Restriction site	Application
*pvdS*_FW	CCCAAGCTTATGTCGGAACAACTGTCTACC	HindIII	Generation of the mini-CTX1-*araC*P_{BAD}*pvdS* construct
*pvdS*_RV	CGGAATTCTGAGGAATGCTCGCCGC	EcoRI	Generation of the mini-CTX1-*araC*P_{BAD}*pvdS* construct
*fur*_FW	CCCAAGCTTATGGTTGAAAATAGCGAACTTC	HindIII	Generation of the mini-CTX1-*araC*P_{BAD}*fur* construct
*fur*_RV	CGGAATTCATGGAACCGTTGCGCGAC	EcoRI	Generation of the mini-CTX1-*araC*P_{BAD}*fur* construct
fur mut_UP_FW	CCGCTCGAGTCGGAACCGGTACCG	XhoI	Generation of the pDM4Δ*fur* construct
fur mut_UP_RV	CGGGATCCATGTCTGCTTTCTCAGCG	BamHI	Generation of the pDM4Δ*fur* construct
fur mut_DOWN_FW	CGGGATCCGCGCGGCTTCGAGCTGG	BamHI	Generation of the pDM4Δ*fur* construct
fur mut_DOWN_RV	GCTCTAGAGTTCCTGGTCAGCGCC	XbaI	Generation of the pDM4Δ*fur* construct
pel mut_UP_FW	ATATCTAGAACGCCGTTACGGCACCCT	XbaI	PCR check of *pel* deletion mutants
pel mut_DOWN_RV	ATACTCGAGGGGCGAAGAGAATCCTCAG	XhoI	PCR check of *pel* deletion mutants
psl mut_UP_FW	ATATCTAGACCGAAATGGCAC GAGGCG	XbaI	PCR check of *psl* deletion mutants
psl mut_DOWN_RV	ATACTCGAGTCAGCGATCATT GTTGACGG	XhoI	PCR check of *psl* deletion mutants
fpvR mut_UP_FW	GCTCTAGAAGGAACTGCGGCAGATG	XbaI	PCR check of *fpvR* deletion mutants
fpvR mut_UP_RV	CCGCTCGAGGGTGTACTGGGCAC	XhoI	PCR check of *fpvR* deletion mutants
*pvdD*_RT_FW	GAAAGGAAGGCATTGGCTG		Real-time PCR
*pvdD*_RT_RV	GTAGACGCAAGACACTCGGG		Real-time PCR
*toxA*_RT_FW	CGACCTCTGGAACGAATGC		Real-time PCR
*toxA*_RT_RV	TTGTCGATGGCCAGCTTG		Real-time PCR
*prpL*_RT_FW	GCCGGCAAGGAAATCTTC		Real-time PCR
*prpL*_RT_RV	CAGGGAGTCGGCGAAATAC		Real-time PCR
*pvdS*_RT_FW	TCGGAACAACTGTCTACCCG		Real-time PCR
*pvdS*_RT_RV	TGCCTTGAACGACGAAGTG		Real-time PCR

[1] Preparative PCRs for cloning were performed using *P. aeruginosa* PAO1 genomic DNA as the template.
[2] Restriction sites are underlined.

Chapter 5

Search for biofilm inhibitors through inhibition of the Gac regulatory system

Chapter 5

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Introduction

The relevance of the Gac system in *P. aeruginosa* pathogenicity has been investigated in many studies, showing that *gac* mutants have reduced ability to cause infection in different models, including plants, nematodes, insects and mice (Rahme *et al*., 1995; Tan *et al*., 1999;Jander *et al*., 2000; Coleman *et al*., 2003). Accordingly, it has been demonstrated that the deletion of the *rsmA* gene, which leads to a constitutively-active state of the Gac system, promotes the development of *P. aeruginosa* chronic infection in a mouse model, likely through stimulation of biofilm formation (Mulcahy *et al*., 2008).

The Gac system has a pivotal role in biofilm formation, as the deletion of *gacS* or *gacA* and their direct effectors *rsmZ* and *rsmY* impairs the ability of *P. aeruginosa* to generate biofilm (Parkins *et al*., 2001; Brencic *et al*., 2009). It has been demonstrated that the Gac system controls biofilm formation by modulating the expression of both the *pel* and *psl* operons, responsible for the production of the exopolysaccharides of the biofilm matrix (Brencic and Lory 2009). With the aim of identifying drugs able to reduce biofilm formation in *P. aeruginosa*, we searched for inhibitors of the Gac system, by employing the drug repurposing approach which was formerly used to identify PvdS inhibitors (Chapter 2). To this aim, a biosensor able to monitor the activation state of the Gac system has been generated and used to screen a commercial library consisting of 1,600 FDA approved drugs (PHARMAKON 1600, MicroSource Discovery Systems). The compounds selected in this screening were then tested for their ability to reduce biofilm formation in *P. aeruginosa*.

