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New insights into Quorum Sensing and other infection-related processes in *Pseudomonas aeruginosa* and *Burkholderia cenocepacia.*

"Nuovi approfondimenti riguardo il Quorum Sensing e altri processi legati all'infezione in *Pseudomonas aeruginosa* e *Burkholderia cenocepacia.*"

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RIASSUNTO ESTESO IN ITALIANO

INTRODUZIONE

Pseudomonas aeruginosa e *Burkholderia cenocepacia*, oltre a essere importanti patogeni nosocomiali, causano infezioni polmonari croniche e letali nella maggior parte dei malati di Fibrosi Cistica (FC). L'infezione polmonare cronica nei malati di FC è impossibile da eradicare mediante le tradizionali terapie antibiotiche (Gibson *et al.*, 2003).

Questo progetto è focalizzato principalmente su *P. aeruginosa*, responsabile della grande maggioranza delle morti nei pazienti affetti da FC (Lyczac *et al.*, 2002). In questo microrganismo la produzione di fattori di virulenza è regolata da un processo di comunicazione tra cellule chiamato Quorum Sensing (QS). Il QS è un fenomeno molto diffuso nei batteri e si basa sulla produzione di specifiche molecole segnale. Durante la crescita i batteri secernono molecole segnale che si accumulano nel mezzo circostante in modo proporzionale alla densità cellulare. Quando viene raggiunta una concentrazione soglia, un recettore viene attivato dal legame con la molecola segnale e regola l'espressione di specifici geni (Whitehead *et al.*, 2001).

P. aeruginosa possiede due sistemi di QS che utilizzano acil-omoserina-lattoni (acil-HSL) come molecole segnale: il sistema *las* ed il sistema *rhl*.

La sintasi LasI, codificata dal gene *lasI*, produce *N*-3-ossi-dodecanoil-omoserina lattone (3-oxo-C12-HSL), in grado di legare il recettore LasR, codificato dal gene *lasR*. I geni *rsaL* e *qscR* codificano per due repressori di *lasI* (RsaL e QscR), che rispondono al 3-oxo-C12-HSL e che quindi costituiscono parte integrante del sistema *las*.

Il sistema *rhl* è organizzato in maniera simile al sistema *las*: i geni *rhlI* e *rhlR* codificano rispettivamente la sintasi (RhlI) e il recettore (RhlR) della molecola segnale *N*-butiril-omoserina lattone (C4-HSL).

I recettori LasR e RhlR, attivati dalle rispettive molecole segnale, regolano centinaia di geni, inclusi quelli di un terzo sistema di QS, basato su un diverso tipo di molecola segnale, e la maggioranza dei geni coinvolti nella virulenza (Schuster & Greemberg, 2006; Smith & Iglewski, 2003).

In *P. aeruginosa* molti regolatori influenzano l'espressione dei geni *las* e *rhl*, tuttavia il loro meccanismo d'azione è stato caratterizzato solo nel caso di Vfr, un attivatore che agisce in modo diretto sulla trascrizione del gene *lasR* (Albus *et al.*, 1997). Esperimenti in modelli murini di infezione hanno dimostrato che, oltre ai mutanti di *P. aeruginosa* inattivati nei geni *las* e *rhl,* anche un mutante inattivato nel gene *vfr* presenta una virulenza fortemente ridotta rispetto al "wild type", (Smith *et al.*, 2004). Ciò suggerisce che anche i regolatori trascrizionali dei geni *las* e *rhl* potrebbero costituire bersagli per lo sviluppo di nuovi farmaci anti-*Pseudomonas*.

B. cenocepacia appartiene al gruppo "*B. cepacia* complex" (Bcc). Tale gruppo è composto da 17 specie di batteri strettamente correlate. I membri del gruppo Bcc infettano solo il 5% dei malati di FC, tuttavia, rispetto a *P. aeruginosa*, causano infezioni con decorso molto più grave, riducendo significativamente le aspettative di vita (Coenye *et al.*, 2001; Isles *et al.*, 1984).

Fra i meccanismi responsabili della elevata resistenza di *B. cenocepacia* alle tradizionali terapie antibiotiche un ruolo rilevante è svolto dalle pompe di efflusso appartenenti alla famiglia RND (Resistance-Nodulation-cell Division; Nikaido & Takatsuka, 2009). Di recente sta emergendo l'idea che le pompe di efflusso RND siano coinvolte, oltre che nella secrezione di composti tossici esogeni, anche nella secrezione di prodotti batterici importanti nella virulenza, inclusi fattori coinvolti nella formazione di biofilm (Piddock, 2006). Il biofilm consiste in una comunità batterica immersa in una matrice extracellulare auto-prodotta che conferisce al batterio resistenza sia agli antibiotici, sia nei confronti del sistema immunitario dell'ospite e gioca un ruolo chiave nelle infezioni croniche (Davies & Bilton, 2009). Le pompe di efflusso RND sono state studiate principalmente per il loro effetto sulla resistenza agli antibiotici, mentre si sa poco riguardo il loro impatto sulla fisiologia della cellula batterica e sull'espressione di fenotipi legati alla virulenza e alla formazione di biofilm.

OBIETTIVI

Dal momento che *P. aeruginosa* e *B. cenocepacia* sono altamente resistenti alle tradizionali terapie antibiotiche, studiarne i principali processi legati all'infezione potrebbe contribuire allo sviluppo di terapie alternative volte a inibire la virulenza anziché la crescita batterica.

Sia il QS che i regolatori dei geni del QS sono considerati buoni bersagli per lo sviluppo di nuove terapie anti-*Pseudomonas*. Per questo, il principale obiettivo di questo progetto è stato l'identificazione e la caratterizzazione funzionale di regolatori trascrizionali che regolano direttamente i geni dei sistemi di QS *las* e *rhl* in *P. aeruginosa*.

Un obiettivo secondario di questa parte del progetto, focalizzata su *P. aeruginosa,* è stato lo screening di una collezione di farmaci già approvati per l'uso nell'uomo, allo scopo di identificare quelli che avessero come attività secondaria l'inibizione del QS in *P. aeruginosa*.

Nella seconda parte di questo progetto è stato studiato il ruolo svolto da due pompe di efflusso RND nell'espressione di fenotipi collegati alla virulenza in *B. cenocepacia*.

RISULTATI

Allo scopo di individuare i regolatori trascrizionali dei geni dei geni *las* e *rhl* di *P. aeruginosa*, è stato usato il metodo della cromatografia per affinità al DNA (Fig. 8). Sei frammenti di DNA, corrispondenti ai promotori dei geni *lasR*, *lasI*, *rsaL*, *qscR*, *rhlR* e *rhlI*, sono stati indipendentemente coniugati a una resina cromatografica e incubati con estratti proteici crudi preparati a partire da colture di *P. aeruginosa* in fase di crescita esponenziale (A_{600}= 2.0) e stazionaria (A_{600}= 5.0). Le proteine in grado di legare in maniera specifica ogni regione promotore sono state eluite, separate su SDS-PAGE e identificate mediante spettrometria di massa MALDI-TOF (Fig. 9A-E).

In tutto sono state identificate 25 proteine, appartenenti a diverse classi funzionali (Tab. 1). Ben diciannove di queste sono classificate come fattori in grado di legare il DNA, di cui tredici sono regolatori trascrizionali (confermati o putativi) e sei sono proteine coinvolte in funzioni generiche collegate al processamento del DNA (come ad esempio le subunità della RNA polimerasi). Mentre queste ultime erano presenti su tutti i promotori usati come ligando, la maggior parte dei tredici regolatori è stata trovata essere associata ad un solo promotore, indicando che l'interazione tra proteina e promotore era specifica. È anche degno di nota il fatto che l'attivatore Vfr, già noto per legare il promotore di *lasR*, è stato identificato usando proprio questo promotore come ligando.

Oltre ai fattori in grado di legare il DNA, sono anche state identificate due proteine classificate come ipotetiche (a funzione sconosciuta) e quattro con funzione diversa dal legame al DNA. Si può supporre, in base alla loro funzione, che quest'ultime abbiano co-purificato con altre proteine (Tab. 1).

Le quindici proteine classificate come regolatori trascrizionali e come ipotetiche proteine sono state selezionate come possibili regolatori del QS e sono state quindi oggetto di ulteriori analisi.

Per verificare *in vivo* l'effetto dei putativi regolatori identificati, sono stati creati quindici mutanti di *P. aeruginosa*, nei quali i geni codificanti i corrispondenti regolatori sono stati indipendentemente deleti (Fig. 10). Mediante l'uso di fusioni trascrizionali, l'attività dei promotori bersaglio è stata misurata durante l'intera curva di crescita nel "wild type" e nei corrispondenti mutanti.

Grazie a questa analisi, è stato dimostrato per la prima volta che le due proteine "histon-like" MvaT e MvaU regolano in modo diretto la trascrizione dei geni del sistema *las*. In particolare MvaT reprime la trascrizione di *lasI* e *qscR* e attiva quella di *lasR* (Fig. 14), mentre MvaU è un repressore sia di *rsaL* che di *lasI* (Fig. 16). MvaT e MvaU sono regolatori globali dell'espressione genica e, in alcuni casi, è stato riportato che possono anche interagire l'una con l'altra (Vallet *et al*., 2004; Castang *et al*., 2008; Li *et al*., 2009). L'interazione tra MvaT e MvaU nella regolazione dei geni del QS è quindi molto complessa e difficile da studiare anche perchè nel ceppo di *P. aeruginosa* preso in esame in questo studio il doppio

mutante *mvaT-mvaU* è letale (Castang *et al.*, 2008). Questo tema sarà oggetto di studi futuri nel nostro laboratorio.

Sorprendentemente, le mutazioni negli altri geni presi in esame in questo studio non hanno causato effetti evidenti sull'attività dei corrispondenti promotori bersaglio, almeno nelle condizioni di crescita standard nelle quali è stato eseguito il saggio di attività promotore. Una spiegazione possibile per questo risultato è che alcune di queste proteine non siano sufficientemente espresse e/o attive nelle condizioni usate, dato che il ruolo di questi regolatori è quella di modulare il QS in risposta a specifiche condizioni ambientali e metaboliche. Per verificare tale ipotesi l'attività dei promotori bersaglio è stata valutata in ceppi ricombinanti di *P. aeruginosa* che sovra-esprimono i putativi regolatori. Come sistema modello, sono stati presi in esame i sei fattori identificati usando come ligando il promotore del gene *lasR*.

I geni codificanti i sei putativi regolatori di *lasR* sono stati clonati in un plasmide di espressione arabinosio-inducibile, i plasmidi risultanti sono stati quindi indipendentemente introdotti in *P. aeruginosa* "wild type", contenente la fusione trascrizionale per la misurazione dell'attività promotore di *lasR*. L'attività promotore di *lasR* è stata quindi misurata durante tutta la curva di crescita in presenza di arabinosio.

I risultati ottenuti mostrano che la sovra-espressione della proteina PA3699 reprime fortemente l'attività promotore di *lasR*, senza inibire la crescita (Fig. 17). La capacità di PA3699 di reprimere l'espressione di *lasR* è stata verificata e confermata anche nell'ospite eterologo *Escherichia coli* (Fig. 20).

La proteina PA3699 è stata purificata e la sua capacità di legare in modo specifico la regione di DNA contenente il promotore di *lasR* è stata dimostrata mediante un saggio di ritardo della motilità elettroforetica del DNA (Fig. 22).

Nel complesso, i risultati ottenuti dimostrano che PA3699 è un repressore diretto dell'espressione di *lasR*. Un aspetto rilevante di questo studio è che, sebbene la struttura cristallografica di PA3699 sia disponibile nelle banche dati dal 2009, questa è la prima volta che gli viene attribuita una funzione specifica.

Poichè la delezione del gene PA3699 non ha effetto sull'espressione di *lasR* nelle condizioni standard di laboratorio, è probabile che tale regolatore venga attivato da specifici stimoli ambientali e/o metabolici. Questo potrebbe essere il caso anche delle altre proteine identificate in questo studio come probabili regolatori del QS. Sono attualmente in corso studi volti all'identificazione degli stimoli ambientali e metabolici in grado di aumentare l'espressione/attività sia di PA3699 che degli altri putativi regolatori identificati.

Un obiettivo secondario di questa parte del lavoro è stato l'identificazione di inibitori del QS. Lo screening è stato effettuato usando un biosensore in grado di misurare i livelli di 3-oxo-C12-HSL. Tra i 1120 composti testati, 9 erano in grado di inibire la produzione di 3-oxo-C12-HSL in *P. aeruginosa* (Tab. 2). Il composto più promettente tra questi è al momento in corso di studio, per verificarne

l'efficacia anti-*Pseudomonas* sia *in vitro* che in modelli murini d'infezione polmonare cronica.

Per quanto riguarda la parte del lavoro che ha riguardato *B. cenocepacia*, studi preliminari di trascrittomica avevano mostrato che i mutanti D4 e D9, inattivati rispettivamente nelle due pompe di efflusso RND-4 e RND-9, e il doppio mutante D4-D9 avevano un'espressione alterata di molti geni correlati al flagello. Dal momento che la motilità flagellare (un fenotipo chiamato "swimming") è strettamente connessa alla virulenza e alla formazione di biofilm, è stato testato l'effetto di queste mutazioni sui fenotipi "swimming" e biofilm. I risultati ottenuti hanno mostrato che, in accordo con quanto ottenuto dai dati di trascrittomica, i mutanti D4 e D9 avevano, rispettivamente, un'aumentata e una diminuita capacità di motilità "swimming" rispetto al "wild type". Il doppio mutante D4-D9 aveva un fenotipo simile al mutante D4 (Fig. 27). Ciò indica che l'inattivazione di diversi sistemi RND può avere effetti drammaticamente diversi su uno specifico fenotipo di virulenza. È interessante notare che tutti i mutanti mostravano una maggiore capacità di formare biofilm, rispetto al "wild type" (Fig. 28). Dal momento che la formazione di biofilm è un fenotipo complesso e pleiotropico non è possibile correlare questo risultato solo con l'espressione dei geni per il flagello.

CONCLUSIONI

I risultati ottenuti nella parte di progetto focalizzata su *P. aeruginosa* hanno permesso di identificare tre nuovi fattori trascrizionali responsabili della regolazione del QS, un processo fondamentale per l'espressione della virulenza in questo batterio. Inoltre, è stata identificata una molecola con spiccata attività anti-QS, quindi potenzialmente in grado di inibire la virulenza di *P. aeruginosa*.

Per quanto riguarda la parte di progetto che ha avuto come oggetto lo studio delle pompe d'efflusso RND, è rilevante notare che molte compagnie farmaceutiche e gruppi di ricerca stanno studiando inibitori delle pompe di efflusso ("efflux pump inhibitors", EPIs) come adiuvanti nelle terapie antibiotiche. In questo studio è stato dimostrato, usando *B. cenocepacia* come sistema modello, che l'inattivazione di pompe di efflusso RND potrebbe aumentare la formazione di biofilm e, in alcuni casi, la motilità, facendo insorgere seri dubbi circa l'opportunità di usare gli EPIs nella terapia. Infatti, l'uso degli EPIs potrebbe da un lato potenziare l'effetto degli antibiotici, ma dall'altro potrebbe promuovere la formazione di biofilm e quindi l'instaurarsi dell'infezione cronica.

ABSTRACT

Pseudomonas aeruginosa and *Burkholderia cenocepacia*, beside their relevance as a nosocomial pathogens, cause lethal chronic lung infections in the vast majority of cystic fibrosis (CF) patients. Once established, the CF lung infection is impossible to eradicate with traditional antimicrobial therapies.

The main body of this project has been focused on *P. aeruginosa*. In this bacterium the Quorum Sensing (QS) system coordinates the production of virulence factors in a cell density-dependent manner via the secretion of specific signal molecules. During growth the bacteria secrete the signal molecules, which accumulate in the surrounding environment as the population density increases. When a threshold concentration is reached, the receptor is activated by the binding with the cognate signal molecule and triggers the expression of several genes, including virulence genes.

P. aeruginosa has two QS systems relying on the production of acylated-homoserine lactone (acyl-HSL) as signal molecules: the *las* and *rhl* systems. The *lasI* gene encodes the LasI synthase producing the *N*-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) signal molecule, that binds to the LasR receptor encoded by *lasR*. The *rsaL* and *qscR* genes encode repressors of *lasI* and constitute an integral part of the *las* QS system, since they respond to 3-oxo-C12-HSL. The *rhl* system is organized similarly to the *las* one: the *rhlI* and *rhlR* genes encode, respectively, for the RhlI synthase and the RhlR receptor of the *N*-butyryl-homoserin lactone (C4-HSL) signal molecule. The signal-activated LasR and RhlR receptors regulate transcription of hundreds of genes, including the genes encoding a third QS system, based on the production of a different signal molecule, and the vast majority of virulence genes.

In *P. aeruginosa* many regulators have been found to affect the expression of the *las* and *rhl* genes, although their mechanism of action remains largely unknown. In particular, only the Vfr protein has been shown to directly activate *lasR* transcription. Besides *las* and *rhl* mutants, also a *vfr* mutant shows reduced virulence in murine models, suggesting that also the QS regulators could be feasible drug-targets.

The main objective of this project has been the identification and functional characterization of transcriptional factors that directly regulate the *las* and *rhl* genes. To achieve this objective, six DNA fragments corresponding to the promoters of *lasR*, *lasI*, *rsaL*, *qscR*, *rhlR* and *rhlI* were independently conjugated to a chromatography resin and incubated with protein crude extracts prepared from *P. aeruginosa* cultures. The proteins able to specifically bind each DNA bait were recovered, separated by SDS-PAGE and identified by MALDI-TOF mass spectrometry.

Overall, 25 proteins bound on the promoter regions of the QS genes were identified. Out of these, fifteen factors were selected for further analysis as possible

QS regulators. Noteworthy, the activator Vfr, already known to directly bind the *lasR* promoter, was recovered using this promoter as a bait.

A set of fifteen *P. aeruginosa* mutants, each one deleted in one of the genes encoding the selected proteins, was generated. In each mutant the activity of correspondent target promoter was compared with that of the wild type, by means of transcriptional fusions between the promoter region of the target genes and the reporter system *lux*. In this system the production of bioluminescence is proportional to the promoter activity. Among the newly identified factors, we showed for the first time that the histon-like proteins MvaT and MvaU directly control the transcription of the *las* genes. In particular, MvaU is a repressor of both *rsaL* and *lasI* transcription, while MvaT represses *lasI* and *qscR* transcription and activates *lasR* transcription. MvaT and MvaU are global regulators of gene expression and on some promoters can also interact each other. The interplay between these two proteins in regulating QS genes is a complex issue that will be the object of future studies.

Unexpectedly, mutations in the other genes here investigated did not cause evident effects on the activity of the corresponding target promoter under the tested laboratory conditions. A possible explanation for this result is that some of these factors are actually QS regulators, but they are not sufficiently expressed and/or active in the standard growth conditions we have used. To verify this hypothesis, we decided to test the activity of target promoters in recombinant *P. aeruginosa* strains over-expressing the putative QS regulators.

We focused on the six factors retrieved on the *lasR* promoter. The results showed that PA3699 over-expression strongly repressed *lasR* promoter activity during the whole growth curve, without affecting cell growth. The ability of PA3699 to repress *lasR* expression was also confirmed in the heterologous host *Escherichia coli*. Moreover, the PA3699 protein was purified and its capability to bind *in vitro* the *lasR* promoter region was demonstrated by electrophoretic mobility shift assay. Overall, these results show that PA3699 is a repressor of *lasR* expression. Since PA3699 mutation has no effect on *lasR* expression under standard cultural conditions, it is likely that this regulator is activated by specific environmental/metabolic stimuli. This could be the case also for the other proteins identified in this study. Environmental and metabolic stimuli increasing the expression/activity of PA3699 and of the other putative regulators identified in this project are currently under investigation.

A minor objective of this part of the project has been the screening of the Prestwick Chemical Library of FDA-approved drugs for the identification of possible secondary activities against *P. aeruginosa* QS. The screening was performed by using a biosensor able to detect 3-oxo-C12-HSL levels. Among the 1120 compounds tested, 9 displayed a QS-inhibitory effect. The anti-*Pseudomonas* activity of the most promising compound is currently under investigation in murine models of chronic lung infection.