Materials and Methods

Bacteria, media and chemicals. Bacterial strains and plasmids used in this work are listed in Table 1. Bacteria were grown in Luria–Bertani medium or M9 minimal medium (Sambrook *et al*., 1989) supplemented with 0.2% glucose as carbon source. Acriflavine hydrochloride, proflavine hemisulfate, and 9-aminoacridine were purchased from Sigma-Aldrich.

General genetic procedures. *E. coli* was routinely used for recombinant DNA manipulations. The P*rpoB*::*lux* construct was generated by cloning the promoter region of *rpoB*, amplified by PCR using the primers P*rpoB*_FW (5'-CCCAAGCTTACAGCCCGAGCGTCAAG-3') and P*rpoB*_RV (5'-CGGGATCCGCAGCGGTACGGCGAAG -3') and digested with the enzymes HindIII and BamHI (restriction sites are underlined in the primer sequences), in the plasmid mini-CTX-*lux* (Becher and Schweizer, 2000) previously digested with the same enzymes. The P*rsmZ*::*lux,* P*rsmY*::*lux and* P*rpoB*::*lux* constructs (Table 1) were integrated into the genome of *P. aeruginosa* strains as described (Massai *et al*., 2011).

Screening for Gac inhibitors. Overnight cultures of PAO1 Pr*smZ*::*lux* were diluted to A_{600} = 0.003 in LB medium and grown at 37°C in microtiter plates in the presence or in the absence of 20 or 200 µM of each compound of the PHARMAKON 1600 library (200 µl final volume).The A_{600} and bioluminescence (LCPS) were measured after 14 hours of growth in a Victor^{3}V plate reader (Perkin-Elmer), and luminescence values were normalized by the A_{600} of the bacterial culture. Criteria used for the selection of hit compounds were: (i) ≥ 50% inhibition of normalized bioluminescence emission and (ii) ≤ 20% alteration of growth relative to the untreated control. Criterion (ii) was aimed at avoiding any unspecific effect of altered growth on bioluminescence emission and/or *rsmZ* expression.

Biofilm assay. Visualization and quantification of biofilm formation was carried out in 12 ml glass tubes. Bacteria were inoculated at an A_{600} = 0.02 in 1 ml of M9 minimal medium supplemented with 0.2% glucose in the presence or in the absence of acriflavine hydrochloride, proflavine hemisulfate, or 9-aminoacridineat 25 or 50 µM concentration. After 14-hours incubation at 37°C under static conditions, the A_{600} of the bacterial cultures (or of appropriate dilutions) was measured in a spectrophotometer (AG22331 Hamburg/ Eppendorf). Planktonic cells were discarded and the tubes were washed with sterile water three times. Attached cells were stained with 0.1% crystal violet and tubes were washed with water to remove unbound dye. Crystal violet was solubilized with 1.2 ml of 95% ethanol, the A_{590} was measured and

the values obtained were normalized to the cell density (A_{600}) of the corresponding bacterial cultures.

Table 1 Bacterial strains and plasmid used in this work

Strain or plasmid	Genotype and/or relevant characteristics	Reference or source
P. aeruginosa		
PAO1 (ATCC15692)	Prototroph	American type culture collection
PAO1 Δ*gacS*	PAO1 deleted of the *gacS* coding sequence	Frangipani *et al*., 2014
PAO1 Δ*rsmZrsmY*	PAO1 deleted of the *rsmZ and rsmY*	Frangipani *et al*., 2014
PAO1 P*rsmZ*::*lux*	PAO1 with a *PrmsZ::lux* fusion integrated into the chromosome in the neutral *attB* site	This work
PAO1 *PrsmY::lux*	PAO1 with a *PrmsY::lux* fusion integrated into the chromosome in the neutral *attB* site	This work
PAO1 *PrpoB::lux*	PAO1 with a *PrpoB::lux* fusion is integrated into the chromosome in the neutral *attB* site	This work
PAO1 Δ*gacS PrsmZ::lux*	PAO1 Δ*gacS* with a *PrmsZ::lux* fusion integrated into the chromosome in the neutral *attB* site	This work
E. coli		
S17.1λ*pir*	*thi pro hsdR hsdM*$^{+}$ *recA RP4-2-Tc::Mu-Km::Tn7 λpir, Gm*^{R}	Simon *et al*., 1983
DH5αF'	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* ∆(*lacZYA-argF*)U169[ϕ*80 dlacZ*∆*M15*], Nal^{R}	Liss, 1987
Plasmid		
pBluescript-II KS+	Cloning vector; ColE1 replicon; Ap^{R}	Stratagene
mini-CTX-*lux*	Promoter-probe vector containing the *luxCDABE* operon; Tc^{R}	Becher and Schweizer, 2000
mini-CTX P*rsmY::lux*	Plasmid to insert a P*rsmY*::*lux* fusion into the chromosome of *P. aeruginosa*	S. Heeb, unpublished
mini-CTX P*rsmZ::lux*	Plasmid to insert a P*rsmZ*::*lux* fusion into the chromosome of *P. aeruginosa*	S. Heeb, unpublished
mini-CTX P*rpoB*::*lux*	Plasmid to insert a P*rpoB*::*lux* fusion into the chromosome of *P. aeruginosa*	This work