The second part of this project has been focused on *B. cenocepacia,* a member of the *B. cepacia* complex (Bcc). This group of strictly related bacteria is the second most important Gram negative pathogen, infecting the CF patients. Like many pathogens, including *P. aeruginosa*, *B. cenocepacia* is highly resistant to a wide range of antibiotics due to the production of drug-efflux pumps belonging to the Resistance-Nodulation-cell Division (RND) family.

It is emerging the notion that, beside exogenous toxic compounds, RND efflux pumps are involved in the secretion of bacterial products important for virulence, including factors involved in biofilm formation. The biofilm is a bacterial community encased in a self-produced extracellular matrix and confers resistance to antibiotics and to the host immune systems, playing a major role in CF lung infection. The RND efflux pumps have been mainly studied for their effect on antibiotic resistance, while little is known about their impact on cell-physiology and virulence-related phenotypes.

A preliminary transcriptomic study showed that mutants inactivated in the D4 and D9 RND- drug efflux systems of *B. cenocepacia* displayed altered expression of flagella-related genes. Since flagellar motility (a phenotype named swimming) is strictly connected to virulence and to biofilm formation, we tested the effect of these mutation on swimming and biofilm. Results showed that, in accordance with transcriptomic data, the D4 and D9 mutants showed increased and decreased swimming motility with respect to the wild type, respectively. Therefore the inactivation of distinct RND systems can have a dramatically different effect on a specific virulence-related phenotype.

Interestingly, both the mutants showed increased biofilm formation with respect to the wild type. Since biofilm formation is a pleiotropic and complex phenotype it is not easy to correlate this result only to flagella genes expression.

Many companies are developing Efflux Pumps Inhibitors (EPIs) as antibiotic therapy adjuvants. In this view, a relevant result of this research is that inactivation of efflux pumps can enhance biofilm formation and, sometimes, motility, raising serious concerns about the use of EPIs in therapy. Indeed, the use of EPIs could be, on one side positive for helping the antibiotic therapy, on the other side, it could promote biofilm formation and chronic infection. More detailed study on the effect of RND efflux pumps in virulence-related phenotypes and chronic infection are therefore strongly desirable.

1. INTRODUCTION

1.1 Cystic fibrosis

Cystic fibrosis (CF) is a recessive genetic disease caused by a mutation in a gene on the long arm of chromosome 7 that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR; Welsh *et al.* 2001). CFTR is an ABC transporter-class ion channel that transports chloride and thiocyanate ions across epithelial cell membranes. Mutations of the CFTR gene affect functioning of the chloride ion channels in these cell membranes. CFTR is required to regulate the components of sweat, digestive juices, and mucus. The inability to regulate sodium and chloride transport due to an aberrant CFTR increases airway secretion viscosity (Welsh *et al.* 2001).

CF is considered one of the most common genetic diseases affecting roughly 70,000 persons worldwide. One child in approximately 2,500 of European carries two defective copies of CFTR (Ratjen & Doring, 2003).

CF primarily affects the airways and submucosal glands with sparing of the interstitium and alveolar spaces until late in the disease (Tomashefski *et al.* 1989). The role of ion transport in the maintenance of lung health is complex. The recent development of a porcine model of CF has provided a further step forward our understanding of the basic mechanisms whereby the loss of CFTR function leads to parenchymal lung damage (Rogers *et al.* 2008). These animals developed lung disease and demonstrated abnormal bacterial clearance in the absence of significant inflammation (Stoltz *et al.* 2010), suggesting that infection of the airways may be the primary event that initiates the progression of CF lung disease.

1.1.1 Chronic infections in cystic fibrosis patients

In almost all patients with CF, bacteria can be isolated from airway secretions at some point in their lives. For the majority, such infections begin in childhood and become chronic by early adulthood.

In the healthy host, bacteria that enter the lungs are cleared rapidly, without the initiation of an inflammatory response. This process involves a variety of innate host defence strategies, both mechanical (mucociliary clearance) and immunological (resident macrophages and antimicrobial peptides). In certain clinical situations this first line of defence fails, and the normally harmless bacteria survive, multiply, and lead to organ damage. Such conditions are encountered in mechanically ventilated patients in intensive care, in those with defective immunity or severe burns, and in patients with CF. The mechanisms underlying the early acquisition of infection in CF are complex and incompletely understood. Briefly, hypotheses include impaired mucociliary clearance related to low airway surface liquid volume, increased availability of cell surface receptors and impaired ingestion of bacteria by epithelial cells. Once within the airway, conditions such as

hypoxia within mucus plugs may attract motile organisms capable of anaerobic survival and encourage biofilm formation (Davies & Bilton 2009).

CF has a unique set of bacterial pathogens that are frequently acquired in an age-dependent sequence and, among them, only *Staphylococcus aureus* may be pathogenic in immunocompetent individuals. *Pseudomonas aeruginosa*, *Burkholderia cepacia*, nontypeable *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans* are all considered opportunistic pathogens. Other organisms seen in CF that are also generally nonpathogenic in the healthy host include *Aspergillus* and nontuberculous mycobacteria.

Early infections in CF airways are most frequently caused by *S. aureus* and *H. influenzae*, organisms that may be seen in other young children with chronic illnesses and in adults with non–CF bronchiectasis. *S. aureus* is often the first organism cultured from the respiratory tract of young children with CF. However, there still is a continue debate about the significance of *S. aureus* in the pathogenesis of CF lung infection. Historically, significant improvements in patient longevity have been associated with the advent of antistaphylococcal therapy. *H. influenzae* is also isolated from the respiratory tract early in the course of CF. The role of *H. influenzae* in progressive airway infection and inflammation in patients with CF has not been clearly demonstrated, although this organism is known to be pathogenic in patients with non–CF bronchiectasis (Gibson *et al.* 2003).

In the CF patients *P. aeruginosa* plays a particularly important role: the inability to regulate sodium and chloride transport due to an aberrant CFTR increases airway secretion viscosity, and within the resulting thick mucus in the lung *P. aeruginosa* find a favourable niche (Lyczak *et al.,* 2000). 80 % to 95 % of CF patients succumb because of respiratory failure brought on by chronic *P. aeruginosa* infection and concomitant airway inflammation. Up to 97 % of CF patients are infected with *P. aeruginosa* by the age of 3 years (Lyczac *et al.*, 2002; Murray *et al.*, 2007).

Among the organisms involved in CF airways *B. cepacia* is particularly relevant, besides *P. aeruginosa*. Although this bacterium infects a small percentage of CF patients (around 5%), the so-called "*B. cepacia* syndrome" leads to high fevers, bacteremia, rapid progression to severe necrotizing pneumonia and death. The majority of *B. cepacia* infected patients have more severe course with decline in lung function and increased mortality with respect to *P. aeruginosa* infection. However, *B. cepacia* is not a single species but rather a group of closely related species, thus the organism should be called *B. cepacia* complex. Seventeen distinctive species of *B. cepacia* have been identified. The vast majority of CF airway infections with *B. cepacia* complex are caused by *B. cenocepacia, B. multivorans* and *B. vietnamiensis* (Gibson *et al.* 2003).

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a highly adaptable bacterium that can colonize various environmental niches, including soil and marine habitats, plants, animals and humans. The completion of the sequencing project of the genome of *P. aeruginosa* PAO1 demonstrated that the ecological versatility is indeed reflected in its gene content, by its relatively large genome (6.3 Mbp) and genetic complexity (5570 open reading frames, ORFs), comparable to that of the simple eukaryote *Saccharomyces cerevisae* (Stover *et al.,* 2000). Compared to the majority of known sequenced bacterial genomes, the genome of *P. aeruginosa* possesses an overall larger number of genes encoding outer membrane proteins, efflux systems and multiple chemotaxis systems, which may contribute to its pathogenesis. Moreover, up to 10 % of the assigned ORFs are classified as transcriptional regulators, reflecting the ability of *P. aeruginosa* to respond and adapt to environmental fluctuations (Stover *et al*., 2000; Goodman & Lory, 2004).

Besides being the major cause of death in CF patients (see above), *P. aeruginosa* is an opportunistic human pathogen that may cause acute infections in hospitalized patients and immuno-compromised individuals, for example those with AIDS, neutropenic patients undergoing chemotherapy, and burn victims (Driscoll *et al.*, 2007).

Infections caused by *P. aeruginosa* are not only very frequent, but they are also associated with high morbidity and mortality rates when compared with infections caused by other bacterial pathogens (Osmon *et al.*, 2004). This is mainly due to the fact that *P. aeruginosa* infections are hard to eradicate because this microorganism is intrinsically resistant to many antibacterials, including β-lactams, macrolides, tetracyclines, co-trimoxazole and most fluoroquinolones and it is particularly prone to acquire new resistances in the hospital environment by horizontal gene transfer (Latifi *et al*., 1995). Also the *P. aeruginosa* tendency to colonize surfaces in organized communities, termed biofilms, makes *P. aeruginosa* less susceptible to antibiotics and disinfectants, anchors the cells to the surface they colonize (for instance to the medical devices) and protects the bacteria from the host defences, such as lymphocytes, phagocytes, antibodies and the ciliary action of the respiratory tract (Greenberg, 2003; Hentzer *et al*., 2004). These characteristics make *P. aeruginosa* a particularly dangerous pathogen.

1.2.1 *P. aeruginosa* virulence

The capacity of *P. aeruginosa* to produce such diverse infections, is due to an arsenal of virulence factors. These factors are collectively capable of causing extensive tissue damages, bloodstream invasion and dissemination in humans and other mammals (Fig. 1; reviewed by Smith & Iglewski, 2003).

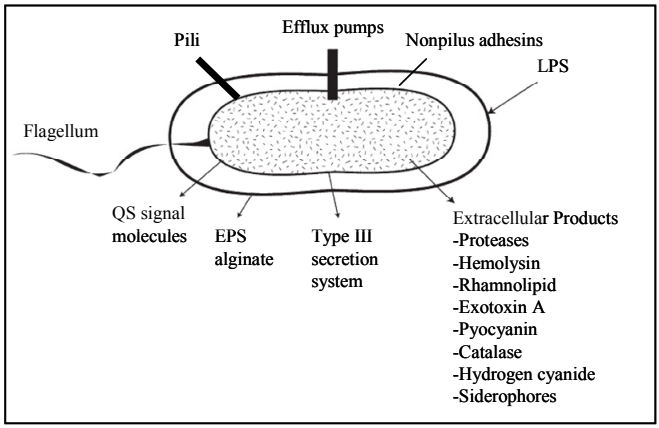

Figure 1. Virulence and antibacterial resistance factors in *P. aeruginosa* (modified from Van Delden & Iglewski, 1999).

Some of the virulence factors that confer to the pathogen the ability to colonize the host are cell surface virulence factors Actually the cell surface itself is a virulence factor because it contains very immunogenic compounds, like the lipopolysaccharide (LPS). Flagella and pili involved in different kind of motility and in chemotaxis, display a critical role in pathogenesis, by adhering to epithelial cells and stimulating an inflammatory response (DiMango *et al*., 1995; Adamo *et al*., 2004). When *P. aeruginosa* chronically infects patients, it adapts to the biofilm mode of growth. The generally accepted definition of a biofilm is "a community of cells attached to a surface or to each other, imbedded in a self-made, protective matrix of extracellular polymeric substances (EPS)" (Fig. 1; Kirisits & Parsek, 2006).

The clinical implications of bacterial biofilms are particularly pronounced (Davies, 2002). Biofilms may form on any foreign object inserted into the human body, and also in the lungs of CF patients *P. aeruginosa* forms biofilm during chronic infection. In the biofilm mode the bacteria are highly tolerant to the action of several antimicrobial agents including antibiotics, disinfectants, and to the action of the immune system (Donlan & Costerton, 2002; Drenkard, 2003).

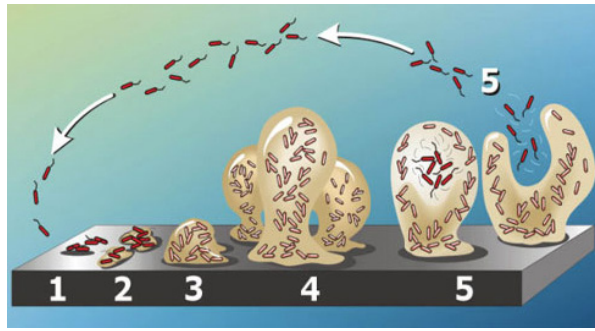

Figure 2. Schematic representation of different stages of biofilm formation. In the colonization stage planktonic cells adhere to the substrate (1) and start to proliferate forming microcolonies (2). During the maturation stage the microcolonies grow (3) forming three-dimensional structures known as "mushrooms" (4). Finally, bacterial cells leave the mushrooms leading to biofilm dispersion (5; modified from Kirisits & Parsek, 2006).

P. aeruginosa also produces several extracellular products that, after the initial step of colonization, cause extensive tissue damage, bloodstream invasion and dissemination. Some of these extracellular products, besides having the role of favoring pathogen dissemination, also provide nutrients to *P. aeruginosa* by causing host tissues damage. These secreted factors are elastases, alkaline proteases, exotoxins and hemolysins that also contribute to the infection in lung disease by destroying the protective glicocalix of the respiratory epithelium (Kipnis *et al*., 2006). Other secreted factors are toxic compounds such as hydrogen cyanide and pyocyanin: hydrogen cyanide is a potent poison that blocks cytochrome oxidase, leading to the inhibition of mitochondrial respiration (Gallagher & Manoil, 2001). Pyocyanin is a blue pigment metabolite of *P. aeruginosa* that has been shown to have numerous pathogenic effects such as increasing IL-8, depressing host-response, and inducing apoptosis in neutrophils (Denning *et al*., 1998; Leidal *et al*., 2001; Allen *et al*., 2005). In animal models of acute and chronic lung infection, pyocyanin was shown to be essential for *P. aeruginosa* virulence (Lau *et al*., 2004). Additionally, due to its known oxidoreductive properties, pyocyanin oxidizes glutathione and inactivates catalase in respiratory epithelial cells thus participating in oxidative-stress related damage (O'Malley *et al*., 2003; O'Malley *et al*., 2004).

The request of iron in *P. aeruginosa* is supported by the production of the two siderophores, pyoverdine and pyochelin, that are small molecules chelating iron from the iron-poor environment encountered in the host, allowing its utilization in *P. aeruginosa* metabolism (Buckling *et al*., 2007).

1.2.2 Quorum sensing

For many years, researchers thought of bacteria as individual cells designed to proliferate under various conditions but unable to interact with each other and to collectively respond to environmental stimuli, as it is typical for multicellular organisms. This view began to change few decades ago with the discovery of the cooperative regulation of luminescence in the Gram-negative marine bacterium *Vibrio fischeri* (Nealson *et al.,* 1970) and regulation of the genetic competence in the Gram-positive bacterium *Streptococcus pneumoniae* (Tomasz, 1965). These bacteria were show to coordinate their behavior via the secretion of specific signaling molecules in a population density-dependent manner. During growth the bacteria secrete these signal molecules that accumulate in the surrounding environment as the population density increases until a critical threshold concentration is reached, which then triggers expression of certain sets of genes. This type of cell-to-cell communication was termed "quorum sensing" (QS) in order to emphasize the fact that a sufficient number of bacteria, the bacterial "quorum", is needed to induce or repress expression of target genes (Fig. 3; Fuqua *et al.*, 1994; Whitehead *et al.,* 2001).

QS has been proven to play an important role in the physiology of various bacterial species. Beside bioluminescence and genetic competence, QS is also involved in the regulation of a wide variety of different physiological processes including antibiotic biosynthesis, motility, plasmid conjugal transfer, biofilm formation, and the production of bacterial virulence factors in plant, animal and human pathogens (Miller & Bassler, 2001; Whitehead *et al*., 2001; Camara *et al*., 2002; Fuqua & Greenberg, 2002; Lazdunski *et al*., 2004).

Furthermore, evidence has accumulated that some bacterial signal molecules are used not only as population density sensors within the same species but also for communication between bacteria of different species or genera occupying the same ecological niche, and even to interact with their eukaryotic hosts.

In Gram-negative bacteria the most common signal molecule used is an acylated homoserine lactone (acyl-HSL) molecule and, in some cases, a single bacterium possesses multiple acyl-HSL signal molecules.

The first acyl-HSL-based QS system that has been studied is the Lux system of *V. fisheri*. It consists of a synthase, LuxI (encoded by *luxI* gene), that produces the 3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL) signal molecule and a signal-receptor protein LuxR (encoded by *luxR* gene) which acts as transcriptional regulator (Nealson *et al.,* 1970).

Since then, a wide number of LuxI-like and LuxR-like proteins have been shown to be involved in acyl-HSL mediated QS systems in a number of Gram-negative bacteria (Whitehead *et al.*, 2001).

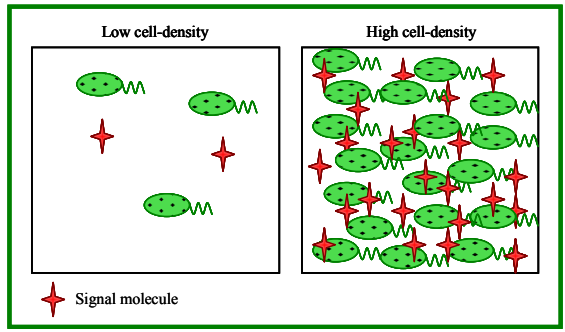

Figure 3. A simple representation of QS. The signal molecule is constitutively produced at a basal level by the bacterial cells. At low-cell densities very little signal molecule is present. As cell density increases, the signal molecule accumulates until a threshold level is reached. This signal molecule concentration is responsible for a coordinate transcriptome reprogramming in the whole bacterial population.

1.2.3 The quorum sensing system of *P. aeruginosa* PAO1

One of the most extensively studied acyl-HSL-dependent cell-to-cell communication systems is the one of *P. aeruginosa.*

P. aeruginosa shows three QS systems, two of which are acyl-HSL-dependent systems.

The first is the *las* system and consists of the LuxR-like transcriptional regulator LasR (codified by *lasR* gene) and of the LuxI-like acyl-HSL synthase LasI (codified by *lasI* gene), which directs the synthesis of *N*-3-oxododecanoyl-homoserine lactone (3-oxo-C12-HSL; Fig.4; Schuster & Greenberg, 2006).

At low-cell density the 3-oxo-C12-HSL molecule is synthesized by LasI at basal levels and is secreted into the surrounding media where it become diluted. In this phase, no QS-response occurs (Pearson *et al*., 1999). With the increasing of cell density, the signal molecule accumulates, until its concentration reaches a threshold level. At this critical concentration, 3-oxo-C12-HSL binds the cognate QS-activator LasR (Fuqua *et al*., 1996). The LasR/3-oxo-C12-HSL complex increases *lasI* transcription, triggering a positive feedback loop that leads to the amplification of 3-oxo-C12-HSL production (Seed *et al*., 1995). Moreover, activated LasR exerts its role of global transcriptional regulator drastically reprogramming the *P. aeruginosa* PAO1 transcriptome.

Among the genes regulated by the LasR/3-oxo-C12-HSL complex there is the second acyl-HSL system: the *rhl* system, that is organized similarly to the *las* one. The *rhl* QS-system is based on the production of *N*-butyryl-homoserine lactone (C4-HSL; Fig.4) by the LuxI-like synthase RhlI, codified by the *rhlI* gene. Also in this case the synthase gene has a cognate LuxR-like transcriptional regulator, RhlR, codified by the *rhlR* gene, that is activated by the binding with the cognate

signal molecule C4-HSL (Latifi *et al*., 1996; Pesci *et al*., 1997). As schematized in figure 5, the *rhl* system, being dependent on LasR/3-oxo-C12-HSL complex for its activation, is hierarchically below the *las* system.

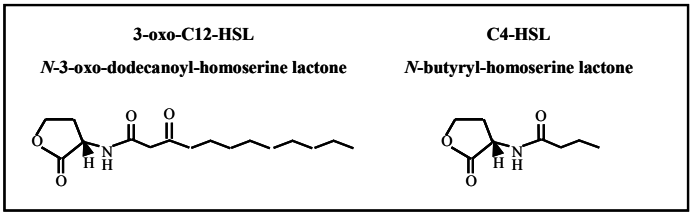

Figure 4. Structure of acyl-HSL molecules exploited by *P. aeruginosa* for cell-to-cell communication (modified from Juhas *et al*., 2005).

LasR and RhlR transcription regulators bind to specific *lux*-box-like sequences, called *las*-boxes in *P. aeruginosa* (Whiteley *et al.,* 1999; Schuster *et al.,* 2004). LasR forms a dimer and binds *las*-boxes only in the presence of 3-oxo-C12-HSL (Kiratisin *et al.,* 2002), whereas RhlR dimerizes and binds DNA both in the presence and absence of C4-HSL, but it is active only as RhlR/C4-HSL complex (Medina *et al.,* 2003; Ventre *et al.,* 2003).

The third QS-response involves another chemical signal, distinct from the above mentioned acyl-HSLs. This signal, 2-heptyl-3-hydroxy-4-quinolone (PQS), adds a further level of complexity to the QS network, providing another link between the *las* and *rhl* QS-systems (Diggle *et al*., 2003).

Briefly, the *phnAB* operon is required for the conversion of 2-amino-2-deoxy-isochorismic acid to anthranilic acid, which is subsequently converted to 2-heptyl-4-quinolone (HHQ) by the enzymes encoded by the *pqsABCD* genes, belonging to the *pqsABCDE* operon. The role of the last gene of this operon, *pqsE*, is not jet known. (Deziel *et al*., 2004; Lepine *et al*., 2004). HHQ is then converted to PQS by the product of the *pqsH* gene. Recent experiments showed that the transcription of *pqsABCDE* operon is negatively regulated by the *rhl* system and positively regulated by the *las* system (Diggle *et al*., 2003). Pesci and co-workers demonstrated that the effects of LasR and RhlR on the *pqsABCDE* operon occur indirectly through the control of the transcriptional regulator PqsR (Pesci *et al.,* 1999). It was demonstrated that *pqsR* transcription is under the positive control of LasR and under the negative control of RhlR. PqsR in turn has a positive effect on the transcription of *pqsABCDE*, directly binding to the promoter of this operon (Wade *et al*., 2005). Thus the production of the PQS signal molecule is dependent on the ratio 3-oxo-C12-HSL/C4-HSL (McGrath *et al*., 2004). This study also demonstrated that PqsR binding to the *pqsABCDE* promoter is augmented by the presence of PQS, implying that PQS acts as a coinducer of PqsR (Wade *et al*.,

2005). Moreover PqsR is a global regulator controlling the expression of several genes of *P. aeruginosa* (Fig. 5).

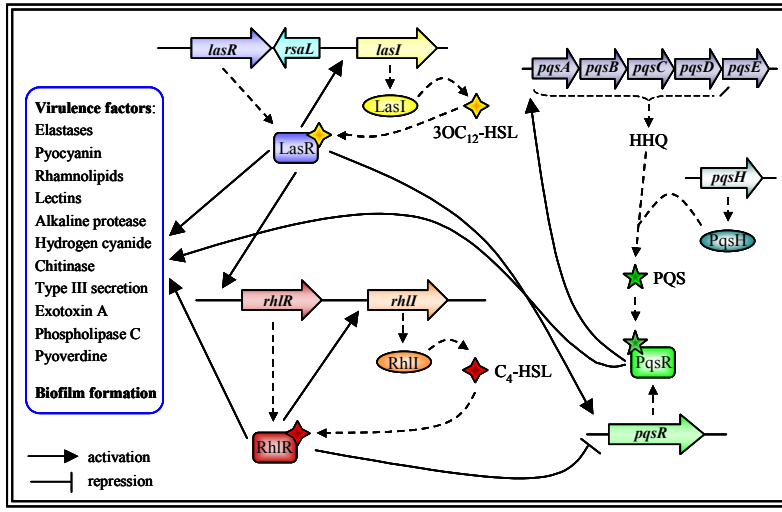

Figure 5. Schematic representation of the central core of the QS network in *P. aeruginosa*, with *las*, *rhl*, and *pqs* systems. Relevant QS-regulated virulence factors are listed (modified from Camara *et al*., 2002 and Diggle *et al*., 2003).

The two regulators RsaL and QscR are considered an integral part of the *las* QS system, since they respond to 3-oxo-C12-HSL signal molecule.

- **RsaL**

In *P. aeruginosa lasR* and *lasI* genes are separated by a 367 bp intergenic region, and are transcribed from independent promoters in the same direction (Seed *et al*., 1995). In this intergenic region, Iglewski and co-workers in late 90's, identified a gene, *rsaL*, located on the opposite strand to *lasR* and *lasI* (Fig. 5). This gene encodes a protein, RsaL, of 80 aminoacids and 9.4 kDa (de Kievit *et al*., 1999).

RsaL represses *lasI* transcription by direct binding to the *lasI* promoter (Rampioni *et al.*, 2006) and it is a member of the tetrahelical superclass of helix-turn-helix proteins (Rampioni *et al.,* 2007a).

Most recent studies highlighted the major role played by RsaL in QS regulation and *P. aeruginosa* physiology. When *P. aeruginosa* is cultured in complex media, levels of 3-oxo-C12-HSL attain a steady state level (homeostasis) in the range of a few μM when the cells are still in the exponential phase, despite the fact that expression of the 3-oxo-C12-HSL synthase gene (*lasI*) is regulated by a positive

feedback through the LasR/3-oxo-C12-HSL complex. This implies the existence of a negative regulatory element balancing the positive feedback exerted by LasR/3-oxo-C12-HSL. The level of 3-oxo-C12-HSL in a *rsaL* mutant culture continues to rise in late logarithmic and stationary phase to a level about 10-fold the level reached in a wild type culture. This result demonstrated that RsaL is the regulatory element responsible of 3-oxo-C12-HSL homeostasis (Rampioni *et al.,* 2007b).

When the quorum concentration of 3-oxo-C12-HSL is reached, the binding of the LasR/3-oxo-C12-HSL complex at a unique site located in the *rsaL-lasI* intergenic region triggers the divergent transcription of both genes. Consequently, the levels of 3-oxo-C12-HSL and of RsaL increase. The binding of RsaL to a unique site on the intergenic region simultaneously represses the transcription of *rsaL* and *lasI*. This generates a negative feedback loop, which counteracts the positive feedback loop mediated by LasR/3-oxo-C12-HSL (Rampioni *et al.,* 2007b).

Homeostasis would allow a population of *P. aeruginosa* cells to maintain 3-oxo-C12-HSL at an appropriate level in a given environment and to change steady-state levels of 3-oxo-C12-HSL as environmental conditions change. However, other mechanisms can play roles in controlling 3-oxo-C12-HSL homeostasis, including for instance, signal-molecule-degrading enzymes and import-export pumps (Köhler *et al*., 2001; Aendekerk *et al*., 2002; Huang *et al*., 2006; Sio *et al*., 2006).

Furthermore, transcription profiling revealed that RsaL regulates 130 genes independent of its effect on QS signal molecule production, including genes involved in virulence. RsaL can repress pyocyanin and hydrogen cyanide virulence genes in two ways: directly, by binding to their promoters, and indirectly, by decreasing levels of the signals for their QS-signal-dependent transcription. These findings highlight the importance of RsaL as a global regulator of *P. aeruginosa* physiology that provides a counterbalance to 3-oxo-C_{12}-HSL-dependent gene activation via multiple mechanisms (Rampioni *et al.*, 2007b).

- **QscR**

The genome of *P. aeruginosa* revealed the presence of a LuxR-like factor, QscR, codified by the *qscR* gene. Differently from the homologous *lasR* and *rhlR* genes, this member of the LuxR family is not genetically linked to a gene encoding for a cognate acyl-HSL synthase. Therefore QscR is classified as an "orphan" QS signal receptor. In a *P. aeruginosa qscR* mutant the *lasI* and *rhlI* genes are prematurely transcribed, producing an increment in the expression of some QS controlled virulence genes, such as those involved in the production of pyocyanin, elastase and hydrogen cyanide. Indeed, the *P. aeruginosa qscR* mutant is hypervirulent (Chugani *et al*., 2001). Several possible mechanisms had been proposed to explain the role of QscR in QS regulation.

At very low concentrations of acyl-HSLs, inactive heterodimers can be formed between QscR and LasR or QscR and RhlR; while increased acyl-HSL concentrations result in QscR-acyl-HSL interaction inducing dissociation of QscR

and relasing LasR and/or RhlR (Ledgham *et al.,* 2003). The current model is therefore that QscR inhibits LasR and RhlR at low concentrations of acyl-HSL through formation of inactive heterodimers with LasR and/or RhlR, thus inhibiting the expression of QS-regulated genes (reviewed by Venturi, 2006).

In accordance with the fact that QscR protein was shown to contain both putative acyl-HSL and DNA-binding domains (Chugani *et al*., 2001), it was recently demonstrated that the 3-oxo-C12-HSL-QscR complex can bind the promoter region of PA1897 gene, indicating that it can directly regulate gene transcription . To exert this function, QscR borrows the signal produced by LasI (Lee *et al*., 2006b).

Comparison of the transcriptional profiles of a *qscR* null mutant and the wild type strain at different stages of culture growth, revealed that more than 400 genes were differentially expressed, many of which were repressed, while a smaller set was activated by QscR (Lequette *et al*., 2006). In the same work, it was also demonstrated that a QscR protein lacking the DNA-binding domain is still able to regulate the majority of these genes, but not all of them. These data on one hand confirm that main role of QscR is that of inhibiting the activity of LasR and/or RhlR, on the other hand provide evidence that QscR can also directly modulate *P. aeruginosa* transcriptome, and suggest that QscR controls its own regulon, which only in part overlaps with the LasR and RhlR ones.

Another significant finding regarding QscR is that this regulator was demonstrated to have broader signal specificity than LasR does. Indeed, QscR can respond to other acyl-HSL molecules. This finding suggests that QscR might also respond to signals produced by other bacteria coexisting with *P. aeruginosa* (Lee *et al*., 2006b). This information is clinically relevant, since the lungs of the CF patients are often co-colonized by *P. aeruginosa* and by the C8-HSL-producing bacterium *B. cenocepacia* (Govan & Deretic, 1996).

1.2.4 Quorum sensing and virulence

In the whole, the QS-circuit regulate about 10 % of all the *P. aeruginosa* PAO1 genome, and has a key role in the infection process regulating the production of many virulence factors and the formation of biofilm (Fig. 7; reviewed in Kirisits & Parsek, 2006 and in Schuster & Greenberg, 2006).

P. aeruginosa strains carrying mutation in the QS genes were less virulent in several mouse models, including acute pneumonia and chronic lung infection models. Similarly, attenuation of virulence was also observed using alternative infection models like *C. elegans*, *A. thaliana, D. melanogaster* and *Dictyostelium discoideum* (reviewed in Smith & Iglewski, 2003, Juhas *et al.,* 2005 and Diggle *et al.,* 2006).

Functional QS systems have been found to be important for establishment of *P. aeruginosa* infection and for its reduced clearance in animal models of virulence. If the animals are infected with QS mutants, the immune response is faster, the polymorphonuclear leukocytes respond by developing stronger oxidative bursts

and antibodies accumulate faster in the infected lung (Rasmussen & Givskov, 2006). Moreover, 3-oxo-C12-HSL also interacts directly with the host organism; indeed it stimulates interferon-γ and IL-8 production, inhibits interleukin-12 and tumour necrosis factor α, promotes immunoglobulin-E production, and causes apoptosis in macrophages and neutrophils. In this respect, the QS signal molecules can be considered as virulence factors themselves (Wagner *et al.,* 2006).

The above data strongly suggest that *P. aeruginosa* QS plays a primary role during infection. As a matter of fact, acyl-HSLs and PQS can be detected at biologically-significant concentrations in sputum, bronchoalveolar and mucopurulent fluids from CF patients and in other clinical samples (Singh *et al.*, 2000; Collier *et al.*, 2002; Middleton *et al.*, 2002; Diggle *et al.,* 2006).

The *P. aeruginosa* QS system regulates functions that strongly affect the structural and chemical-physical features of the biofilm, including the production of rhamnolipids, the secretion of DNA, swarming motility and the pyoverdine-mediated uptake of iron. Moreover, QS mutants have defects in biofilm structure and produce biofilms that are more susceptible to detergents and antibiotics (Kirisits & Parsek, 2006).

Therefore, the ultimate role of the *P. aeruginosa* QS system is to coordinate the expression of virulence factors and biofilm production at the level of the whole bacterial population.

In summary, the QS system is important in both acute and chronic infections. In the first case it is likely that QS enable *P. aeruginosa* to overcome host defence mechanisms, since isolated production of extracellular virulence factors by a small number of bacteria would probably lead to an efficient host response, neutralizing these compounds. In the chronic infection, the role of QS is strictly linked with the biofilm mode of growth. In both cases, the QS signal molecules participate to the cross-talk between host and parasite enhancing the fitness of the *P. aeruginosa* community in the host environment.

1.2.5 Quorum sensing regulation in *P. aeruginosa*

As previously mentioned, *P. aeruginosa* is an organism with complex genome. This genetic complexity is highlighted by the presence of a large proportion of regulatory genes, which make up almost 9 % of the total number of ORFs, further highlighting metabolic plasticity and capability of adaptation to several environments. Several recent studies have shown that QS in *Pseudomonas* is integrated with certain aspects of cell physiology and that it responds to other environmental signals, beside cell density. Production of QS signal molecules and virulence factors must be finely controlled and timed to achieve a productive and persistent infection. This is also stressed by the overlap of the acyl-HSL QS regulon with other regulons controlled by global regulators, providing a high degree of interconnectivity among different signalling networks. The integration of QS into additional regulatory circuits increases the range of environmental and

metabolic signals that affect QS gene expression beyond cell density as well as further tuning the timing of the QS response. At least 17 gene products that have an impact on the production of QS signal molecules have been identified. These regulators are important in modulating the production of signal molecules and virulence factors likely in response to stimuli deriving from the environment (*e.g.* from the host during infection; Fig. 6; reviewed in Venturi, 2006). Most of these QS regulators have been identified by genetic analysis and only in few cases their mechanism of action is known.

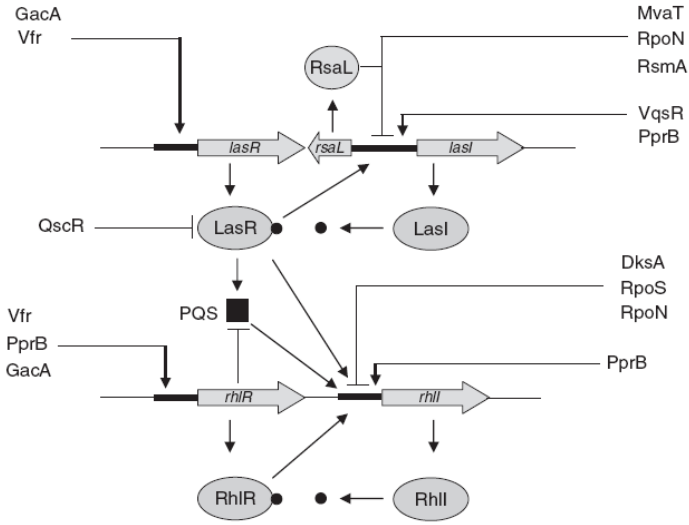

Figure 6. Regulation of the *las* and *rhl* quorum-sensing systems in *P. aeruginosa*. The *las* system is at the top of the hierarchy, regulating the *rhl* system and PQS production. Arrows on the promoter regions of the indicated genes mean positive regulation, whereas a short parallel line indicates negative regulation (Venturi, 2006).

- **Vfr**

The QS and cyclic AMP (cAMP) signalling networks are linked via the transcriptional regulator Vfr, a member of the cAMP receptor protein (CRP) family. Vfr was first identified in *P. aeruginosa* as a virulence factor regulator, due to its positive effect on the production of several virulence factors, such as proteases and exotoxin A. This effect is probably mediated by LasR, since Vfr positively regulates transcription of *lasR* by directly binding to a CRP consensus sequence present in the *lasR* promoter region (Albus *et al*., 1997). Also the *rhlR* promoter has been shown to be positively regulated by Vfr, but it is not known

whether Vfr directly activates the *rhlR* promoter through a Vfr-binding site or if it acts indirectly via other regulators (Medina *et al*., 2003).

As mentioned above, Vfr is homologous to the cAMP receptor protein family and contains a cAMP binding domain. However, differently from *Escherichia coli* and other enterobacteriaceae, cAMP levels in pseudomonads are not related to glucose uptake and utilization (West *et al.,* 1994). Therefore the role of cAMP in *Pseudomonas*, as well as the environmental stimuli to which Vfr respond, are still far to be understood.

- **MvaT**

The MvaT protein belongs to the H-NS family (histon-like nucleoid structuring). The proteins of this family are involved in processes of compacting chromosomal DNA, working as global gene expression regulators in Gram-negative bacteria (Dorman, 2004). The structure of these proteins includes two domains, with a flexible linker between them. The *N*-terminal domain is involved in the formation of oligomers, while the *C*-terminal domain is involved in the DNA-binding (Dorman *et al.*, 1999; Tendeng & Bertin, 2003).

In *P. aeruginosa* MvaT regulates the expression of genes involved in virulence, in arginine metabolism, in antibiotic resistance and in biofilm formation, through direct binding to the promoters or through indirect regulation (Diggle *et al.*, 2002; Vallet *et al.*, 2004; Westfall *et al.*, 2004 and 2006; Li *et al.*, 2009).

Interestingly a *mvaT* mutant was shown to over-produce 3-oxo-C12-HSL and C4-HSL signal molecules and to regulate some QS-dependent virulence phenotypes (Diggle *et al.*, 2002).

1.3 *Burkholderia cepacia* complex

Burkholderia cepacia was described in 1950 as *Pseudomonas cepacia*, the phytopathogen responsible for the sour skin disease in onions (Burkholder, 1950). Molecular taxonomic analysis of this bacterium and closely related species, recovered from diverse environments, including pathogens of plants, animals, and humans, led to their inclusion into the new genus *Burkholderia* (Yabuuchi *et al.*,1992), now comprising over 50 species. Since then, impressive advances have been achieved in the taxonomy of *B. cepacia* and related species, now collectively known as the *B. cepacia* complex (Bcc), comprising of at least 17 different but closely related species, phenotypically similar: non-fermenting, aerobic, Gram-negative β-proteobacteria (Coenye *et al.*, 2001). The genome of these strains consists of three chromosomes and many of them contain also plasmids. The progress in the taxonomy of these bacteria has been mainly due to their emergence as important opportunistic pathogens, capable of causing life-threatening infections in immunocompromised patients, in patients with chronic granulomatous disease, and especially in 2 to 8% of CF patients (Gibson *et al.*, 2003).

Although strains from all the Bcc species are capable of causing infections to CF patients, their prevalence varies geographically. For example, while *B. multivorans* is predominant in Europe, *B. cenocepacia* predominates in North America (Govan *et al.*, 2007).

The *B. cenocepacia* epidemic ET12 lineage that originated in Canada and spread to Europe has been one of the most prevalent Bcc genotypes isolated from CF patients, with strain J2315 being studied in depth as model strain (Foweraker, 2009). The genome of *B. cenocepacia* J2315, a multidrug-resistant CF isolate was recently published (Holden *et al.*, 2009).

The 8.06-Mb genome of this highly transmissible pathogen, consisting of three circular chromosomes and a plasmid, encodes a broad array of functions typical of metabolically versatile genus *Burkholderia*, as well as several virulence and drug resistance functions (Holden *et al.*, 2009).

In CF patients, antibiotics are used to clear early infection, treat acute exacerbations of chronic infection and reduce their relapse frequency. Despite the heavy use of antibiotics in CF, over the last decades, *B. cepacia* complex has emerged as an important respiratory pathogen in the CF community. After colonization with a Bcc strain, few patients experience an asymptomatic carriage, while the majority experiences an increased decline of pulmonary function, associated with chronic infection and exacerbation episodes. Dramatically, a significant percentage of the Bcc-infected patients will develop a rapid and fatal necrotizing pneumonia known as the cepacia syndrome (Isles *et al.*, 1984).

1.3.1 *B. cepacia* complex virulence

Compared to the advances achieved in the taxonomy, knowledge on the molecular mechanisms underlying Bcc pathogenicity and progress on the development of new therapeutic agents are still limited.