Results and Discussion

Construction and validation of the biosensor for Gac inhibitors

When the Gac system is active, the sensor kinase GacS activates GacA which in turn triggers the transcription of the *rsmZ* and *rsmY* genes, encoding the small RNAs RsmZ and RsmY. The Gac system directly regulates, at the transcriptional level, only the expression of these two small RNAs (Brencic *et al*., 2009). In order to generate a reporter system to monitor the activation state of the Gac system, the promoter region of *rsmZ* was fused to the *luxCDABE* operon and inserted into a neutral site of the chromosome in the wild type strain *P. aeruginosa* PAO1. As negative control, the construct was also inserted in a isogenic *gacS* deletion mutant, in which the Gac system is constitutively inactive. As expected, the expression of *rsmZ*, measured as bioluminescence emission normalized to the cell density of the bacterial culture, was strongly reduced in the *gacS* mutant compared to the wild type (Figure 1A).

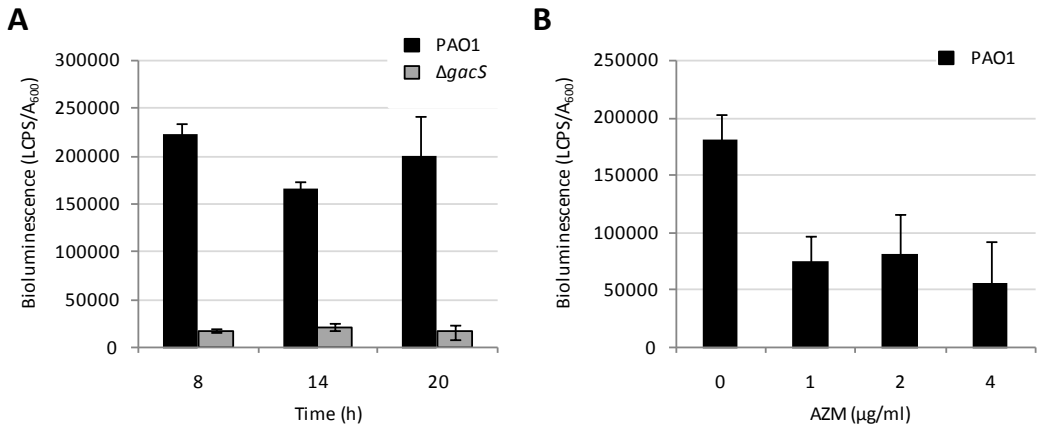

Fig 1. Validation of the reporter system.
(A) Bioluminescence emission (LCPS/A_{600}) by PAO1 P*rsmZ::lux* and Δ*gacS* P*rsmZ::lux* grown for 14 h in LB medium at 37°C under static conditions. (B) Bioluminescence emission (LCPS/A_{600}) by PAO1 P*rsmZ::lux* in the presence of increasing concentrations of AZM (0-4 µg/ml) after 14 h of growth in LB medium at 37°C under static conditions. Values represent the mean (± SD) of three independent assays.

To further verify the reliability of this reporter system, the expression of *rsmZ* was measured in the presence of different sub-MIC concentrations of azithromycin (AZM), since it has been recently reported that AZM at sub-MIC concentrations reduces the expression of *rsmZ* and *rsmY* by affecting the translation of regulatory proteins involved in the activation of the small RNA transcription (Pérez-Martínez and Haas, 2011). AZM treatment caused a 50% decrease in bioluminescence emission compared

with the untreated control (Figure 1B). Overall, these results confirmed the suitability of the PAO1 P*rsmZ*::*lux* reporter system to monitor the activation state of the Gac system.

Screening for Gac inhibitors

The PAO1 P*rsmZ*::*lux* reporter system was used to screen the commercial library PHARMAKON 1600. This library consists of 1,600 chemical compounds with known biological activities, selected for their high chemical and pharmacological diversity and safety in humans (http://www.msdiscovery.com/pharma.html).

The aim of the screening was to identify compounds which are able to inhibit the bioluminescence emission but that have little or no effect on bacterial growth. Only three of the 1,600 compounds tested (*i.e.* acriflavine hydrochloride, proflavine hemisulfate, and 9-aminoacridine) showed reproducible inhibitory activity on *rsmZ* transcription with minor effects on bacterial growth. These compounds correspond to different flavine derivatives, and are generally used as local antiseptics, although *P. aeruginosa* is intrinsically resistant to the growth inhibitory activity of these drugs likely due to active efflux (Sekiya *et al*. 2003).

To verify the results of the screening assay, the three compounds were purchased from an alternative supplier (Sigma-Aldrich) and retested at different concentrations for their inhibitory activity on *rsmZ* expression. This new experiment confirmed that flavines have little effect on bacterial growth and strong inhibitory activity on *rsmZ* expression in a dose-dependent manner (Figures 2A and 2B). In order to assess the specificity of the selected compounds, they were also tested on *P. aeruginosa* PAO1 recombinant strains containing either a P*rsmY::lux* transcriptional fusion or a *PrpoB::lux* fusion (Table 1). The housekeeping gene *rpoB* encodes the β subunit of the RNA polymerase, and was therefore used as control to rule out any generalized effect on gene expression. A reduction of *rsmY* promoter activity (40-70% in the presence of 25-50 μM compound concentrations) was observed for all compounds, although it was more evident for acriflavine and proflavine compared to 9-aminoacridine (Figure 2C) and, in general, it was less pronounced than that observed with the P*rsmZ::lux* fusion (Figure 2A). On the other hand, flavines did not show any relevant inhibitory effect on *rpoB* transcription, while 9-aminoacridine and, to a lesser extent, proflavine actually induced P*rpoB* activity (Figures 2C e 2D).

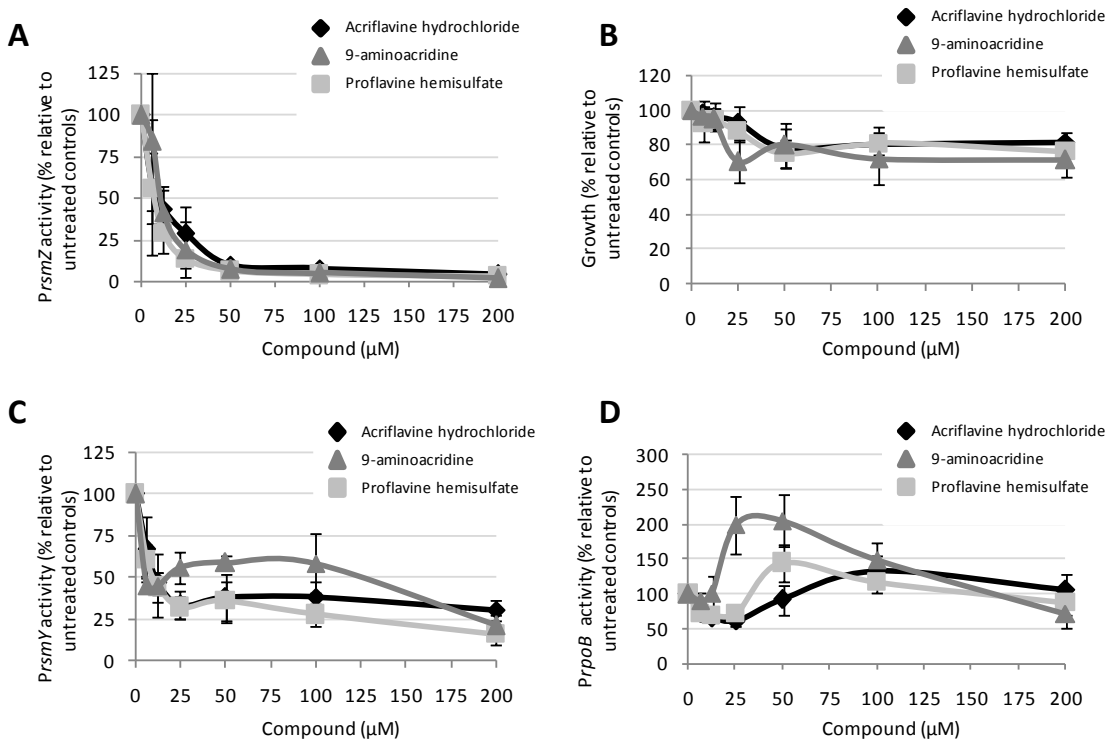

Fig 2. Flavines negatively affect the activity of the *rsmZ* and *rsmY* promoters
Bioluminescence emission (LCPS/A_{600}), expressed as percentage relative to untreated controls, by (A) PAO1 P*rsmZ::lux*, (C) PAO1 P*rsmY::lux* and (D) PAO1 P*rpoB::lux* grown for 14 h in LB medium at 37°C under static conditions in the presence of increasing concentrations (0-200 µM) of acriflavine hydrochloride, proflavine hemisulfate or 9-aminoacridine. (B) Growth (A_{600}), expressed as percentage relative to untreated controls, of PAO1 P*rsmZ::lux* grown as described above in the presence of increasing concentrations (0-200 µM) of acriflavine hydrochloride, proflavine hemisulfate or 9-aminoacridine. Values represent the mean (± SD) of at least three independent assays.