In order to successfully establish an infection, after entering into the respiratory tract of the CF patient, bacteria have to adhere to host mucosal or epithelial surfaces. In the case of the CF lung, the thickened mucus layer provides an ideal environment for microbial colonization, due to defective mucus clearance, reduced efficacy of antimicrobial peptides, and enhanced inflammatory response (Boucher 2007). The ability to cross the epithelial barrier and gain access to the blood stream seems to be restricted to Bcc strains, as other CF pathogens usually do not cause bacteremia.

During the interaction with the CF host, several virulence factors are thought to play critical roles for the success of the pathogen, although their precise contribution to the overall Bcc pathogenicity remains to be thoroughly elucidated.

Extracellular lipase, metalloproteases and serine proteases are thought to play roles directly related to the interaction with epithelial cells (McClean & Callaghan 2009).

Bacterial surface structures like the lipopolysaccharide (LPS), flagella and pili are also important in the interaction with the CF host. Flagella are required for

many biological processes, for example motility, production of biofilms, adherence and invasion into host cells (Mahenthiralingam *et al.*, 2005; Moens & Vanderleyden, 1996; O'Toole & Kolter 1998). Indeed *B. cepacia* complex bacteria are motile and they possess one or longer polar flagella responsible for swimming motility. Flagella represent one of the virulence factors which contribute to the development of disease caused by these bacteria as shown by *in vivo* data (Urban *et al.*, 2004). They have been described as a major factor contributing to host inflammatory responses to bacteria due to the interaction of bacterial flagellin with the Toll-like receptor 5 (TLR5) (Hayashi *et al.*, 2001; Liaudet *et al.*, 2003). The production and assembly of these multi-component structures involve more than 40 genes. In particular, members of the Bcc express one of the two types of flagellin that can be distinguished by size: 55 kDa for type I and 45 kDa for type II (Hales *et al.*, 1998).

The production of siderophores such as pyochelin, salicylic acid, cepabactin, and ornibactin, also contribute to Bcc pathogenesis (Agnoli *et al.*, 2006).

Protein secretion is also an important mechanism by which bacteria are able to deliver proteins to the environment and to host cells, being able to influence the host response and being crucial for virulence and survival. Several transport systems have been implicated in the secretion of many virulence factors by Bcc strains such as proteases, hemolysins, and adhesins, among others. Type I and type II secretion systems were shown to be responsible for the secretion of proteins with hemolytic activity in isolates of the *B. cenocepacia* J2315 (Whitby *et al.*, 2006).

Another important feature of Bcc is their ability to form biofilms, communities within which bacteria live in a sessile lifestyle, protected from environmental insults and aggression from the immune system defences of the host. In addition, Bcc bacteria in biofilms have been demonstrated to be more resistant to antibiotics than planktonic cells, contributing to their persistence in the CF lung (Caraher *et al.*, 2007). The characterization of the chemical structure and composition of the EPS showed that it is composed of glucose, mannose, rhamnose, galactose, and glucuronic acid (Cescutti *et al.*, 2000).

1.3.2 The RND efflux transporters in *B. cepacia* complex

B. cenocepacia and other members of the Bcc demonstrate high-levels of intrinsic resistance to most clinically relevant antibiotics, complicating the treatment of the infection (Waters & Ratjen, 2006). In CF isolates, multi-drug resistance (MDR) is defined as resistance to all of the agents belonging to at least two of three classes of antibiotics, such as quinolones, aminoglycosides, and β-lactam agents, including monobactams and carbapenems (Saiman & Siegel, 2003).

Particularly interesting among mediators of MDR in Gram-negative bacteria are transporters belonging to the RND (Resistance-Nodulation-Cell Division) family, whose members catalyze the active efflux of many antibiotics and chemotherapeutic agents (Nikaido & Takatsuka, 2009).

RND transporters are protein complexes that span both the cytoplasmic and outer membrane. The complex comprises the inner membrane RND transporter protein, a periplasmic-exposed membrane protein, belonging to the membrane fusion protein (Mfp) family; and an outer membrane channel protein (Omp; Fig. 7). RND pumps and accessory proteins form large multi-protein assemblies that traverse both the inner and outer membranes of Gram-negative bacteria (Lomovskaya & Totrov, 2005; Poole, 2004; Zgurskaya, 2002).

The *Escherichia coli* AcrAB-TolC and the *P. aeruginosa* MexAB-OprM complexes are well characterized examples of RND transporters; the resolution of the three-dimensional structures of various RND components supported the model according to which these efflux systems form a channel for the extrusion of substrates/drugs from within the cell envelope back into the external environment (Akama *et al.*, 2004a and 2004b; Higgins *et al.*, 2004; Koronakis *et al.*, 2000; Murakami *et al.*, 2002).

The RND transporters, unlike all other known drug pumps, are capable of capturing their substrates in the periplasmic space rather than in the membrane, or from the cytoplasm. RND pumps can recognize and extrude positively or negatively charged and neutral molecules, substances as hydrophobic as organic solvents and lipids, and compounds as hydrophilic as aminoglycoside antibiotics. In essence, high-affinity substrate binding induces high-affinity ATP-binding, which in turn induces substrate release, ATP hydrolysis and subsequent release of ADP to re-set the system (Lomoskaya *et al.*, 2007).

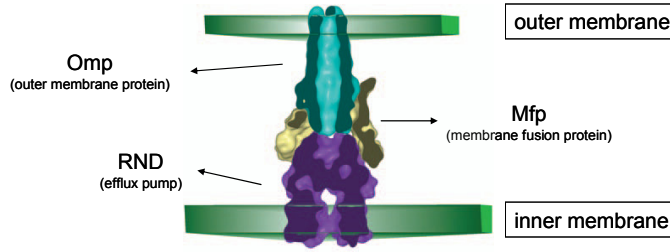

Figure 7. Schematic representation of the RND efflux pumps tripartite complex (modified from Lomovskaya *et al.*, 2007).

1.3.3 Quorum sensing of *B. cepacia* complex

The first indication that cell-cell communication was present in the Bcc was the observation that *P. aeruginosa* concentrated stationary phase spent culture fluids markedly affected the production of siderophore, protease and lipase in a Bcc strain (McKenney *et al.*, 1995). Indeed, Bcc bacteria can recognize and respond to *P. aeruginosa* QS molecules (Riedel *et al.*, 2001), highlighting a possible role for

inter-species communication in the course of the disease of CF patients co-infected with *P. aeruginosa* and *B. cepacia*.

All species of the Bcc possess at least one LuxI/LuxR-like QS system, named CepIR, which provides a mechanism for rapid adaptation to environmental changes. In Bcc, QS regulates the expression of various virulence factors, like toxins, proteases, lipase, and siderophores. Swarming motility and biofilm formation are also regulated by QS in Bcc (reviewed in Venturi *et al.*, 2004).

The proteins, gene organization and structure of acyl-HSLs in Bcc QS systems are highly identical. In *B. cenocepacia* K56-2 CepI synthesizes two HSL molecules, a *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-octanoyl-L-homoserine lactone (C8-HSL). Other Bcc strains may have an additional QS system, responsible for the production of *N*-decanoyl-L-homoserine lactone, *N*-dodecanoyl-L-homoserine lactone and *N*-3-oxo-decanoyl-L-homoserine lactone (reviewed in Venturi *et al.*, 2004).

2. GENERAL RATIONALE AND AIMS

Bacterial infections are the major cause of death in CF patients. Two opportunistic Gram-negative pathogens produce very dangerous infections in these patients, principally for their high resistance to traditional antimicrobial therapies: *P. aeruginosa* and *B. cepacia* complex.

An innovative approach to combat infections could be the use of drugs able to inhibit the production of virulence factors, without inhibiting growth. Selective targeting of the pathogenic potential has the advantage of reducing the bacterial adaptability to the host environment and the severity of the infection but imposing a lower selective pressure toward the emergence of resistant clones with respect to conventional growth-inhibitory compounds (i.e. antibiotics). Moreover, the reduction of the virulent potential of a pathogen provides the host immune system with a better chance of clearing the infection (Cegelski *et al.*, 2008). A deep knowledge of the mechanisms involved in pathogenesis could give an invaluable contribute to the identification of suitable targets for the development of a new generation of drugs aimed to decrease the pathogenic behaviour of the bacteria at the level of the whole bacterial community.

In bacteria, adaptation to the host environment and virulence factor production is regulated by complex global regulative networks.

An increasing body of evidence indicates that in many bacteria, including *P. aeruginosa*, the pathogenic potential mainly relies on the ability to control the expression of an array of different virulence traits, rather than on the presence/expression of a specific set of factors (Lee *et al.*, 2006a).

This PhD work is aimed at giving new insights into virulence related processes that control virulence in *P. aeruginosa* and *B. cenocepacia*.

The first and the second chapter of this study are focused on *P. aeruginosa* QS systems. This global regulative network is critical for virulence factors production and biofilm formation, constituting an ideal target for the development of anti-*Pseudomonas* drugs.

In particular the first part is aimed at identifying the transcriptional regulators of the two acyl-HSLs based QS systems of *P. aeruginosa*.

The main goal of the second part is to identify molecules able to inhibit the *las* QS system of *P. aeruginosa*.

The last chapter is focused on the RND transporters of *B. cenocepacia*. The specific aim of this part of the work is to investigate the role played by these transporters in processes different from antibiotic resistance, such as virulence-related phenotypes.

3. CHAPTER I
Picking up novel *P. aeruginosa* quorum sensing regulators

3.1 Background and rationale

As described above, the QS communication system enables bacteria to act as a community by coordinating gene expression in response to environmental changes. This kind of regulation is thought to give to pathogenic bacteria a selective advantage over host defences, and thus it is important for their virulence potential.

An increasing amount of data concur to the knowledge that the three QS systems of *P. aeruginosa* (i.e. *las*, *rhl* and *pqs*) are strictly interconnected and integrated in a complex regulatory network aimed to coordinate *P. aeruginosa* group behaviour in response to external stimuli (Schuster & Greenberg, 2006; Venturi, 2006). Indeed, as described in the introduction, the expression of genes involved in synthesis and perception of QS signal molecules is finely regulated at the transcriptional and post-transcriptional level in response to metabolic and environmental stimuli (Soberón-Chávez *et al*., 2005; Dunn & Stabb, 2006; Duan & Surette, 2007). However, the proteins involved in this regulation, their mechanisms of action and actual impact on *P. aeruginosa* pathogenic potential are in most cases still unknown. In particular, only the Vfr protein has been shown to directly activate *lasR* transcription (Albus *et al*., 1997).

Besides *las* and *rhl* mutants, also a *vfr* mutant shows a strongly reduced virulence in murine models (Smith *et al.*, 2004), suggesting that also the QS regulators could be feasible drug-targets for the development of new anti-*Pseudomonas* therapies.

3.2 Specific aim

This part of the project is aimed at identifying transcriptional factors that directly regulate the QS genes, focusing on the *las* and *rhl* QS systems of *P. aeruginosa*. The following genes have been taken into consideration: *lasR* and *lasI,* encoding the 3-oxo-C12-HSL receptor and synthase, respectively; *rsaL* and *qscR* encoding repressors of *lasI* and constituting an integral part of the *las* QS system (since they respond to 3-oxo-C12-HSL); *rhlR* and *rhlI,* encoding the C4-HSL receptor and synthase, respectively. A schematic representation of the *las* and *rhl* genes is given in figure 5.

A DNA-affinity chromatography combined with MALDI-TOF mass spectrometry approach was used to recover and identify, from a protein crude extract derived from *P. aeruginosa* PAO1 cultures, proteins able to bind specific DNA fragments corresponding to the promoters of the gene above indicated.

The effect of each selected protein on its target promoter/s was tested *in vivo* in *P. aeruginosa* cells.

3.3 Results and discussion

3.3.1 Retrieving regulators by DNA-affinity chromatography

To the aim of picking up QS regulators from *P. aeruginosa* protein crude extracts, a DNA-affinity chromatography approach was used. Briefly five different biotinylated DNA, corresponding to the *rsaL-lasI* bidirectional promoter (P*rasL-lasI*) and to the promoters of *lasR*, *qscR*, *rhlR* and *rhlI* genes (P*lasR*, P*qscR*, P*rhlR* and P*rhlI* respettively) were immobilized on a streptavidin-conjugated chromatography resin. The five resins were then incubated with protein crude extracts prepared from *P. aeruginosa* cultures grown in Luria-Bertani broth until A_{600} = 2.0 (exponential growth phase) and A_{600}= 5.0 (stationary growth phase). After several washes only the proteins specifically bound to the promoter were eluted, separated by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE), and identified by MALDI-TOF mass spectrometry (Fig. 8).

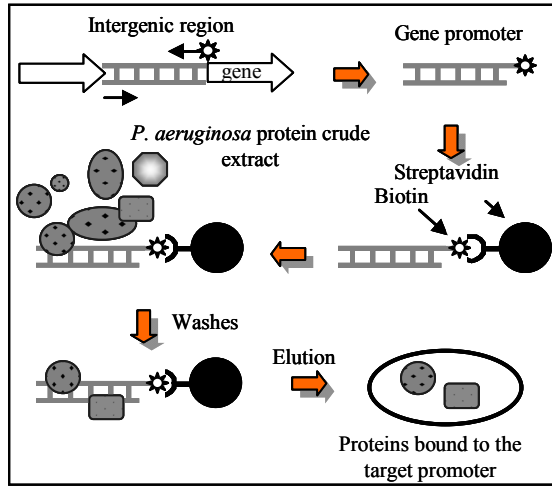

Figure 8. Schematic representation of the DNA-affinity chromatography procedure. Experimental steps (represented as orange arrows) are as follows: 1, PCR amplification of the promoter region of the gene with unlabelled and biotin-labelled primer (white star); 2, binding of the DNA bait to streptavidin-coupled magnetic beads (armed black circle); 3, incubation of the DNA bait with whole cell proteins (grey shapes); 4, magnetic separation of DNA-binding proteins; 5, salt-based elution of the DNA-binding proteins (modified from Imperi *et al.*, 2010).

Details regarding the length and the position of the used DNA fragments in *P. aeruginosa* sequenced genome are given in Materials and Methods. In figure 9A-E examples of SDS-PAGE gels from different DNA-affinity chromatography experiments are shown. In the majority of the cases the experiment was performed in duplicate, obtaining similar results and no protein bands were visible in control samples corresponding to beads uncoupled to DNA (data not shown).

Figure 9A

DNA-affinity chromatography using P*lasR* as ligand.

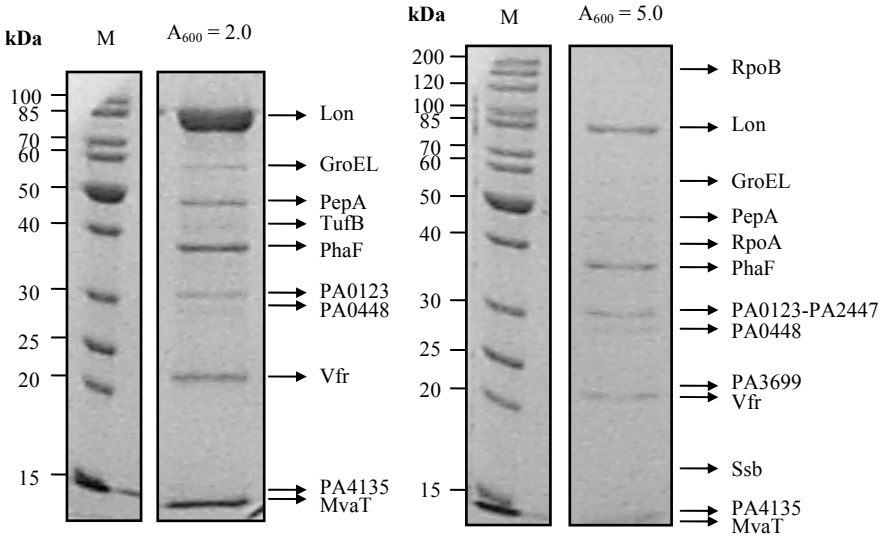

Figure 9B

DNA-affinity chromatography using P*rsaL-lasI* as ligand.

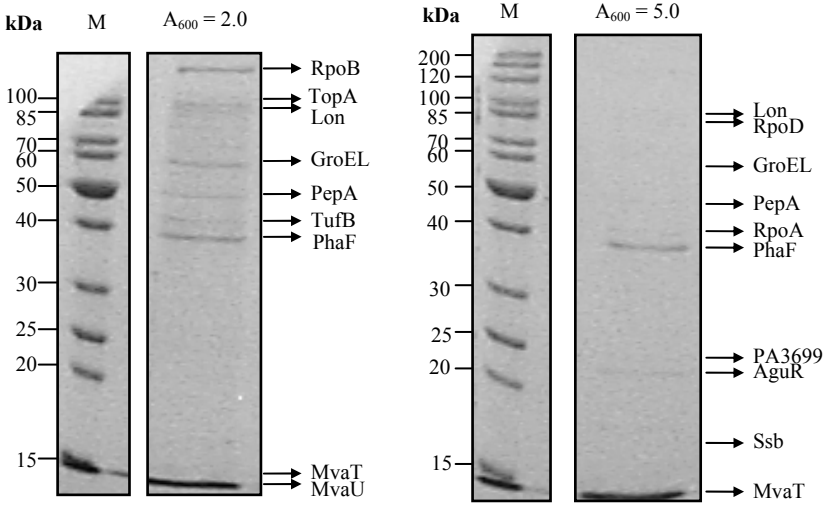

Figure 9C

DNA-affinity chromatography using P*qscR* as ligand.

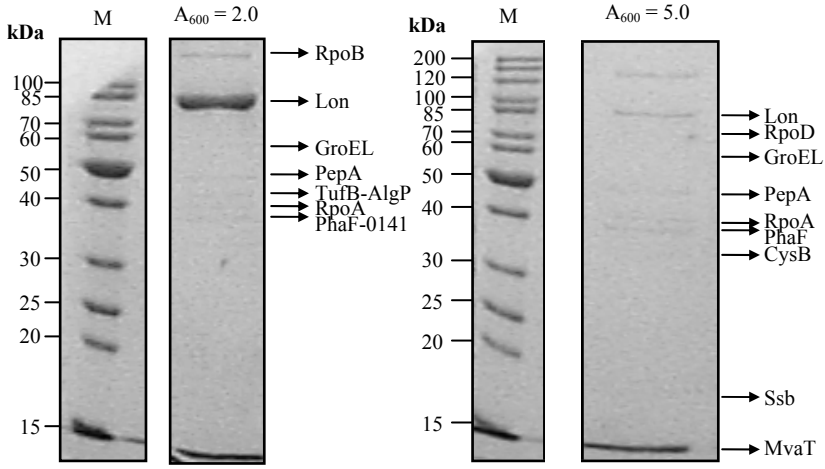

Figure 9D

DNA-affinity chromatography using P*rhlR* as ligand.

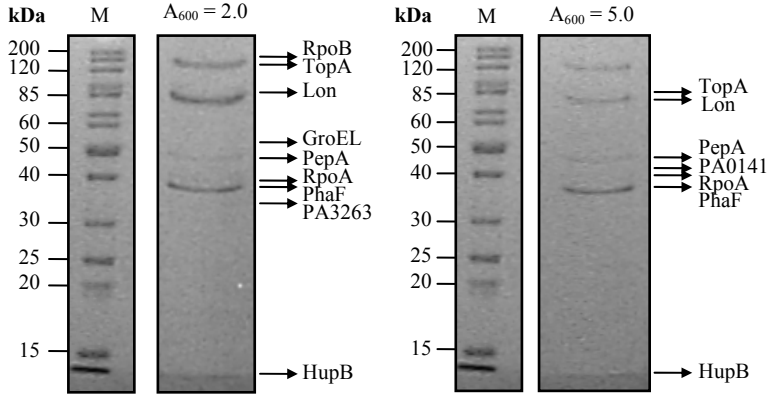

Figure 9E

DNA-affinity chromatography using P*rhlI* as ligand.