Effect of flavines on biofilm formation

The Gac system is involved in the regulation of the switch from the planktonic to the biofilm lifestyle, and indeed *P. aeruginosa* mutants impaired in Gac signaling (*gacA* or *rsmZ rsmY* mutants) were found to be strongly defective in biofilm formation (Brencic *et al*., 2009; Parkins *et al*., 2001; Sall *et al*., 2014, Figure 3A). We therefore investigated whether flavines could reduce or inhibit biofilm formation by negatively affecting the activation state of the Gac system. Surprisingly, the addition of each flavine to the growth medium caused an increase in biofilm formation in the wild type strain (Figure 3B), indicating that, irrespective of their inhibitory effect on the Gac system, these compounds actually promote biofilm formation in *P. aeruginosa*, likely in a Gac-independent manner. The latter hypothesis was verified by testing the effect of the flavine compounds on biofilm formation by a *rsmZ rsmY* deletion mutant, in

which the Gac system is constitutively inactive (Brencic *et al*. 2009; Moscoso *et al*. 2011; Frangipani *et al*. 2014). The addition of flavines to the growth medium caused a strong increase in the ability of *rsmZ rsmY* mutant to generate biofilm (Figure 3C), confirming that the unexpected positive effect of flavines on biofilm formation by *P. aeruginosa* is independent of the activity of the Gac system.

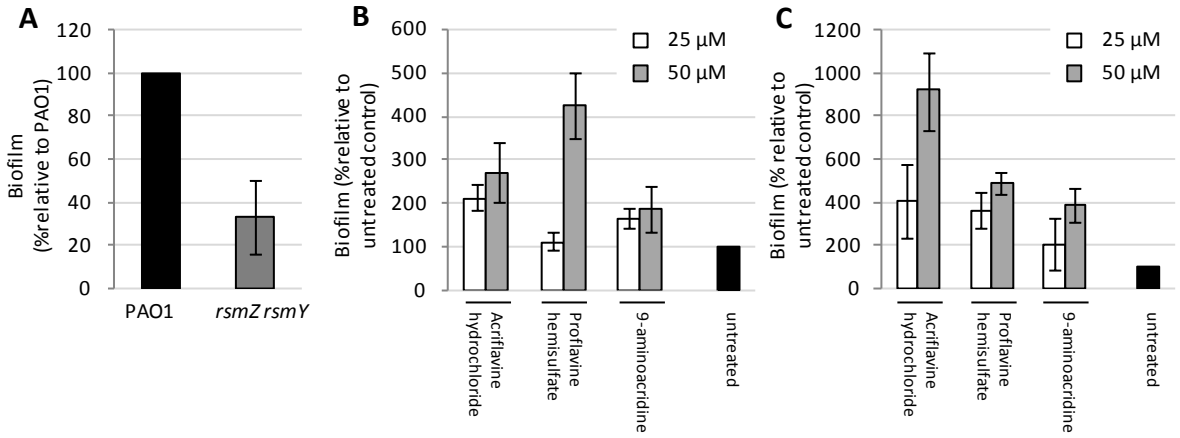

Fig 3. Effect of flavins on biofilm formation.
(A) Biofilm formation (A_{590}/A_{600}) on glass tubes by the wild type strain PAO1 and an isogenic *rsmZ rsmY* double mutant after 14 h of growth in M9 with 0.2% glucose under static conditions. Biofilm formation is expressed as percentage relative to PAO1. B-C) Biofilm formation (A_{590}/A_{600}) on glass tubes by PAO1 (B) or the *rsmZ rsmY* mutant (C) in the presence of 25 or 50 µM of acriflavine hydrochloride, proflavine hemisulfate or 9-aminoacridine. The values are expressed as percentage relative to untreated controls. Strains were cultured as described in panel A. Values represent the mean (± SD) of three independent assays. Comparable results were obtained in LB medium (data not shown).

Conclusions

In this study the drug repurposing approach was used to search for inhibitors of the Gac system, with the final goal of identifying anti-biofilm drugs active against *P. aeruginosa*. Three compounds showing a potent inhibitory effect on *rsmZ* and *rsmY* promoter activity were found, but unexpectedly they all caused a strong (Gac-independent) increase in biofilm formation, clearly indicating that these compounds cannot be further developed as anti-biofilm drugs against *P. aeruginosa*. Thus, although the drug repurposing approach can represent a promising strategy to search for anti-virulence side activities, as demonstrated by our flucytosine study (Chapter 2) and some other works (Imperi *et al*., 2013; Ho Sui *et al*., 2012; Gi *et al*., 2014), it failed in identifying anti-biofilm compounds among those showing Gac inhibitory activity. This could be at least partially related to the multifactorial nature of the biofilm phenotype, which is controlled by a several cellular and environmental factors (Coggan and Wolfgang, 2012; Balasubramanian *et al*., 2013). Thus, a compound with activity against a specific regulatory network (*e.g.* the Gac system) could also have other general effects on *P.* aeruginosa physiology which could ultimately influence the biofilm mode of growth, in an unpredictable manner.

References

Balasubramanian D, Schneper L, Kumari H, Mathee K (2013) A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. Nucleic Acids Res. **41**:1-20.

Becher A, Schweizer HP (2000) Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *Biotechniques* **29**:948-952.

Brencic A, Lory S (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. Mol Microbiol.**72**:612-32.

Brencic A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, Lory S (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusivelythrough its control over the transcription of the RsmY and RsmZ regulatory small RNAs. MolMicrobiol. **73**:434-45.

Coggan KA, Wolfgang MC (2012) Global regulatory pathways and cross-talk control Pseudomonas aeruginosa environmental lifestyle and virulence phenotype. Curr Issues Mol Biol. **14**:47-70.

Coleman FT, Mueschenborn S, Meluleni G, Ray C, Carey VJ, Vargas SO, Cannon CL,Ausubel FM, Pier GB (2003) Hypersusceptibility of cystic fibrosis mice to chronic *Pseudomonas aeruginosa* oropharyngeal colonization and lung infection. ProcNatlAcadSci U S A. **100**:1949-54.

Frangipani E, Visaggio D, Heeb S, Kaever V, Cámara M, Visca P, Imperi F (2014) The Gac/Rsm and cyclic-di-GMP signalling networks coordinately regulate iron uptake in *Pseudomonas aeruginosa*. Environ Microbiol. **16**:676-88.

Gi M, Jeong J, Lee K, Lee KM, Toyofuku M, Yong DE, Yoon SS, Choi JY (2014) A drug-repositioning screening identifies pentetic acid as a potential therapeutic agent for suppressing the elastase-mediated virulence of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. **58**:7205-14

Ho Sui SJ, Lo R, Fernandes AR, Caulfield MD, Lerman JA, Xie L, Bourne PE, Baillie DL, Brinkman FS (2012) Raloxifene attenuates *Pseudomonas aeruginosa* pyocyanin production and virulence. Int J Antimicrob Agents. **40**:246-51.

Jander G, Rahme LG, Ausubel FM (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J Bacteriol. **182**:3843-5.

Imperi F, Massai F, Ramachandran Pillai C, Longo F, Zennaro E, Rampioni G, Visca P, Leoni L (2013) New life for an old drug: the anthelmintic drug niclosamide inhibits *Pseudomonas aeruginosa* quorum sensing. Antimicrob Agents Chemother. **57**:996-1005. doi: 10.1128/AAC.01952-12. Epub 2012 Dec 17.

Imperi F, Massai F, Facchini M, Frangipani E, Visaggio D, Leoni L, Bragonzi A, Visca P (2013) Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity. ProcNatlAcadSci U S A. **110**:7458-63

Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ (2012) The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev. **76**:46-65

Liss L (1987) New M13 host: DH5 F' competent cells. Focus **9**:13.

Massai F, Imperi F, Quattrucci S, Zennaro E, Visca P, Leoni L (2011) A multi task biosensor for micro-volumetric detection of N-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal. Biosens Bioelectron. **26**:3444-9.

Mulcahy H, O'Callaghan J, O'Grady EP, Maciá MD, Borrell N, Gómez C, Casey PG, Hill C, Adams C, Gahan CG, Oliver A, O'Gara F (2008) *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence,persistence, and pulmonary inflammation. Infect Immun. **76**:632-8.

Parkins MD, Ceri H, Storey DG (2001) *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. Mol Microbiol. **40**:1215-26.

Pérez-Martínez I, Haas D (2011) Azithromycin inhibits expression of the GacA-dependent small RNAs RsmY and RsmZ in *Pseudomonas aeruginosa*. AntimicrobAgents Chemother. **55**:3399-405.

Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM (1995) Commonvirulence factors for bacterial pathogenicity in plants and animals. Science. 268:1899-902.

Reimmann C, Beyeler M, Latifi A, Winteler H, Foglino M, Lazdunski A, Haas D (1997)The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol Microbiol. **24**:309-19.

Sall KM, Casabona MG, Bordi C, Huber P, de Bentzmann S, Attrée I, Elsen S (2014) A gacS deletion in *Pseudomonas aeruginosa* cystic fibrosis isolate CHA shapes its virulence. PLoS One. **9**:e95936.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), 2nd Ed.

Sekiya H, Mima T, Morita Y, Kuroda T, Mizushima T, Tsuchiya T (2003) Functional cloning and characterization of a multidrug efflux pump, mexHI-opmD, from a *Pseudomonas aeruginosa* mutant. Antimicrob Agents Chemother. **47**:2990-2.

Simon, R., Priefer, U. and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1**: 784-790.

Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM (1999) *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. ProcNatlAcadSci U S A. **96**:2408-13