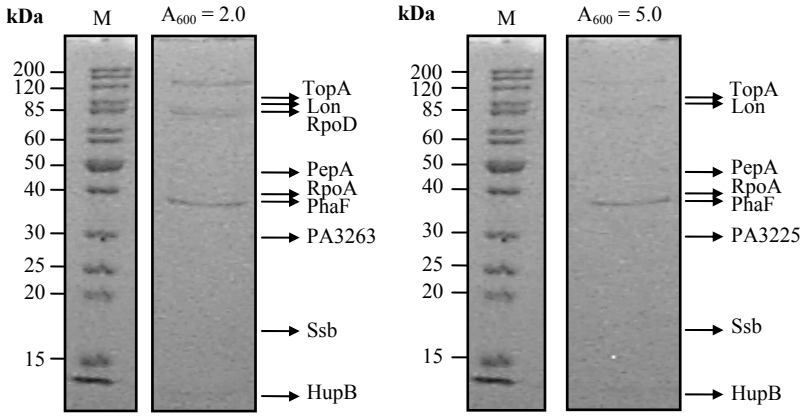

Figure 9. SDS-PAGE resolution of proteins bound to (A) P*lasR* (B) P*rsaL-lasI* (C) P*qscR* (D) P*rhlR* (E) P*rhlI*. Whole cell proteins were prepared from PAO1 cultures grown in Luria-Bertani broth until a cell density corresponding to A_{600}= 2.0 (exponential growth phase) and A_{600}= 5.0 (stationary growth phase). Bands subjected to mass spectrometry are highlighted by arrows and the corresponding identified protein is indicated. M, molecular weight marker with size (kDa) on the left.

Overall, twenty-five proteins were identified, belonging to different functional classes (Tab. 1). Among these nineteen were annotated as DNA-binding proteins: thirteen confirmed or predicted transcriptional regulators and six general transcriptional factors, such as RNA-polymerase subunits. The latter were recovered from almost all the analyzed promoters, on the contrary most of the transcriptional regulators were found associated to a single specific promoter, ensuring specificity of the protein-promoter interaction. Indeed the Vfr activator, known to bind P*lasR,* was able to bind this promoter, but not other promoters.

Two hypothetical proteins with unknown function and four proteins with function different from DNA-binding were also retrieved from the DNA-affinity chromatography. The latter are mainly protease and chaperon proteins that likely co-purified with the other eluted proteins (Tab. 1).

Fifteen different proteins were selected for further investigations as good candidates as transcriptional regulators of the main *las* and *rhl* genes: the thirteen confirmed or predicted transcriptional regulators and the two hypothetical proteins.

The complete list of the identified proteins, their function, predicted or confirmed, and the promoter from which they were recovered are shown in table 1. More information about the *in silico* analysis of MALDI-TOF results are given in "Supplementary Data" (Tab. S1-S5). It is known that the DNA-affinity chromatography can generate artifacts, leading to the identification of proteins that *in vivo* do not interact with the target promoter. In our experiments, most of the confirmed or predicted transcriptional regulators were recovered only from one promoter. For instance Vfr regulator was recovered only from P*lasR*, as expected given that it is the only regulator known to directly bind P*lasR* region. This evidence strengthened the reliability of our experimental approach, and encouraged further studies. Therefore, all the confirmed or predicted transcriptional regulators and the conserved hypothetical proteins with unknown function were selected for further analysis as putative QS regulators. All the selected proteins are highlighted in bold in the table 1.

Table 1. List of all the proteins identified by DNA-affinity chromatography (Fig. 9A-E).

PA no.[a]	Gene name[b]	Function[c]	Target promoter [d]	
			A_{600}=2.0	A_{600}=5.0
		DNA-binding proteins (involved in processes different from transcription regulation)		
PA0576	*rpoD*	sigma factor RpoD	*rhlI*	*rsaL-lasI, qscR*
PA1804	*hupB*	DNA-binding protein HU	*rhlI, rhlR*	*rhlI,rhlR*
PA3011	*topA*	DNA topoisomerase I	*rsaL-lasI, rhlI, rhlR*	*rhlI, rhlR*
PA4232	*ssb*	single-stranded DNA-binding protein	*rhlI*	*rsaL-lasI, qscR, lasR, rhlI*
PA4238	*rpoA*	DNA-directed RNA polymerase alfa chain	*qscR, rhlI, rhlR*	*rsaL-lasI, lasR, qscR, rhlI, rhlR*
PA4270	*rpoB*	DNA-directed RNA polymerase beta chain	*rsaL-lasI, qscR, rhlR*	*lasR*
		Transcriptional regulators (confirmed or predicted)		
PA0123		probable transcriptional regulator	*lasR*	*lasR*
PA0294	*aguR*	Probable transcriptional regulator AguR		*rsaL-lasI*
PA0448		probable transcriptional regulator	*lasR*	*lasR*
PA0652	*vfr*	Transcriptional regulator Vfr	*lasR*	*lasR*
PA1754	*cysB*	Transcriptional regulator CysB		*qscR*
PA2447		probable transcriptional regulator		*lasR*
PA2667	*mvaU*	Transcriptional regulator MvaU	*rsaL-lasI*	
PA3225		probable transcriptional regulator		*rhlI*
PA3699		probable transcriptional regulator		*rsaL-lasI, lasR*
PA4135		probable transcriptional regulator	*lasR*	*lasR*
PA4315	*mvaT*	transcriptional regulators MvaT	*rsaL-lasI, lasR*	*rsaL-lasI, lasR, qscR*
PA5060	*phaF*	polyhydroxyalkanoate synthesis protein PhaF	*rsaL-lasI, lasR, qscR, rhlI, rhlR*	*rsaL-lasI, lasR, qscR, rhlI, rhlR*
PA5253	*algP*	alginate regulatory protein AlgP	*qscR*	
		Conserved hypothetical proteins		
PA0141		conserved hypothetical protein	*qscR*	*rhlR*
PA3263		conserved hypothetical protein	*rhlI, rhlR*	
		Others		
PA0779	*lon*	probable ATP-dependent protease	*rsaL-lasI, lasR, qscR, rhlI, rhlR*	*rsaL-lasI, lasR, qscR, rhlI, rhlR*
PA3831	*pepA*	leucine aminopeptidase	*rsaL-lasI, lasR, qscR, rhlI, rhlR*	*rsaL-lasI, lasR, qscR, rhlI, rhlR*
PA4277	*tufB*	elongation factor Tu	*rsaL-lasI, lasR, qscR*	
PA4385	*groEL*	GroEL protein	*rsaL-lasI, lasR, qscR, rhlR*	*rsaL-lasI, lasR, qscR*

a. PA number refers to Pseudomonas Genome Database (PGD) annotation (http://www.pseudomonas.com).
b. Gene name, if assigned in the PGD.
c. Annotated function in PGD.
d. Genes which promoters were used as baits in the DNA-affinity chromatography; the optical density of *P. aeruginosa* cultures from which protein crude extracts were obtained are indicated.

3.3.2 *In vivo* characterization of the putative QS regulators

In order to verify the *in vivo* effect of the identified QS putative regulators, a set of fifteen *P. aeruginosa* mutants, each one deleted in the gene coding for one of the selected proteins, was generated and the promoter activity of the target genes was compared in the wild type and in the corresponding mutant.

The fifteen mutants were constructed with the method summarized in figure 10 (more details are in Materials and Methods, Hoang *et al*., 1998).

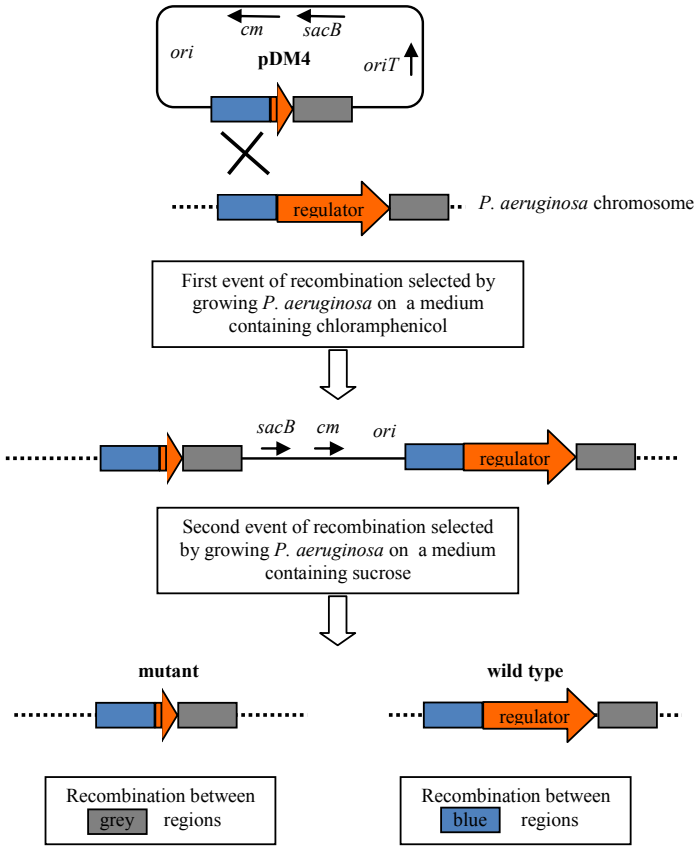

Figure 10. Schematic representation of the mutagenesis method described by Hoang *et al*., 1998. The upstream and downstream regions of the target gene are represented in blue and grey respectively. The orange arrows represent the target gene (wild type) that will be deleted. *sacB*, toxic gene in presence of sucrose; *cm*, gene for the resistance to the chloramphenicol; *ori*, replication origin; *oriT*, transfer origin.

Promoter activity was measured in *P. aeruginosa* PAO1 wild type and in the mutants by means of transcriptional fusions. Recombinant strains carrying a transcriptional fusion between the promoter region of the target gene and the *luxCDABE* operon from *Xenorhabdus luminescens* were generated. To do this, the *lasR*, *lasI*, *rsaL*, *qscR*, *rhlR* and *rhlI* promoter regions were cloned upstream the *luxCDABE* operon in the mini-CTX-*lux* plasmid (Hoang *et al*., 2000, see Materials and Methods for more details), generating six different genetic cassettes (P*lasR*::*luxCDABE,* P*lasI*::*luxCDABE,* P*rsaL*::*luxCDABE,* P*qscR*::*luxCDABE,* P*rhlR*::*luxCDABE,* P*rhlI*::*luxCDABE* respectively). These cassettes were independently introduced in *P. aeruginosa* PAO1 wild type and in the mutants generated as described in the figure 10. In the constructed recombinant strains the target promoter controls the expression of the reporter operon *luxCDABE.* This operon encodes the luciferase enzyme and the enzymatic complex required for the synthesis of its substrate; therefore in the recombinant strain the activity of the promoter can be easily monitored during bacterial growth by measuring light emission as LCPS (Light Counts Per Second). Moreover the transcriptional fusion was inserted in a neutral site of the chromosome in single copy, avoiding possible problems related to plasmid stability and multicopy-effect, often reported when working with plasmid-encoded transcriptional fusions (Becher & Schweizer, 2000). None of the fifteen mutations and the insertion of none of the genetic cassettes affected the growth of *P. aeruginosa* (data not shown).

Promoter activity of the genes mentioned above was measured every hour for 16 hours of growth in LB in microtiter plates in the wild type and in the corresponding mutants, all carrying the different genetic cassettes (details in Materials and Methods). The correspondence between a putative regulator and the target promoters is shown in table 1.

- ***vfr* mutant**

In the *vfr* mutant P*lasR* activity was decreased in comparison to the wild type, reaching about 80% of reduction of the maximal promoter activity (Fig. S1). LasR is the main activator of *lasI* expression and Vfr was already known to activate *lasR* transcription (Albus *et al.*, 1997). However the actual impact of such an activation on *lasI* expression and consequently on 3-oxo-C12-HSL production has never been investigated. For this reason the effect of *vfr* mutation on *lasI* expression was tested by introducing the P*lasI*::*luxCDABE* transcriptional fusion in the *vfr* mutant (Δ*vfr*).

The P*lasI* activity in the *P. aeruginosa* PAO1 wild type and in the Δ*vfr* was monitored every one hour for 16 hours by growing the two strains in LB in microtiter plates. The two strains showed the same growth curve and the trend of P*lasI* activity was the same (data not shown). The maximal promoter activity showed by the two strains is reported in figure 11.

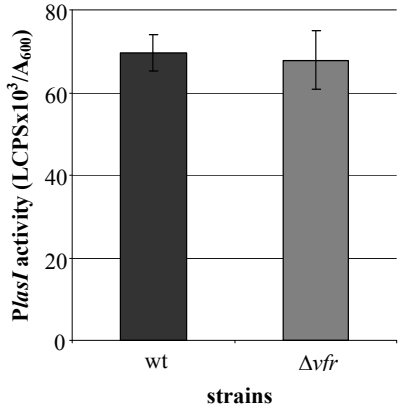

Figure 11. Histogram representing P*lasI* maximal promoter activity measured in *P. aeruginosa* PAO1 wild type (wt; dark grey bar) and in the Δ*vfr* mutant (light grey bar), both carrying the P*lasI*::*lucCDABE* cassette in the chromosome. The average of three independent experiments is reported with standard deviations.

P*lasI* activity was not affected by *vfr* mutation. This result is interesting, because, Vfr increases *lasR* expression and LasR/3-oxo-C12-HSL complex activates *lasI* transcription.

However LasR also activates *rsaL* expression and RsaL represses *lasI* transcription (Rampioni *et al.*, 2006). It is possible that Vfr activating *lasR* expression increases RsaL intracellular concentration and this counteracts the increased activation by LasR/3-oxo-C12-HSL complex on the output gene *lasI*.

To verify this hypothesis we determined the effect of Vfr on P*lasI* activity in the absence of RsaL. To do this, the Δ*rsaL* deletion mutant and Δ*rsaL*Δ*vfr* double deletion mutant were generated and the P*lasI*::*luxCDABE* transcriptional fusion was introduced in these two strains. We also performed control experiments proving that the *rsaL* mutation does not affect *lasR* expression (data not shown).

As shown in figure 12, the maximal P*lasI* promoter activity in the Δ*rsaL*Δ*vfr* double mutant is decreased in comparison with the P*lasI* activity in the Δ*rsaL* mutant. The effect of these mutations on P*lasI* activity was maintained along the whole growth curve (data not shown). This finding demonstrates that, in the absence of the RsaL repressor, the Vfr-dependent activation of *lasR* transcription determinates an activation of *lasI* expression and hence of 3-oxo-C12-HSL production. The fact that the wild type and the *vfr* mutant disclosed the same P*lasI* activity is likely due to the simultaneous increase of the *lasI* repressor RsaL.

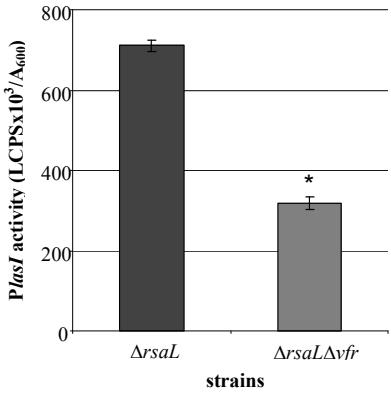

Figure 12. Histogram representing P*lasI* maximal promoter activity measured in the Δ*rsaL* mutant (dark grey bar) and in the Δ*rsaL*Δ*vfr* double mutant (light grey bar), both carrying the P*lasI*::*luxCDABE* cassette in the chromosome. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to Δ*rsaL* is indicated with one asterisk (* = $p<0.01$).

This could be interesting in the QS-regulated phenotypes. Indeed there are some virulence-related genes that are regulated by both the activator complex LasR/3-oxo-C12-HSL and the repressor RsaL.

For instance *phz*A-G1 operon, that provide for the pyocyanin production, is directly repressed by RsaL (Rampioni *et al*. 2007) and directly activated by the LasR/3-oxo-C12-HSL complex (Whiteley *et al.*, 1999).

To clarify the impact of *vfr* and *rsaL* mutations on pyocyanin production, we determined pyocyanin concentration in culture supernatants of *P. aeruginosa* wild type, Δ*vfr*, Δ*rsaL* and Δ*rsaL*Δ*vfr* mutants .

As expected pyocyanin production was not affected by *vfr* mutation, but was decreased in a Δ*rsaL*Δ*vfr* double mutant in comparison with the Δ*rsaL* single mutant (Fig. 13).

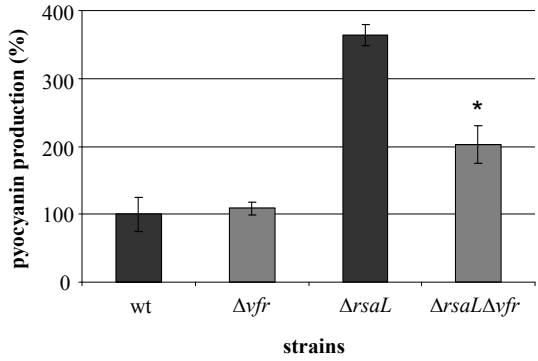

Figure 13. Histogram representing pyocyanin production measured in the wild type (wt, dark grey bar), in the Δ*vfr* (light grey bars), in the Δ*rsaL* mutant (dark grey bar) and in the Δ*rsaL*Δ*vfr* double mutant (light grey bar). Pyocyanin production is reported in % with respect to the wild type. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to the wild type is indicated with one asterisk (* = $p<0.01$).

In the whole, our results show that there is a clear regulatory interplay between Vfr, LasR and RsaL. It is not easy to understand the physiological role of Vfr within this regulatory circuit. Indeed the activity of Vfr is dependent on the availability of cAMP and the real role of this intra-cellular signal in *P. aeruginosa* is not yet clear. Probably, in different environmental conditions and in the host environment Vfr, affecting LasR expression, plays an important role in determining the steady state level of the 3-oxo-C12-HSL signal molecule and consequently on the regulation of QS-dependent genes. Moreover, it is important to underline that RsaL also controls the expression of genes that are not QS-dependent. Thus, Vfr, affecting RsaL levels, could have a broader role in cell metabolism.

- ***mvaT* and *mvaU* mutants**

In figure 14 the maximal P*lasR*, P*lasI* and P*qscR* promoter activities measured in *P. aeruginosa* PAO1 wild type and in the *mvaT* mutant are reported in % with respect to the wild type. The *mvaT* mutation affected the promoter activity of *lasR*, *lasI* and *qscR* genes during the whole growth curve, while it didn't affect *rsaL* promoter activity. In the *mvaT* mutant maximal P*lasR* activity was 35% decreased with respect to the wild type, while maximal P*lasI* and P*qscR* activities were 30% and 95% respectively increased with respect to the wild type. Since LasI produces the 3-oxo-C12-HSL signal molecule, the presented result is in accordance with previous data, in which the *mvaT* mutant was shown to overproduce the 3-oxo-C12-HSL (Diggle *et al.* 2002). Moreover the effect of *mvaT* mutation on *qscR* expression is in accordance with previous transcriptomic analysis performed on the

mvaT mutant in which *qscR* was up-regulated (Vallet *et al.*, 2004). However MvaT had never been described as a regulator of QS genes expression and this is the first evidence that MvaT directly controls the transcription of the *las* system.

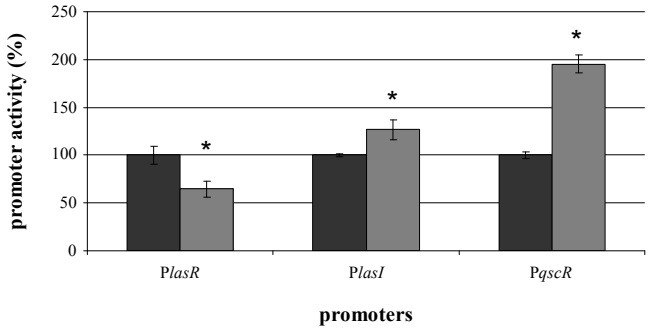

Figure 14. Graph representing P*lasR*, P*lasI* and P*qscR* maximal promoter activity in *P. aeruginosa* PAO1 wild type (dark grey bars) and in the *mvaT* mutant (light grey bars). Promoter activity was measured by means of transcriptional fusions between the indicated promoter and the *luxCDABE* operon and is reported in % with respect to the wild type. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to the wild type is indicated with one asterisk (* = $p < 0.01$).

The expression of *lasI* is enhanced by LasR and repressed by QscR. If MvaT affects *lasI* expression through LasR and QscR, a strong decrease of *lasI* promoter activity would be expected n the *mvaT* mutant. Conversely the *mvaT* mutation causes an increase of *lasI* promoter activity, suggesting that MvaT regulates *lasR*, *lasI* and *qscR* in an independent way (Fig. 15).

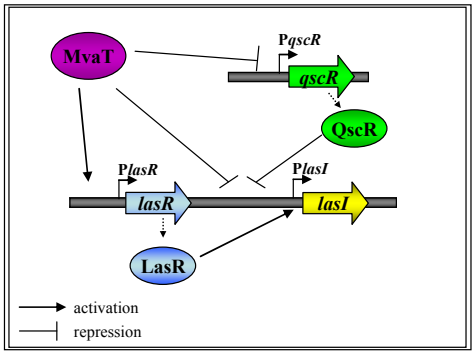

Figure 15. MvaT independently regulates *lasR*, *lasI* and *qscR* transcription.

The *mvaU* mutation increased the promoter activity of *lasI* and *rsaL* genes during the whole growth curve. In figure 16 the maximal P*lasI* and P*rsaL* promoter activities, measured in *P. aeruginosa* PAO1 wild type and in the *mvaU* mutant, are reported in % with respect to the wild type.

As shown in figure 16, in the *mvaU* mutant maximal P*lasI* and P*rsaL* activities were respectively 70% and 100% increased with respect to the wild type. This result is very interesting since MvaU has never been shown before to have an effect on the QS response and on the QS genes.

The data obtained on the *mvaT* and *mvaU* mutants are in agreement with what previously suggested by chromatin immunoprecipitation experiments (ChIP-on-chip) in which MvaT and MvaU were identified as proteins that could bind the *rsaL-lasI* bidirectional promoter and the *lasR* promoter (Castang *et al*., 2008).

MvaT and MvaU are global regulators of gene expression and they are interconnected by complex relationships, not yet clarified. They can act as activators or repressors: some genes can be regulated by MvaT-MvaT or MvaU-MvaU homodimers and other genes by MvaT-MvaU eterodimers (Vallet *et al*., 2004; Castang *et al*., 2008; Li *et al*., 2009). For instance, the *cupA* gene expression is repressed by MvaT, but it is not affected by MvaU. However an over-expression of MvaU can complement the effect of an *mvaT* mutation on *cupA* expression (Vallet *et al*., 2004). Other genes, such as *lecA*, are repressed by both MvaT and MvaU (Diggle *et al.*, 2002).

Our data suggest that MvaT and MvaU could unbalance the final output of the LasR-mediated positive loop and the RsaL-mediated negative loop by independently activating or repressing single *las* genes. However, understanding the interplay between these two proteins in regulating QS genes is a complex issue also because *mvaT mvaU* double mutants are in the majority of the cases lethal (Castang *et al.*, 2008; Li *et al.*, 2009).

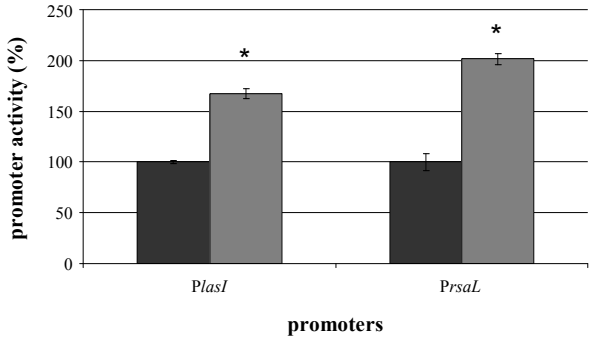

Figure 16. Graph representing P*lasI* and P*rsaL* maximal promoter activity in *P. aeruginosa* PAO1 wild type (dark grey bars) and in the *mvaU* mutant (light grey bars). Promoter activity was measured by means of transcriptional fusions between the indicated promoter and the *luxCDABE* operon and is reported in % with respect to the wild type. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to the wild type is indicated with one asterisk (* = $p < 0.01$).

- **Mutants in the other putative regulators**

Unexpectedly, the inactivation of the other novel putative regulators identified to bind *las* and *rhl* genes *in vitro* did not affect the activity of these promoters *in vivo* (Fig. S1-S6). A possible explanation for this result is that some of these factors are actually QS regulators, but they are not sufficiently expressed and/or active under the conditions used *in vivo* in the promoter activity assay.

3.3.3 Over-expression of *lasR* putative regulators

Often, the difficulties experienced in the assessment of the role of a single regulator in complex and interconnected regulatory circuits are solved by over-expressing that regulator.

Therefore, we decided to over-express the putative regulatory proteins that, as a consequence of the mutation of the corresponding genes, did not show any effect on the activity of the target promoter under our experimental conditions.

Since LasR triggers the activation of both the *las* and *rhl* QS systems, up to now the attention was focused on the six new factors retrieved on the *lasR* promoter: PA0123, PA0448, PA2447, PA3699, PA4135 and PhaF. To elucidate the role of these proteins in modulating P*lasR* activity, their genes were cloned in the pHERD plasmid under the control of an L-arabinose-inducible promoter (Qiu *et al*., 2008), resulting in the generation of the six plasmids pR0123, pR0448, pR2447, pR3699, pR4135 and pR*phaF*. These plasmids were independently introduced by conjugation in the *P. aeruginosa* PAO1 wild type strain carrying in the

chromosome the transcriptional fusion between the P*lasR* promoter region and *luxCDABE* operon. In order to verify that the presence of the pHERD empty plasmid and of the inducer did not affect P*lasR* activity, this activity was monitored in *P. aeruginosa* wild type carrying or not the pHERD empty plasmid, in the presence and in absence of L-arabinose. The presence of both the pHERD empty plasmid and L-arabinose did not affect promoter activity or bacterial growth (data not shown).

In figure 17 it is reported the maximal P*lasR* promoter activity (A) and the corresponding cell density (B) measured in the *P. aeruginosa* PAO1 strains carrying the pHERD-based plasmids for protein over-expression previously described. As a control, the *P. aeruginosa* PAO1 carrying the pHERD empty plasmid was used. All the strains were grown in LB supplemented with 0.1 % L-arabinose to induce the over-expression of the target protein. Unfortunately, it was not possible to test the effect of PA2447 over-expression on P*lasR* activity, due to the lethal effect of pR2447 once conjugated in *P. aeruginosa* PAO1.

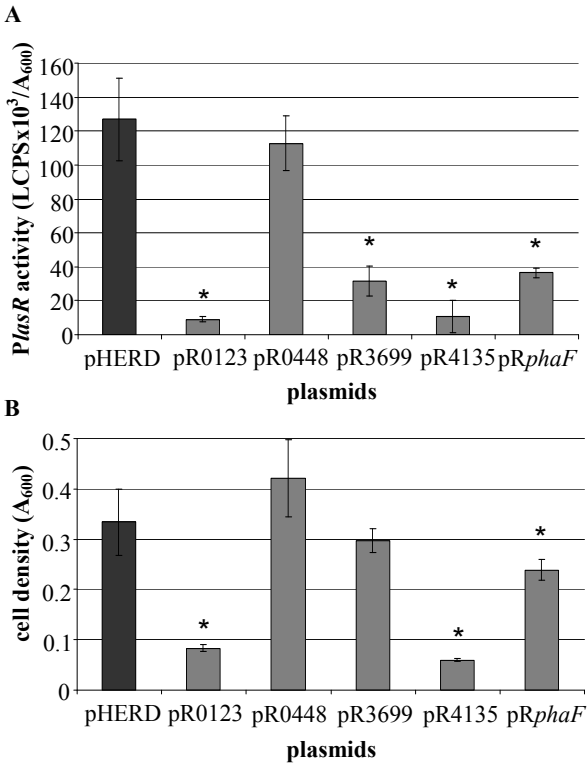

Figure 17. Histograms representing (A) P*lasR* maximal promoter activity and (B) maximal cells density measured in *P. aeruginosa* PAO1 P*lasR::lucCDABE* strains carrying the plasmids indicated in the graphs, grown in LB broth supplemented with 0.1 % (w/v) L-arabinose. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to *P. aeruginosa* PAO1 P*lasR::luxCDABE* (pHERD) is indicated with one asterisk (* = $p < 0.01$).

As shown in figure 17A, P*lasR* maximal activity was drastically reduced in the strains over-expressing PA0123, PA3699, PA4135 and PhaF with respect to the control strain carrying pHERD, while the effect of PA0448 over-expression on P*lasR* transcription was negligible. However, as reported in figure 17B, the strains carrying the pR0123, pR4135 and pR*phaF* plasmids displayed a maximal cell density lower than *P. aeruginosa* PAO1 pHERD, suggesting that the over-expression of PA0123, PA4135 and PhaF negatively impacts *P. aeruginosa* energy metabolism.

Considering that luciferase requires ATP for its enzymatic activity, the decrease in light emission observed in the strains displaying a slower growth rate could be due to a reduced activity of the *lux* enzymes, rather than to a decreased activity of

P*lasR* (Meighen & Dunlap, 1993). Moreover the QS communication system is based on the sensing of bacterial cell density, and this implies that every physiological or metabolic perturbation that impacts cell growth (in this case the over-expression of some tested proteins), leading to different cell densities at the different time points of the growth curve, might result in an alteration of the QS response, and consequently of P*lasR* activity. For these reasons, our study on PA0123, PA4135 and PhaF could lead to non-conclusive and unreliable data. Therefore these proteins were no further investigated in this work.

By comparing figure 17A and 17B, it is possible to conclude that only the over-expression of PA3699 has a significant effect on P*lasR* activity. Moreover, PA3699 is annotated as a probable transcriptional regulator (www.pseudomonas.com) for which no data on its physiological role has been reported in the letterature. Even if the PA3699 function was not investigated before, the crystal structure of the protein has been determined and deposited in the Protein Data Bank (PDB; www.rcsb.org/pdb; accession number, 3KKD) by Minor and co-workers in 2009. According to its structural features, the PA3699 protein belongs to the TetR-family of transcriptional regulators. This group of transcriptional regulators typically activated upon the binding of a specific ligand to an allosteric site (Ramos *et al*., 2005). Thus, it is likely that when expressed at a physiological level, PA3699 could regulate *lasR* transcription only in the presence of a specific ligand. Considering that the interaction of a transcriptional regulator with its DNA target sequence is governed by a thermodynamic equilibrium between the bound and unbound form, the over-expression of PA3699 could shift this equilibrium to the bound state, resulting in P*lasR* regulation also in the absence of the actual stimulus/ligand. In our laboratory experiments are currently in progress to identify the cultural conditions leading to PA3699 full activity/expression.

3.3.4 PA3699 represses *lasR* expression

As reported in figure 18, PA3699 over-expression (growth in LB medium supplemented with 0.1 % L-arabinose) strongly repressed P*lasR* activity during the whole growth curve, without affecting cell growth. Moreover, as reported in figure 19 in which the maximal P*lasR* promoter activity is reported, the repression exerted by PA3699 on P*lasR* transcription was proportional to the amount of L-arabinose present in the growth medium.

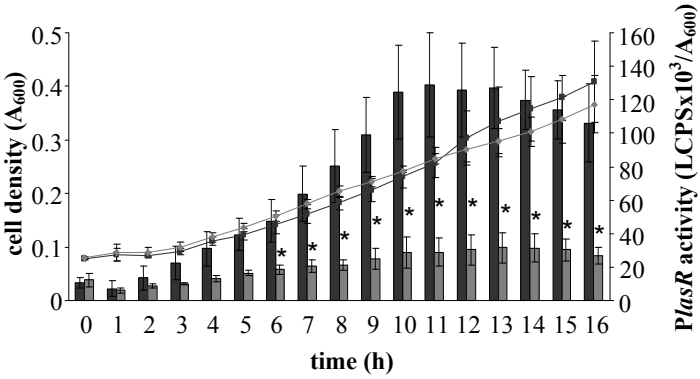

Figure 18. Graph representing cell density (lines) and P*lasR* promoter activity (histograms) in *P. aeruginosa* PAO1 P*lasR*::*lucCDABE* carrying pHERD (dark grey line and dark grey bars) or pR3699 (light grey line and light grey bars). Strains were grown in LB broth supplemented with 0.1 % (w/v) L-arabinose. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to *P. aeruginosa* PAO1 P*lasR::luxCDABE* (pHERD) is indicated with one asterisk (* = p<0.01).

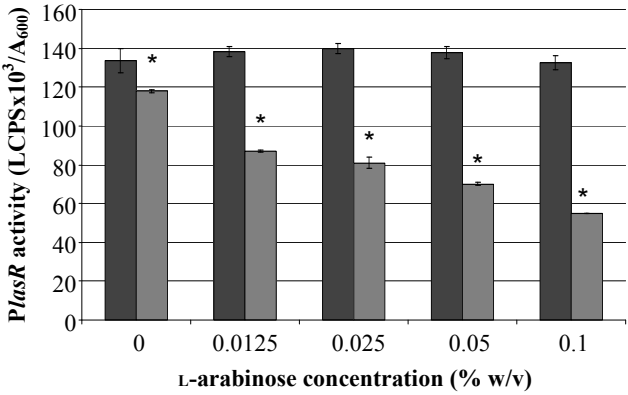

Figure 19. Histogram representing P*lasR* maximal promoter activity measured in *P. aeruginosa* PAO1 P*lasR*::*lucCDABE* strains carrying pHERD (dark grey bars) or pR3699 (light grey bars) grown in LB broth supplemented with different L-arabinose concentrations (%, w/v), as indicated in the figure. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to *P. aeruginosa* PAO1 P*lasR::luxCDABE* (pHERD) is indicated with one asterisk (* = p<0.01).

Since PA3699 was identified by the DNA-affinity chromatography method, it likely represses *lasR* expression by the direct binding to the *lasR* promoter region. In order to strengthen this hypothesis, the effect of PA3699 on *lasR* transcription was also tested in *E. coli*. The pR3699 plasmid, over-expressing PA3699 protein was introduced in *E. coli* MC4100 carrying the pMP*lasR* plasmid. In this plasmid P*lasR* controls the expression of the reporter gene *lacZ*, that encodes the enzyme β-galactosidase; therefore the production of β-galactosidase will be dependent on the promoter activity of P*lasR*. β-galactosidase activity in bacterial cells can be easily measured by a simple biochemical assay (see "Materials and methods" section for further details). *E. coli* MC4100 is a strain in which the own *lacZ* gene is completely deleted, thus the quantity of β-galactosidase measured correspond to that produced by pMP*lasR*. *E. coli* MC4100 carrying both pR3699 and pMP*lasR* was grown at 37°C in LB supplemented with 0.1 % L-arabinose, in order to induce the over-expression of PA3699. As a control *E. coli* MC4100 carrying both pHERD empty plasmid and pMP*lasR* was used. Samples were withdrawn every 2 hours during the entire growth curve and β-galactosidase activity was measured (Miller, 1972). Results showed that PA3699 expression represses P*lasR* activity along the whole growth curve (data not shown). As shown in figure 20, P*lasR* maximal activity was decreased in *E. coli* over-expressing PA3699 in comparison with the activity in *E. coli* carrying pHERD empty plasmid. This result strongly supports the direct regulation of PA3699 on *lasR* expression.

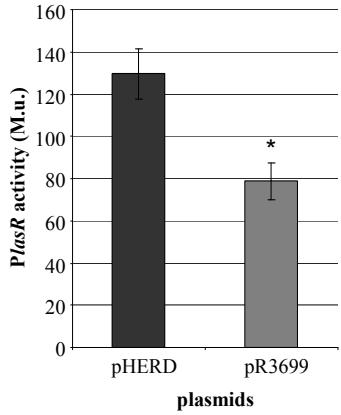

Figure 20. Histogram representing P*lasR* maximal promoter activity measured in *E. coli* MC4100 pMP*lasR* strains carrying pHERD (dark grey bar) or pR3699 (light grey bar) grown in LB broth supplemented with different 0.1 % L-arabinose. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to *E. coli* MC4100 pMP*lasR* pHERD is indicated with one asterisk (* = $p < 0.01$).

3.3.5 Purified PA3699 binds the P*lasR* promoter region

Once demonstrated that PA3699 over-expression represses *lasR* transcription, the protein has been purified to investigate its molecular mechanism of action.

To this aim, the PA3699 gene was cloned in the expression plasmid pET-28b(+) (Novagen) in frame with a sequence coding for a six histidine tag (6xHis) at the 5'-terminus of the gene. The resulting pE3699-N6 plasmid, that allows expression of PA3699 with the 6xHis tag at the *N*-terminal domain of the protein (PA3699-N6), was transformed in *E. coli* BL21 (DE3, pLysS), the election host for over-expression of heterologous proteins with pET-based plasmids (Novagen).

As a first step, different cultural conditions (composition of the medium, temperature and induction time) were tested for their effect on PA3699-N6 expression and solubility. This preliminary analysis revealed that PA3699-N6 was maximally expressed in the soluble fraction of the protein crude extract when cells were grown in LB medium supplemented 0.2 % (w/v) glucose at 37°C, and induced with 0.5 mM IPTG at a cell density of 0.8 (absorbance of the culture at 600 nm wavelength) for 4 hours (data not shown). PA3699-N6 was over-expressed in these conditions in a 250 ml culture, and subsequently purified from the protein soluble fraction by nickel-nitrilotriacetic acid (Ni-NTA)-affinity chromatography, exploiting the high affinity of the 6xHis tag for nickel resin (for more details see Materials and Methods).

The pET28b(+) plasmid carries a sequences encoding thrombin cleavage site between the 5' tag and the target sequence, enabling optional removal of the 6xHis tag after purification. In order to avoid problems due to the presence of the 6xHis tag at the *N*-terminal domain of the protein, the 6xHis tag was subsequently cleaved by thrombin digestion.

As shown in the SDS-PAGE reported in figure 21A, a protein with a molecular weight consistent with PA3699-N6, ~ 26 kDa, was over-expressed in induced conditions (lane 2), and was visible in the protein soluble fraction (lane 3); when purified, this protein was virtually free from contaminants (lane 4).The cleaved protein showed a decreased molecular weight with respect of the purified protein with 6xHis (lane 5). The Western-blot analysis shown in figure 21B, performed with anti-6xHis primary antibody and peroxidase-conjugated secondary antiboby, confirmed the identity of the purified protein as PA3699-N6 and the absence of the tag in the cleaved protein.

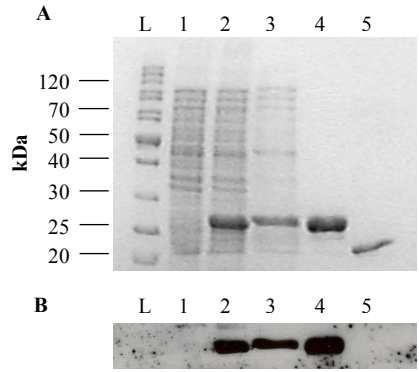

Figure 21. (A) SDS-PAGE analysis of samples withdrawn at different steps of PA3699-N6 purification. Lane L, PageRuler Unstained Protein Ladder (Fermentas): lane 1, non-induced protein crude extract; lane 2, induced protein crude extract; lane 3, soluble fraction of the induced protein crude extract; lane 4, purified protein; line 5, purified protein after thrombin cleaveage. The molecular weight of same proteins of the ladder is reported on the left as 10^3 Dalton (kDa). (B) Western blot analysis performed with anti-6xHis primary antibody and peroxidase-conjugated secondary antiboby on a gel identical to the SDS-PAGE shown in (A).

The DNA-binding properties of purified PA3699 were investigated by means of electrophoretic motility shift assay (EMSA) using a DNA probe (P*lasR* probe) encompassing the central promoter region of *lasR* (from nucleotides -359 to -150 relative to the *lasR* start codon). The EMSA is a technique based on the lower electrophoteric motility on a native polyacrylamide gel of a DNA/protein complex with respect to the free DNA probe. The DNA probe containing the P*lasR* promoter was labelled by fill-in with the Klenow enzyme and deossi-adenosine triphosphate labelled with radioactive ^{32}P([α-^{32}P]dATP) for rapid and high-sensitive detection, and run on a native polyacrylamide gel after incubation with different concentrations of purified PA399 (more details are given in Materials and Methods).

As shown in Fig. 19, incubation of the DNA probe with purified PA3699 led to the formation of a complex endowed with lower electrophoretic motility with respect to the free DNA probe. The concentration of PA3699 required to shift the probe was 1.87 μM, and only a faint band was detectable at the same level when using 1.25 μM protein.

In this assay, an unspecific DNA probe was also added as a competitor. The electrophoretic motility of this DNA probe did not change by adding increasing concentrations of the protein, indicating that purified PA3699 was not able to bind this probe and ensuring the specificity of the P*lasR* probe/PA3699 interaction (Fig. 22).