Chapter 6

Concluding remarks

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P. aeruginosa is one of the most dreaded opportunistic pathogens in the hospital setting and represents the first cause of morbidity and mortality in cystic fibrosis patients (Driscoll *et al*., 2007). The success of this opportunistic pathogen is mainly due to its ability to resist to several antimicrobial agents and to produce a multiplicity of virulence factors (Gellatly and Hancock, 2013). Among the virulence factors produced by *P. aeruginosa*, the siderophore pyoverdine is one of the most interesting because it plays a dual role during the infection; as an iron chelator, it allows the bacteria to survive under iron depleted conditions while, as a signal molecule, it promotes the expression of other virulence factors (Visca et al., 2007). Moreover, it has been demonstrated that this siderophore is also important for biofilm formation under low-iron conditions (Banin *et al*., 2005). In *P. aeruginosa* biofilm formation is mainly regulated by two different signaling networks, namely the Gac system and c-di-GMP signaling (Hickman *et al*., 2005; Brencic and Lory 2009). They control biofilm formation by regulating, at the transcriptional or translational level, the expression of the operons encoding the enzymes involved in the synthesis of the exopolysaccharides Pel and Psl. Different transcriptomic studies suggested that the Gac system could interplay with the pyoverdine system, although results were not conclusive (Burrowes *et al*., 2006; Brencic and Lory 2009). In this PhD thesis, the role of the Gac system in the regulation of pyoverdine production has been clarified. It has been shown that the Gac system, as well as the intracellular signaling molecule c-di-GMP, promote pyoverdine production by increasing the expression of the two aggregative exopolysaccharides Pel and Psl. These exopolysaccharides are an important component of the biofilm extracellular matrix, but are also essential for bacterial cell aggregation in liquid culture, as it has been shown in this thesis and previously suggested by Klebensberger and colleagues (Klebensberger *et al*., 2007). Notably, we have demonstrated that the effect of Pel and Psl on pyoverdine production does not depend on the exopolysaccharides *per se*, but on their ability to support the formation of planktonic aggregates, as confirmed through the simulation of cellular aggregation in an exopolysaccharide-independent manner. The mechanism by which aggregation promotes pyoverdine production remains an open issue, but our results rule out the involvement of well-known regulators of pyoverdine gene expression (i.e. the ferric uptake regulator Fur and pyoverdine signaling). On the other hand, the role of the alternative sigma factor PvdS in aggregation-mediated regulation of pyoverdine genes

remains controversial. Although we have observed a strong effect of the Gac system and c-di-GMP and consequently, of the Pel and Psl exopolysaccharides, on *pvdS* transcription (Chapters 3 and 4), the constitutive expression of this alternative sigma factor is not sufficient to restore pyoverdine production in the exopolysaccharide-null mutant. This evidence indicates that other mechanisms downstream of *pvdS* gene expression could be involved in the regulation of pyoverdine mediated by cellular aggregation. Our study strongly suggests the presence of a new regulatory system, activated during planktonic aggregate formation, which controls the expression of pyoverdine genes and, consequently, the production of pyoverdine-dependent virulence factors. Planktonic aggregates could represent an intermediate way of growth between the dispersed single cells and the mature biofilm , and cellular aggregation is also the first committed step of the biofilm formation. A transcriptomic analysis suggested that during the mature biofilm the virulence genes expression is attenuated (Waite *et al*., 2006). This PhD thesis suggests that the virulence potential of *P. aeruginosa* may be actually increased during the first stages of biofilm formation, when siderophores, extracellular enzymes and toxins would provide cells with essential nutrients for the energy-demanding biofilm development process. Further studies are clearly required to identify and characterize the system(s) involved in the regulation of virulence during the formation of the planktonic aggregates.

The knowledge of the regulatory networks controlling the expression of virulence genes is essential not only for better understanding the pathogenicity of this bacterium but also for the development of other possible strategies to fight *P. aeruginosa* infections. *P. aeruginosa* infections are generally difficult to treat due to resistance to many antimicrobial agents (Moore and Flaws, 2011). The antimicrobial agents commonly used in clinical practice cause a strong selective pressure which promotes the development of resistance. A promising strategy to reduce the emergence of drug resistance is to inhibit virulence factor production rather than inhibit bacterial growth (Clatworthy *et al*., 2007). In this thesis, a drug repurposing approach was used to search for anti-pyoverdine and anti-Gac compounds, by screening two different libraries of compounds already approved for use in humans. The first screening led to the identification of an antimycotic drug, flucytosine, which showed promising anti-virulence activity both *in vitro* and *in vivo* (Chapter 2). Flucytosine inhibits pyoverdine-dependent virulence by reducing the expression of the alternative sigma factor PvdS. To exert its inhibitory activity on pyoverdine production, flucytosine has to be converted into 5-fluorouracil by the cellular enzyme

cytosine deaminase. Although flucytosine by itself is not toxic, 5-fluorouracil is highly cytotoxic since it inhibits protein synthesis and DNA replication in eukaryotic cells (Pan et al., 2011). Although our work led to exclude that flucytosine and, thus, 5-fluorouracil affect pyoverdine production through Fur or pyoverdine signaling, the mechanism by which 5-fluorouracil impairs the transcription of *pvdS* remains unknown. Nevertheless the anti-virulence activity of flucytosine, both *in vitro* and *in vivo,* confirms that the pyoverdine system represents an excellent target to disarm *P. aeruginosa*.