These data clearly demonstrate that PA3699 is able to specifically bind the *lasR* promoter region, showing for the first time that PA3699 is a DNA-binding protein. Moreover, these data strongly support the notion that PA3699 directly represses *lasR* transcription by physically interacting with the *lasR* promoter.

The over-expression of a transcriptional factor can also lead to the aspecific regulation of certain promoters that are not controlled when the regulator is expressed at physiological levels, generating artifacts. For PA3699 this possibility was ruled out by demonstrating the direct binding of purified PA3699 to the P*lasR* DNA probe in EMSA experiments.

This result strengthens the hypothesis that PA3699 is actually a *lasR* regulator, but the effect of PA3699 mutation on *lasR* expression was not evident in the tested condition. It is possible that also the other regulators identified by the DNA-affinity chromatography are actually QS regulators, but in the tested condition they were not sufficiently expressed and/or active to affect promoter activity in the corresponding mutant.

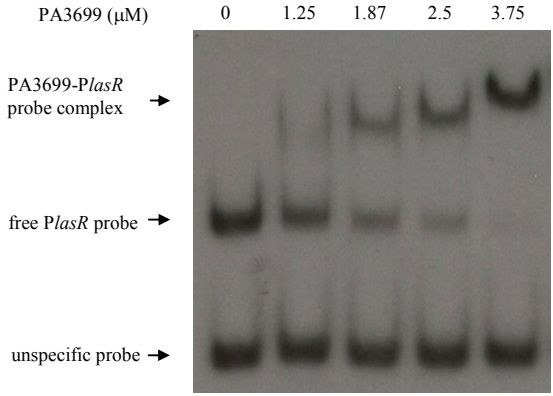

Figure 22. Autoradiography of an EMSA performed with purified PA3699 on the DNA probe encompassing the *lasR* promoter region. PA3699 concentration is indicated; the concentration of the probe was 0.2 nM. Unspecific probe was added at the same concentration. The PA3699/DNA complex and the free DNA probe are indicated.

3.3.6 PA3699 represses pyocyanin production

It was interesting to notice that the *P. aeruginosa* PAO1 P*lasR*::*luxCDABE* strain carrying the pR3699 plasmid had a different colour with respect to the *P. aeruginosa* PAO1 strain carrying pHERD when incubated on *Pseudomonas* Isolation Agar (PIA) plates at 37°C for 1 day. In order to verify that the effect was not related to the presence of the P*lasR*::*luxCDABE* transcriptional fusion

integrated in the chromosome of these strains, the pHERD and pR3699 plasmids were also introduced in *P. aeruginosa* PAO1 wild type. The difference in the colour was still detectable.

As shown in figure 23A, the wild type strain, over-expressing PA3699 was less green then the strain containing the empty plasmid also when PA3699 expression was not induced with L-arabinose.

Pyocyanin production in *P. aeruginosa* PAO1 is controlled by the QS system, since the LasR/3-oxo-C12-HSL complex is required for full expression of some *phzA1* operon, coding for the enzymes involved in pyocyanin biosynthesis (Whiteley *et al*., 1999; Schuster *et al*., 2003). Since pyocyanin is a green-blue pigment, we investigated the effect of PA3699 over-expression on pyocyanin production. To this aim, pyocyanin was extracted from culture supernatants of *P. aeruginosa* PAO1 wild type, ΔPA3699 mutant and ΔPA3699 complemented with pR3699 plasmid grown in LB broth,and of *P. aeruginosa* PAO1 strains carrying the pHERD or pR3699 plasmids, grown LB broth supplemented with 0.1 % L-arabinose to induce PA3699 over-expression.

As shown in figure 23B, pyocyanin extracted was 40 % increased in the ΔPA3699 mutant in comparison with the wild type strain and it was merely detectable from the strain carrying the pR3699 plasmid in comparison with the strain carrying the pHERD empty plasmid. Also in the mutant complemented by pR3699 pyocyanin production was strongly decreased in comparison with the wild type, indicating that, also without inducing the over-expression of the protein, the multiple copies of PA3699 are sufficient to complement and overcome the PA3699 mutation .

This data identify PA3699 as a strong repressor of pyocyanin production. The repression exerted by PA3699 on pyocyanin production could be ascribable to an indirect effect mediated by the reduced transcription of the *lasR* gene. However, the effect of PA3699 over-expression on P*lasR* promoter activity is not as strong as the effect on pyocyanin production. In fact, ΔPA3699 produces 40 % more pyocyanin with respect to the parental strain in LB medium, while the P*lasR* activity in the ΔPA3699 was not affected, suggesting that the level of active PA3699 under standard growth conditions is sufficient to repress pyocyanin promoters, but it is not high enough to repress P*lasR*.

These data suggest that the repression exerted by PA3699 on pyocyanin biosynthesis is not only mediated by LasR but could directly be exerted on *phz* genes. This hypothesis is supported by the observation that the *P. aeruginosa* strain carrying the pR3699 plasmid accumulated a red pigment after 2 days of incubation at 37°C, while in the same conditions the strain carrying the pHERD empty plasmid displayed the typical green pigmentation (Fig. 24A).

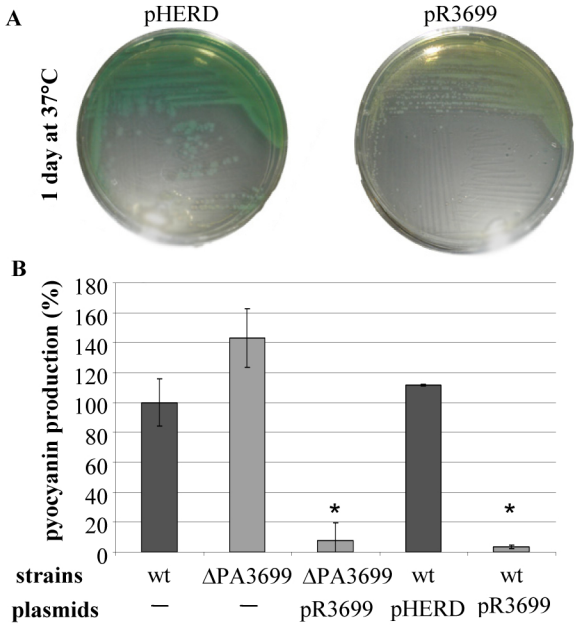

Figure 23. (A) PIA plates with *P. aeruginosa* PAO1 carrying pHERD, on the left, or pR3699, on the right, grown for 1 day at 37°C. (B) Histogram representing pyocyanin production measured in the wild type (wt, dark grey bar), in the ΔPA3699 mutant and in the complemented mutant carrying pR3699 (light grey bars). On the right of the graph there is pyocyanin production in *P. aeruginosa* PAO1 carrying pHERD (dark grey bar) or pR3699 (light grey bar). Pyocyanin production is reported in % with respect to the wild type. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to the wild type is indicated with one asterisk (* = $p < 0.01$).

The red pigment was extracted from culture supernatant and its absorption spectrum was determined. As shown in figure 24B, typical absorption of pyocyanin at 310 nm and 370 nm was observed in the supernatant of the strain carrying pHERD (green line), while only an absorption peak at 370 nm was detected in the supernatant collected from the strain over-expressing PA3699 (red line). The latter spectrum is typical of phenazine-1-caboxylic acid (PCA), a precursor of pyocyanin that is converted to 5-methylphenazine-1-carboxylic acid by the methyltransferase PhzM (Mavrodi *et al*., 2001; Parsons *et al*., 2007).

This result suggests that, among the different factors involved in pyocyanin production, PA3699 repress the expression of PhzM, thus leading to accumulation of PCA precursor.

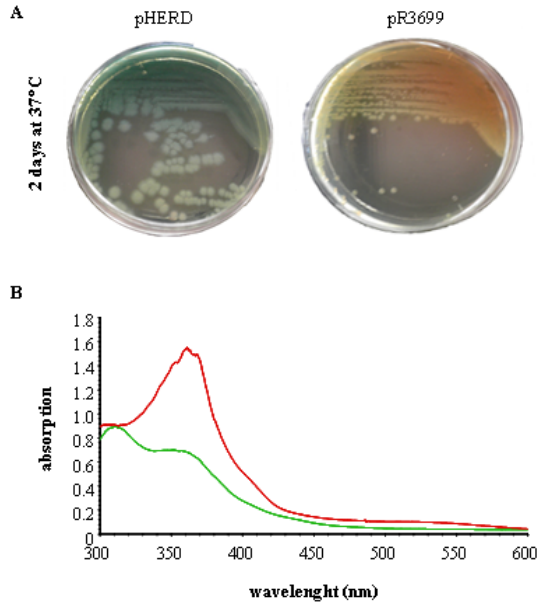

Figure 24. (A) PIA plates with *P. aeruginosa* PAO1 strains carrying pHERD, on the left, or pR3699, on the right, grown for 2 day at 37°C. (B) Graph representing the absportion spectra of the supernatants from *P. aeruginosa* PAO1 carrying pHERD (green line) or pR3699 (red line). Strains were grown in LB broth supplemented with 0.1 % (w/v) L-arabinose.

3.4 Conclusion

In this part of the project, the technique of the DNA-affinity chromatography was used to pick up from *P. aeruginosa* protein crude extracts novel putative regulators of the main *las* and *rhl* QS genes. Fifteen good candidates as QS regulators were identified and mutants of the corresponding genes, as well strains that over-expressed such proteins, were obtained.

The results obtained provide new insights on the role of Vfr, of the histon-like proteins MvaT and MvaU and of the TetR-like protein PA3699.

The *vfr* mutation decreased the P*lasR* activity, confirming the literature data (Albus *et al.*, 1997) and strengthening our experimental approach. In the *vfr* mutant, the decrease in P*lasR* activity did not led to a corresponding decrease in *PlasI* activity (that is strongly dependent by LasR). We showed that there is a clear interplay between Vfr and RsaL and demonstrated that, in the absence of RsaL, Vfr increases *lasI* expression and the production of pyocyanin. It is difficult to understand the physiological conditions that affect Vfr activity since the activity of this regulator is dependent on the availability of cAMP and the real role of this co-

factor in *P. aeruginosa* is not yet established (Collier *et al.*, 1996). Overall, the above results strengthen the notion that RsaL is a master controller of 3-oxo-C12-HSL signal molecule homeostasis; due to RsaL-mediated homeostasis, a change in LasR expression not always correlate to a change in *lasI* transcription, despite its promoter is strongly dependent upon LasR. Considering that 3-oxo-C12-HSL levels in a certain environmental niche depend upon the balance between synthesis and degradation/dilution, a change in LasR expression that is not coupled to a correspondent change in *lasI* expression and 3-oxo-C12-HSL production would provide a very fast way to affect the number of genes actually controlled by LasR, allowing the QS regulon to shrink or enlarge, depending on environmental conditions.

Mutants in the *mvaT* and *mvaU* genes showed a complex regulatory pattern. Our data suggest that both these proteins mainly act by unbalancing the level of LasR and RsaL regulators, which control the homeostasis of the signal molecule, while MvaT can directly affect *lasI* expression.

P. aeruginosa possesses 38 TetR-family proteins and only in seven cases their function is known (Ramos *et al.*, 2005; Morita *et al*., 2006; MacEachran *et al*., 2008; Kazakov *et al*., 2009; Förster-Fromme & Jendrossek , 2010; Ghosh *et al*., 2011). In this experimental work it has been attributed for the first time a function to the TetR-like protein PA3699. We showed that PA3699 is a transcriptional regulator that represses the expression of the *lasR* gene by direct interaction with its promoter region. Moreover, the repressive effect of PA3699 on the biosynthesis of the virulence factor pyocyanin has been demonstrated.

To our knowledge, beside the activator Vfr, only PA3699 has been reported to directly affect *lasR* transcription up to now. The presented data contribute to the understanding of the complex regulatory network underlying QS and virulence in *P. aeruginosa*.

Interestingly, mutations in the majority of the other transcriptional regulators here identified did not cause relevant effects on the promoter activity of the corresponding target genes under standard laboratory growth conditions. However, our results on the PA3699 protein suggest that at least some of these factors could be expressed and/or active only under specific conditions.

The high number of new putative QS regulators individuated and the interplay of RsaL and Vfr in QS regulation could be a good example of the high interconnectivity of a complex regulatory circuit that is able to integrate different stimuli at different hierarchical levels. The integration of several signalling pathways at this level makes sense given the fact that QS regulates hundreds of genes, therefore switching on the QS system is a large commitment for the bacterial cell that must be tightly controlled.

The data presented in this chapter allow to clarify the complex regulatory QS circuit governing *P. aeruginosa* physiology and at the same time they highlight how far is a comprehensive knowledge of their molecular mechanisms and implications.

4. CHAPTER II
Screening for new *P. aeruginosa* quorum sensing inhibitors

4.1 Background and rationale

Although the establishment of *P. aeruginosa* lung infection in CF patients can be delayed by aggressive early antibiotic treatment, eradication of established infection is currently impossible. Moreover, frequent antibiotic courses in chronically infected individuals, besides exerting selective pressure for antibiotic resistance, often results in cumulative toxicity (Frerichs & Smyth 2009).

An innovative approach is the use of drugs able to specifically inhibit the bacterial capability to establish the infection rather than bacterial growth. Targeting of the bacterial pathogenic potential has the advantage of reducing the bacterial adaptability to the host environment and the severity of the infection without creating, in principle, the selective pressure generally caused by conventional antibiotics. Moreover, in the immunocompetent CF patients, the use of virulence inhibitors could provide the host immune system with a better chance of clearing the infection (Cegelski *et al.* 2008).

QS signal molecules can be detected in sputum of CF patients, indicating that these systems are active during *P. aeruginosa* colonization of CF lung (Massai *et al.* 2011). Moreover, as described in the introduction, QS-defective mutants are strongly impaired in pathogenesis in different animal models of infection, indicative of the importance of QS in *P. aeruginosa* pathogenicity (Winstanley & Fothergill 2009). Overall, these data suggest that QS could constitute an ideal target for the development of non conventional therapies.

Researches aimed at identifying inhibitors of the *P. aeruginosa* QS signalling system started several years ago. High-throughput screening of thousands of natural and synthetic compounds has shown that furanone C30 and garlic extracts have QS inhibitor (QSI) activity. Both have been shown to render *P. aeruginosa* biofilms susceptible to antibiotics and to the host immune system, and proved to inhibit chronic *P. aeruginosa* lung infection in mouse models (Hentzer *et al.* 2003; Smyth *et al.* 2010). Due to its high toxicity, furanone C30 has not been tested in humans. Conversely, a pilot study in a small number of CF patients has been undertaken to investigate the effect of garlic oil macerate on chronic *P. aeruginosa* lung infection. Although the results were not statistically significant, a trend towards improvement of patient clinical outcome was observed (Smyth *et al.* 2010). The identification of the QSI compound present in the garlic extract and its use at a physiological-active concentration could probably lead to a further improvement of garlic-based therapy. However, once this compound will be identified and characterized, time-consuming toxicology studies will be necessary prior to clinical trials.

Taken together, the above results support the relevance of QS as an anti-*P. aeruginosa* drug target and encourage the research of novel compounds with high QSI activity and low toxicity for humans.

4.2 Specific aim

The inefficacy of existing therapies in eradicating *P. aeruginosa* chronic infections calls for the identification and development of new anti-QS compounds.

An intelligent and potentially more efficient strategy than high-throughput screening for the development of novel drugs is searching for new side activities in old drugs for which bioavailability and toxicity studies have already been performed and efficacy in humans has been confirmed. This approach has been named SOSA (Selective Optimization of Side Activities; Wermuth, 2006).

A commercial library of drug-like compounds, containing about 1000 molecules with known biological activities, the majority of which being established drug molecules, have been screened with the aim of identifying drugs with novel QSI activity.

4.3 Results and discussion

4.3.1 Screening of a library of FDA-approved compounds

The past researches for QSIs have been mainly focused on searching for inhibitors of the major QS system of *P. aeruginosa,* by using screening systems conceiving the 3-oxo-C12-HSL signal receptor (LasR) as the only target (Persson *et al.*, 2005). In contrast, very few attempts have been made to identify inhibitors of other steps of the *las* regulatory circuit for instance signal synthesis and transport.

The screening system used in this study has been previously developed in our laboratory and is based on the co-cultivation of two *P. aeruginosa* strains: a QS signal producer (*P. aeruginosa* PA14 wild type) and a signal production-defective reporter strain (PA14-R3), which emits bioluminescence in response to 3-oxo-C12-HSL (Massai *et al.*, 2011). The addition of a molecule with QS inhibitory activity towards any process related to 3-oxo-C12-HSL-dependent QS reduces the emission of luminescence by the reporter strain with respect to the control co-culture without any inhibitor added (Fig. 25). This approach has the advantage of allowing the identification of compounds targeting, besides 3-oxo-C12-HSL signaling, any cellular process critical for QS response, including 3-oxo-C12-HSL synthesis and secretion (Massai *et al.* 2011).

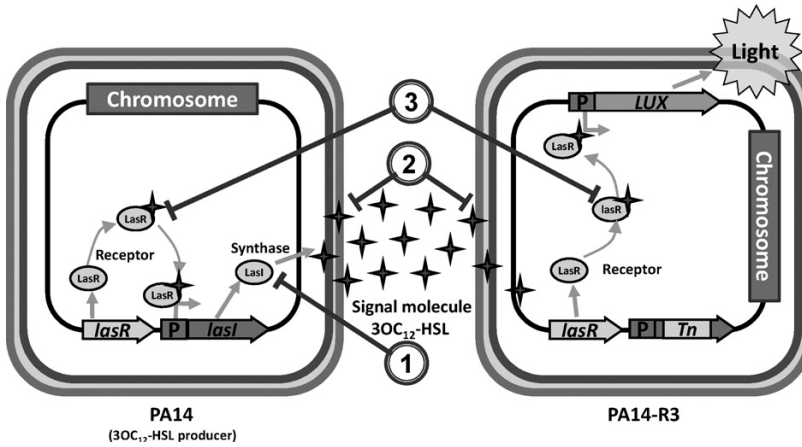

Figure 25. Schematic representation of the PA14/PA14-R3 co-cultivation screening system. The wild type PA14 produces the 3-oxo-C12-HSL signal molecule that in turn activates PA14-R3 bioluminescence emission. Molecules interfering with (1) synthesis of the signal molecule, (2) transport of the signal molecule across the cell envelope, and (3) activity of the signal molecule receptor will cause a reduction of luminescence with respect to the untreated control (Massai *et al.* 2011).

About 1000 chemical compounds with known biological activities of a commercial library were added to the co-culture at 100, 10, 1 μg/ml final concentration in microtiter plates. The bioluminescence emitted by the latter co-cultures were compared with a control co-culture without any compound added.

Seven compounds produced more than 50 % reduction of bioluminescence, but less than 25 % variation of growth with respect to the control sample. These compounds are listed in table 2 as probable QSIs. The "inhibitory concentration $_{50}$" (IC_{50}) is considered the minimum concentration needed to obtain an inhibition of bioluminescence of 50 % and it was calculated on the reduction rate of bioluminescence in the samples after the addition of the compounds (Tab. 2).

The assay was repeated for the seven compounds, obtaining the same result.

Four of the selected compounds were antibiotics (Tab. 2) and at the concentrations tested, they did not affect *P. aeruginosa* growth, confirming that sub-inhibitory antibiotic concentrations can affect QS (Skindersoe *et al.*, 2008). The anti-parasitic drug (compound QSI-1) showed the highest QSI activity and it is of very low toxicity to humans.

A patent application for compound QSI-1 is in preparation. For this reason the name of the chemical library screened in this study, as well as the identity of the identified compounds cannot be revealed. The patent will likely be deposited within few months (early 2012) and a study will be published afterwards.

Table 2. Compounds identified as putative QSI.

Compound	IC_{50} (μM)	Properties
QSI-1	10	Anti-parasitic drug
QSI-2	20	Aminoglycoside antibiotic
QSI-3	150	Antineoplastic agent
QSI-4	50	Bactericidal antibiotic of the rifamycin group
QSI-5	50	Macrolide glycopeptide antibiotic
QSI-6	60	Ammonium salt from the group of benzylisoquinoline alkaloids
QSI-7	10	Bactericidal antibiotic of the rifamycin group

4.4 Conclusion

Past studies demonstrated that QS inhibitory compounds were effective in *P. aeruginosa* clearance and in reducing mortality in animal models of pulmonary infection (Rasmussen & Givskov, 2006).

With the selective optimization of side activities of drug molecules (the SOSA approach) hold drugs, which use in humans has already been approved, could be screened for side activities of interest. Once the screening has generated a hit, this could be either tested directly in clinical studies or used as the starting point for a drug optimization program. This strategy has a high probability of yielding safe and bioavailable drug-like compounds, and it is thus expected to reduce the time and the cost generally associated with standard drug discovery processes. The suitability of the SOSA approach for the development of novel pharmaceutically active compounds has been extensively proven in the literature (Wermuth 2006).

By screening thousand of FDA-approved drugs, seven new QSIs were discovered. Next purpose will be the functional characterization of these compounds, starting with QSI-1. These studies will make it possible to assess the feasibility of the identified QSI compounds as anti-*Pseudomonas* drugs. Given the high probability of yielding safe and bioavailable drug-like compounds, this result is an invaluable contribute to put the basis for the development of new anti-*Pseudomonas* drugs.

5. CHAPTER III
Deciphering the role of RND efflux transporters in *B. cenocepacia*

5.1 Background, rationale and aims

Antimicrobial therapy against *B. cepacia* complex (Bcc) infections is often ineffective since Bcc members are highly resistant to most clinically relevant antimicrobial agents and disinfectants (Peeters *et al.*, 2009).

As described in the introduction, a very important role in the resistance of *B. cepacia* complex is played by the RND transporters.

In the sequenced genome of the multidrug-resistant CF isolate *B. cenocepacia* J2315 sixteen operons encoding RND efflux pumps were identified and named RND-1 to RND-16 (Fig. 26; Buroni *et al.*, 2009).

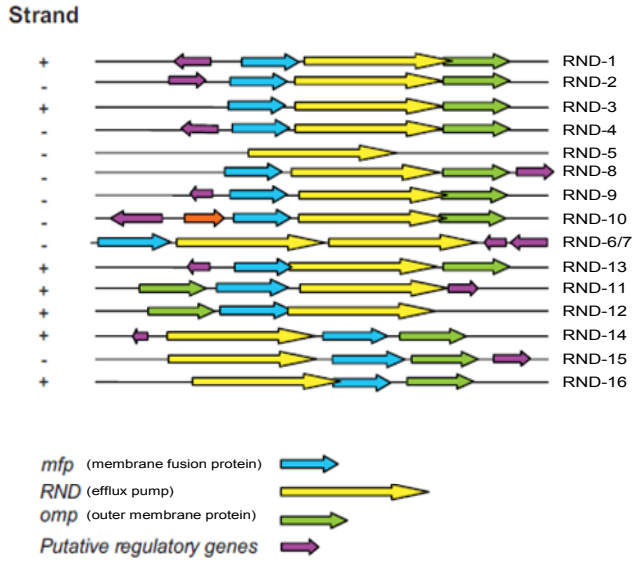

Figure 26. Schematic representations of the organization of the 16 gene clusters encoding RND efflux pumps in *B. cenocepacia* J2315 genome**.** RND transporter-encoding genes are depicted as yellow arrows (RND), outer-membrane protein-encoding genes as green arrows (*omp*), periplasmatic membrane fusion protein-encoding genes as pale blue (*mfp*), and putative regulatory genes (physically linked) as pink arrows (modified from Perrin *et al.*, 2010 and Buroni *et al.*, 2009).

Several studies carried out on different Gram negative bacteria suggest that, beside the active efflux of exogenous toxic compounds (e.g. antibiotics), RND efflux systems play important roles in bacterial pathogenesis, participating in colonization and persistence of bacteria in the host, as well as in metal ion homeostasis (Piddock, 2006; Ma *et al.*, 2009).

The significance of RND efflux systems in *B. cenocepacia*, their role in the infection related processes and the complex transcriptional networks in which they are inserted are still largely unexplored.

In order to give new insights in RND efflux systems functions and in *B. cenocepacia* pathogenesis, the role of the RND efflux systems not only in antibiotic resistance but also in other metabolic pathways, including those relevant for pathogenesis was investigate by comparing the transcriptome of *rnd* mutants with that of the wild type strain using microarray analysis (Bazzini *et al.*, 2011).

To this aim, the two operons encoding the putative RND-4 and RND-9 transporters (Fig. 26) were deleted in *B. cenocepacia* J2315, generating the corresponding mutants strains D4, D9 and D4-D9. RND-4 and RND-9 have been selected among the other RND systems of *B. cenocepacia* J2315 because they could be relevant in the CF infection. Indeed, the RND-4 transporter confers resistance to a wide range of antibiotics (Buroni *et al.*, 2009), while the RND-9 operon is over-expressed in the sputum of CF patients (Drevinek *et al.*, 2008). Moreover, the RND-9 whole operon shares significant amino acid identity with the MexEF-OprN efflux system of *P. aeruginosa* (Köhler *et al.*, 2001; Poole, 2001).

The microarray analyses showed that, in comparison to the wild type strain, 216, 168 and 550 genes were differentially expressed in the D4, D9 and D4-D9 mutants, respectively. The majority of the differentially expressed genes belonged to a wide range of different functional classes. However, a significant proportion of such genes was involved in flagellar-dependent motility and chemotaxis (Fig. S1 and Tab. S1 in Bazzini *et al.*, 2011). In particular, the D4 mutant over-expressed 38 flagellum-related genes and 17 chemotaxis-related genes. Within these two groups, 26 and 14 genes were also up-regulated in the D4-D9 mutant, respectively (Tab. 4 in Bazzini *et al.*, 2011). Conversely, the D9 mutant showed 10 flagellum-related genes and 4 chemotaxis-related genes in the down-regulated gene list (Tab. 5 in Bazzini *et al.*, 2011). Although the overall validity of the microarray analysis was confirmed by real time PCR analysis (Bazzini *et al.*, 2011), the specific aim of this part of the experimental work was to validate the microarray results through phenotypic assays.

5.2 Results and discussion

5.2.1 *rnd* mutation affects *B. cenocepacia* motility and biofilm

Transcriptomic profiles of the three mutants suggested that the RND-4 and RND-9 efflux pumps could play opposite roles in flagellum-dependent functions, like swimming and chemotaxis. To assess this hypothesis, these phenotypes were analyzed in the wild type and in the RND-mutated strains.

Swimming, a flagellum-mediated motility, was evaluated inoculating bacteria with a toothpick in a Petri dish containing a growth medium with a low percentage of agar. In this condition, bacteria can swim through the soft agar and produce an halo. The diameter of the halo produced by the four analyzed strains is a measure of their ability to swim (Fig. 27).

Results showed that the D4 and D4-D9 mutants had enhanced swimming motility with respect to the wild type, while the D9 mutant was less motile than the wild type (Fig. 27). These data are in full agreement with the microarray analysis, showing that flagellum-related genes are up-regulated in the D4 and D4-D9 mutants and down-regulated in the D9 mutant. Moreover, D4 has 12 more up-regulated genes involved in motility than D4-D9, this could be an explanation to the fact that this mutant is slightly more motile than the double mutant D4-D9 (Fig. 27).

To the best of our knowledge, this is the second time that the effect of RND efflux pumps mutation on motility-related phenotypes has been described in the literature. Indeed, the absence of RND components, such as AcrB, in *Salmonella enterica* caused widespread repression motility genes in the mutant and this was associated with decreased motility (Webber *et al.*, 2009).

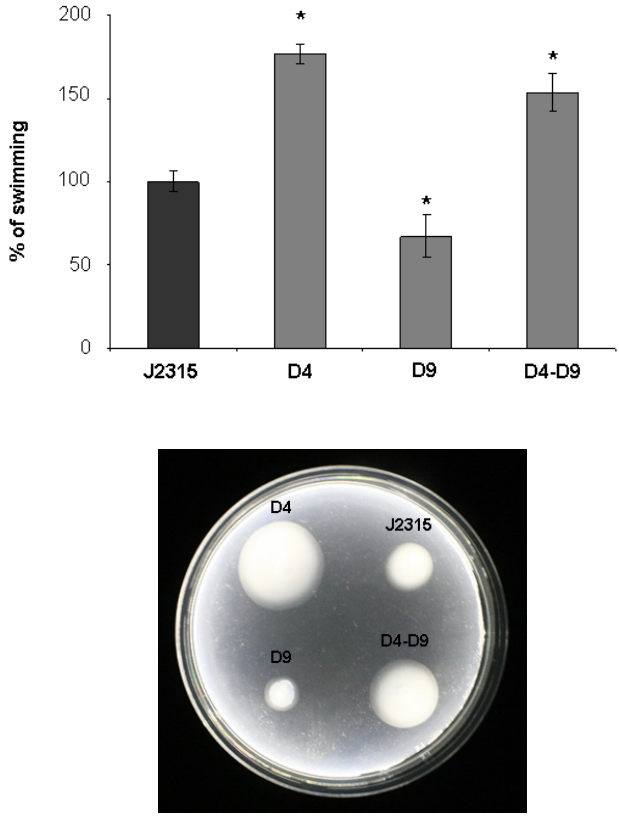

Figure 27. Histogram representing swimming halos measured in the wild type (J2315) and in the D4, D9, D4-D9 mutants. Swimming halos are reported in % with respect to the wild type. The average of three independent experiments is reported with standard deviations. Statistical significance with respect to the wild type is indicated with one asterisk (* = $p<0.01$). The photo below the graph shows one representative experiment.

Concerning chemotaxis, preliminary experiments using different attractant/repellents were performed. The three mutants and the wild type were grown 18 hours in presence of casaminoacids and LB as attractants and aztreonam and chloramphenicol as repellents. Despite the differences observed in the microarray analysis, the three mutants and the wild type showed the same positive chemotactic phenotype versus the used attractants and absence of chemotactic response versus the used repellents (data not shown). However it is possible that differences in chemotaxis might be appreciated by the use of specific attractant or repellent molecules.

It is known that in many bacteria flagella could play a role also in adhesion and biofilm formation (Anderson *et al.*, 2010). Therefore, an investigation about the ability of the four strains to produce biofilm was performed by using two standard methods: adhesion to polyvinyl chloride (PVC) microplates and Congo red binding (details are given in Materials and Methods). The two methods gave comparable results and, surprisingly, demonstrated that all the mutants showed enhanced biofilm formation, with respect to the wild type (Fig. 28).

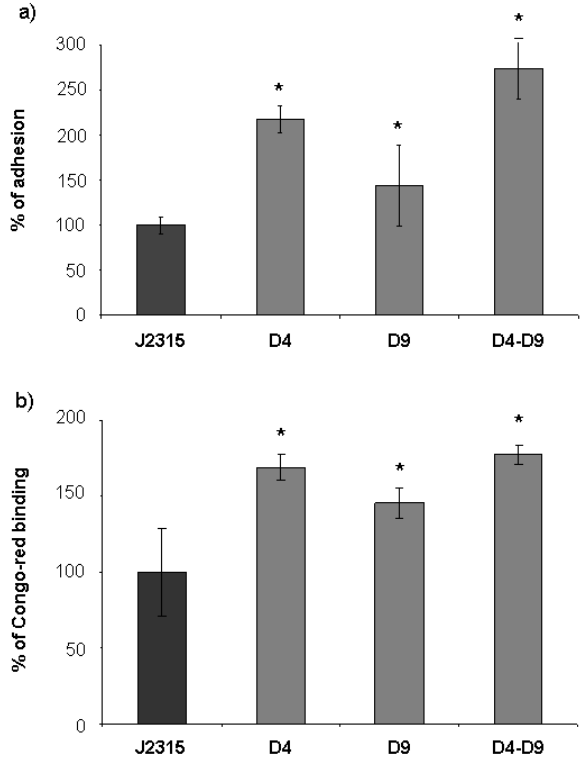

Figure 28. (A) Adhesion to polyvinyl chloride mitrotiter plates measured by crystal violet staining. (B) Congo red dye binding ability. In both cases, results are given as a percentage, considering *B. cenocepacia* wild type (J2315) as 100%. The mean of three different experiments with standard deviation is reported. Statistical significance with respect to the wild type is indicated with one asterisk (* = $p<0.01$).

The above results indicate that differences in flagella expression in the D4 and D9 strains, with respect to the wild type, play a minor role in biofilm formation, at least in these experimental conditions. However the biofilm experiment also showed that D9 produces less biofilm than D4 and D4-D9 and this difference might be explained, at least in part, by the up-regulation of flagellar-related genes in the D4 and D4-D9 mutants (Tab. 4 in Bazzini *et al.*, 2011).

Biofilm formation is a complex pleiotropic phenotype, strongly dependent upon experimental conditions and growth media (Coenye, 2010a and 2010b). Therefore, it is not easy to correlate the microarray data derived from planktonic cultures with the increased biofilm production of the RND-mutants, with respect to the wild type. Actually, the increased biofilm production of the three RND-mutants was unexpected since it is not possible to identify genes obviously involved in biofilm formation among the 33 having the same expression pattern in the three microarray experiments (Tab. S1 in Bazzini *et al.*, 2011). However, 19 out of the 24 genes up-regulated in all the microarray experiments, are phage-related genes. Over-expression of phage-related genes in sessile cells compared with planktonic cells and/or increased expression in response to stress has been observed in several species. Bacterial stress response can increase the mobility of bacteriophages, and it has been proposed that prophage production may play a role in generating genetic diversity in the biofilm (Coenye, 2010a). It is tempting to speculate that cytoplasmic accumulation of toxic metabolites and/or metabolic signals due to the lack of RND-4 and/or RND-9 efflux pumps could produce a general stress response triggering the expression of genes involved in biofilm formation. Therefore it is conceivable that the cytoplasmic accumulation of efflux pump-specific metabolites (different for each mutant) could act as signals triggering opposite behavioural response in the two mutants. For instance, the D4 mutant was previously showed to accumulated 30% less QS signal molecules in the medium as compared to J2315 (Buroni *et al.*, 2009).

In order to determine if also the inactivation of RND-9 efflux pump may affect the transport of QS signal molecules, the accumulation of this molecules in the D9 and D4-D9 mutants was evaluated. Acyl-HSLs production was measured using a biosensor. Briefly *E. coli* DH5α carrying the plasmid pSCR1, in which *lacZ* is under the control of a acyl-HSLs responsive *B. cenocepacia* promoter, was used to determine β-galactosidase activity in the presence of culture supernatants derived from J2315 wild type and D4, D9 and D4-D9 mutants.

Results confirmed previous data showing that the D4 mutant accumulates in the growth medium less acyl-HSLs than the wild type (Buroni *et al.*, 2009), moreover, a reduction of acyl-HSLs concentration was observed also in the supernatant derived from the D4-D9 double mutant. On the contrary, the amount of acyl-HSLs in supernatant derived from strain D9 did not differ from the parental strain (Fig. 29).

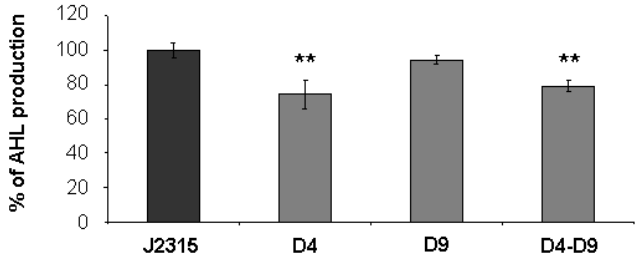

Figure 29. Values of acyl-HSLs accumulated in the supernatant of the indicated strains are in percentage in relation to the *B. cenocepacia* wild type (J2315). The mean of three different experiments with standard deviation is reported. Statistical significance with respect to the wild type is indicated with two asterisks (** = $p<0.05$).

In accordance with the low impact of D4 and D9 mutations on acyl-HSLs production, only few genes known to be acyl-HSL regulated were also differentially regulated in the microarray analysis (Tab. S9 in Bazzini *et al.*, 2011).

Overall, these observations suggest that it is unlikely that the phenotype of the D4, D9 and D4-D9 mutants is due to an unbalance in acyl-HSLs import/export rates. However, the results of this experiments support the hypothesis that other molecules acting as metabolic signals or constituting the biofilm matrix could accumulate in the D4, D9 and D4-D9 mutants and account for the motility and biofilm phenotypes of these strains. This finding stimulates future studies on the role played by RND pumps in the efflux of endogenously produced molecules potentially involved in virulence and host colonization (e.g. biofilm matrix components, biologically active secondary metabolites, signal molecules), besides their role in drug resistance.

5.3 Conclusion

It is well established that bacterial multidrug-resistance efflux pumps can confer clinically relevant resistance to antibiotics, but it is now understood that these efflux pumps also have a role(s) in bacterial physiology. Some efflux pumps of RND family have been shown to have a role in the colonization and the persistence of bacteria in the host (Piddock, 2006). For these reasons, understanding the role of RND efflux transporters is fundamental to highlight their involvement in functions different from drug resistance. Moreover, since many companies are developing Efflux Pumps Inhibitors (EPIs) as antibiotic therapy adjuvant, this knowledge is very important in this ongoing area of drug development.

Here, by integrating transcriptomics and a set of different phenotypic assays, the knowledge on the role of the clinically important RND protein family was

expanded. In particular, the RND-4 and RND-9 pumps were shown for the first time to be involved in motility and biofilm formation of *B. cenocepacia* J2315. The opposite impact of RND-4 and RND-9 on motility showed that the inactivation of distinct RND systems can have a different effect on a specific virulence-related phenotype. This is one of the few cases in the literature in which RND transporters have been characterized for their participation to bacterial processes different from antibiotic and toxic compounds resistance.

A mutation in the RND-4 pump enhanced motility and biofilm formation, while a mutation in the RND-9 pump enhanced biofilm formation, but inhibited swimming motility. If this is true also in the host, the use of EPIs could be, on one side positive for helping the antibiotic therapy, on the other side, it could promote biofilm formation and thus chronic infection, raising serious concerns on the use of EPIs in therapy.

The results of this study have been published in Bazzini *et al.*, 2011.

LIST OF ABBREVIATIONS

[α-^{32}P]ATP: adenosine triphosphate labelled with ^{32}P in alfa position
3-oxo-C12-HSL: *N*-3-oxo-dodecanoyl-homoserin lactone
3-oxo-C6-HSL: 3-oxohexanoyl-homoserine lactone
6×His: six histidine tag
A_{600}: absorbance at 600 nm
acyl-HSL: acyl-homoserin lactone
ADP: adenosine diphosphate
ATP: adenosine-5'-triphosphate
Bcc: *Burkholderia cepacia* complex
bp: base pair
C_4-HSL: *N*-butyryl-homoserine lactone
C6-HSL: *N*-hexanoyl-L-homoserine lactone
C8-HSL: *N*-octanoyl-L-homoserine lactone
cAMP: cyclic adenosine monophosphate
CF: cystic fibrosis
CFTR: cystic fibrosis transmembrane conductance regulator
CRP: cAMP receptor protein
DNA: deoxyribonucleic acid
EMSA: electrophoretic mobility shift assay
EPI: efflux pumps inhibitor
EPS: extracellular polymeric substances
HHQ: 2-heptyl-4-quinolone
H-NS: histon-like nucleoid structuring
IC_{50}: inhibitory concentration $_{50}$
IPTG: isopropyl-β-D-thiogalactopyranoside
LB: Luria-Bertani broth
LPS: lipopolysaccharide
MDR: multi-drug resistance
Mfp: membrane fusion protein
Omp: outer membrane protein
ORF: open reading frame
PCA: phenazine-1-caboxylic acid
PDB: protein data bank
PIA: *Pseudomonas* isolation agar
P*lasI*: promoter of the *lasI* gene
P*lasR*: promoter of the *lasR* gene
PQS: 2-heptyl-3-hydroxy-4-quinolone
P*qscR*: promoter of the *qscR* gene
P*rhlI*: promoter of the *rhlI* gene
P*rhlR*: promoter of the *rsaL* gene

P*rsaL*:	promoter of the *rsaL* gene
P*rsaL-lasI*:	bidirectional promoter of *rsaL-lasI* genes
PVC:	polyvinyl chloride
QS:	quorum sensing
QSI:	quorum sensing inhibitor
RND:	resistance-nodulation-cell division
SDS-PAGE:	Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis
SOSA:	Selective Optimization of Side Activities
TLR5:	Toll-like receptor 5
WT:	wild type

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