The results obtained in the screening for Gac inhibitors are less encouraging. Although the screening led to the identification of three inhibitors of the activation of the Gac system, these drugs were found to promote, rather than inhibit, biofilm formation in *P. aeruginosa*, in a Gac-independent manner. Consequently, these compounds were not investigated further as anti-biofilm compounds. Biofilm formation is a multi-factorial process (Jimenez *et al*., 2012) and it cannot be excluded that a given compound with activity against a specific regulatory network (*e.g.* the Gac system) could also have other (unpredictable) effects on *P. aeruginosa* physiology, which could ultimately affect the switch from the planktonic to the biofilm lifestyle.

In conclusion, in this thesis the drug repurposing approach has been used to search for pyoverdine and Gac inhibitors, but the outcomes of these two screenings were completely different. In one case, a new promising drug to fight *P. aeruginosa* infections was identified, while in the second one the hit compounds failed in the suppressing biofilm formation. Despite the discouraging results obtained with the anti-Gac screening, flucytosine and other anti-virulence compounds identified by other research groups (Ho Sui *et al*., 2012, Gi *et al* 2014) using the drug repurposing approach represent the proof that this strategy can be considered a fast and cheap way to identify new potential anti-virulence drugs.

References

Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci U S A. **102**:11076-81.

Brencic A, Lory S (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. Mol Microbiol.**72**:612-32.

Burrowes E, Baysse C, Adams C, O'Gara F (2006) Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. Microbiology. **152**:405-18.

Clatworthy AE, Pierson E, Hung DT (2007) Targeting virulence: a new paradigm for antimicrobial therapy. Nat. Chem. Biol. **3**:541–548

Driscoll JA, Brody SL, Kollef MH (2007) The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs. **67**:351–368.

Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ (2012) The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev. **76**:46-65

Gellatly SL, Hancock RE (2013) *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis. **67**:159-73.

Gi M, Jeong J, Lee K, Lee KM, Toyofuku M, Yong DE, Yoon SS, Choi JY (2014) A drug-repositioning screening identifies pentetic acid as a potential therapeutic agent for suppressing the elastase-mediated virulence of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. **58**:7205-14

Ho Sui SJ, Lo R, Fernandes AR, Caulfield MD, Lerman JA, Xie L, Bourne PE, Baillie DL, Brinkman FS (2012) Raloxifene attenuates *Pseudomonas aeruginosa* pyocyanin production and virulence. Int J Antimicrob Agents. **40**:246-51.

Hickman JW, Tifrea DF, Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A. **102**:14422-7.

Klebensberger J, Lautenschlager K, Bressler D, Wingender J, Philipp B (2007) Detergent-induced cell aggregation in subpopulations of *Pseudomonas aeruginosa* as a preadaptive survival strategy. Environ Microbiol. **9**:2247-59.

Moore NM, Flaws ML (2011) Treatment strategies and recommendations for *Pseudomonas aeruginosa* infections. Clin Lab Sci. **24**:52-6.

Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML (2002) GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. Mol Microbiol. **45**:1277-87.

Pan X, Wang C, Wang F, Li P, Hu Z, Shan Y, Zhang J (2011) Development of 5-Fluorouracil derivatives as anticancer agents. Curr Med Chem. **18**:4538-56.

Visca P, Imperi F, Lamont IL (2007) Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. **15**:22-30.

Waite RD, Paccanaro A, Papakonstantinopoulou A, Hurst JM, Saqi M, Littler E, Curtis MA (2006) Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. BMC Genomics. **7**:162.

List of publications

Imperi F, Massai F, Facchini M, Frangipani E, Visaggio D, Leoni L, Bragonzi A, Visca P (2013) Repurposing the antimycotic drug flucytosine for suppression of Pseudomonas aeruginosa pathogenicity. Proc Natl Acad Sci U S A. **110**:7458-63.

Frangipani E, Visaggio D, Heeb S, Kaever V, Cámara M, Visca P, Imperi F (2014) The Gac/Rsm and cyclic-di-GMP signalling networks coordinately regulate iron uptake in Pseudomonas aeruginosa. Environ Microbiol. **16**:676-88.

Daniela Visaggio, Martina Pasqua, Carlo Bonchi, Volkhard Kaever, Paolo Visca , and Francesco Imperi (2014) Exopolysaccharide-mediated cell aggregation promotes pyoverdine dependent iron uptake and virulence in *Pseudomonas aeruginosa.*Submitted to Environmental microbiology

Additional papers not included in the PhD thesis

Gherardi G, D'Ambrosio F, Visaggio D, Dicuonzo G, Del Grosso M, Pantosti A (2012) Serotype and clonal evolution of penicillin-nonsusceptible invasive *Streptococcus pneumoniae* in the 7-valent pneumococcal conjugate vaccine era in Italy. Antimicrob Agents Chemother.**56**:4965-8.

Gherardi G, Angeletti S, Panitti M, Pompilio A, Di Bonaventura G, Crea F, Avola A, Fico L, Palazzo C, Sapia GF, Visaggio D, Dicuonzo G (2012) Comparative evaluation of the Vitek-2 Compact and Phoenix systems for rapid identification and antibiotic susceptibility testing directly from blood cultures of Gram-negative and Gram-positive isolates. Diagn Microbiol Infect Dis. **72**:20-31.

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