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Quorum sensing and pyoverdine signaling: possible targets for the development of inhibitors of *Pseudomonas aeruginosa* virulence

"Quorum sensing" e "pyoverdine signaling": possibili bersagli per lo sviluppo di inibitori della virulenza di *Pseudomonas aeruginosa*

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The only explanation I can think of is that a multitude of the bacteria are stronger than a few, and thus by union are able to overcome obstacles too great for the few.

[Smith 1905]

RIASSUNTO

Introduzione

Pseudomonas aeruginosa è un batterio Gram-negativo, aerobio, dotato di grande versatilità metabolica e capacità adattativa. Nonostante sia stato isolato in ecosistemi terrestri, marini, fluviali e lacustri, oltre che in associazione con organismi superiori, *P. aeruginosa* è soprattutto noto come patogeno opportunista umano in quanto agente di infezioni in pazienti ospedalizzati e immunocompromessi. In particolare, l'infezione polmonare cronica causata da *P. aeruginosa* è la principale causa di morbosità e mortalità nei soggetti affetti da Fibrosi Cistica (FC), malattia genetica ereditaria che colpisce circa 1 nato su 3.000. La capacità di questo organismo di adattarsi all'ambiente offerto dall'ospite e causare infezioni è probabilmente la risultante della produzione di un gran numero di fattori di virulenza e dei meccanismi di resistenza alle difese immunitarie ed agli agenti antimicrobici. In alcuni processi infettivi i batteri possono costituire delle comunità ed il loro comportamento sociale può influenzare l'espressione dei geni di virulenza. Il sistema di "quorum sensing" (QS) ed il sistema del "pyoverdine signaling" sono dei meccanismi di regolazione globale dell'espressione genica che controllano la capacità di adattamento di *P. aeruginosa* nell'ospite. Il QS è un sistema di comunicazione cellula-cellula dipendente dalla densità cellulare, basato sulla produzione e secrezione di una specifica molecola segnale che si accumula nell'ambiente. Raggiunta una concentrazione soglia, proporzionale alla densità della popolazione batterica, il legame di questa molecola ad un recettore citoplasmatico con funzione regolatrice determina l'attivazione della trascrizione di geni bersaglio, inclusi geni per la biosintesi della molecola segnale stessa, generando così un circuito di autoinduzione. *P. aeruginosa* possiede tre sistemi di QS, *las*, *rhl* e *pqs*, ciascuno caratterizzato da una tipica molecola segnale, C_4-HSL, $3OC_{12}$-HSL e PQS, rispettivamente (Fig. 7), e organizzati in maniera gerarchica, con il sistema *las* al vertice (Fig. 6). Questi sistemi di QS regolano circa il 10% dei geni di *P. aeruginosa*, fra cui numerosi geni di virulenza. La pioverdina (Fig. 4) è un sideroforo, ovvero un chelante batterico del ferro, capace di veicolare tale metallo essenziale dall'ambiente extracellulare all'interno della cellula. La pioverdina agisce anche come molecola segnale all'interno di in un circuito di segnalazione, attivato mediante il legame della ferri-pioverdina con il suo recettore di

membrana esterna FpvA. Questa interazione dà luogo alla trasmissione di un segnale al fattore antisigma FpvR, localizzato nella membrana citoplasmatica, con la conseguente attivazione del fattore sigma alternativo PvdS, in grado di interagire con la RNA polimerasi ed attivare la trascrizione di vari geni bersaglio, fra i quali geni di virulenza e geni per la biosintesi della pioverdina stessa (Fig. 5).

Obiettivi della ricerca

P. aeruginosa rappresenta un bersaglio impegnativo per lo sviluppo di nuovi trattamenti anti-infettivi, in quanto è in grado di produrre un arsenale di fattori di virulenza e integrare differenti meccanismi di resistenza. Sotto la pressione selettiva esercitata dai trattamenti tradizionali, basati su farmaci che inibiscono la crescita, *P. aeruginosa* è in grado di sviluppare resistenze, rendendo necessaria la ricerca di nuovi bersagli e stategie per contenere o eliminare le infezioni sostenute da questo batterio patogeno. Lo sviluppo di farmaci in grado di inibire specifici determinanti di virulenza, piuttosto che inibire la crescita, potrebbe rivelarsi una efficace alternativa/integrazione ai trattamenti convenzionali. Questi farmaci avrebbero il duplice vantaggio di ridurre l'adattabilità del patogeno all'ambiente dell'ospite, favorendo così il sistema immunitario nel combattere l'infezione, e di prevenire l'emergere di ceppi resistenti[1]. I sistemi del QS e del "pyoverdine signalling" sono necessari per l'espressione ottimale di fattori di virulenza[2,3] e per la produzione di biofilm[4,5], e pertanto rappresentano promettenti bersagli per una terapia farmacologica basata sull'inibizione della virulenza.

Gli obiettivi primari del mio dottorato sono stati la messa a punto, la validazione e l'utilizzo di nuovi sistemi di screening ad alta processività per l'identificazione di molecole in grado di inibire il QS ed il "pyoverdine signalling" in *P. aeruginosa*. Nella prima parte dello studio sono stati sviluppati dei biosensori per la determinazione della risposta batterica al QS ed al "pyoverdine signalling". Tali biosensori sono stati impiegati nello screening di una libreria chimica composta da farmaci commercializzati, selezionati per la diversità delle loro proprietà chimiche e farmacologiche. Lo screening ha reso possibile l'identificazione di due composti non tossici, attivi rispettivamente contro il QS e il "pyoverdine signalling" a concentrazioni biologicamente significative. Questi composti sono stati saggiati per determinarne l'impatto sulla produzione di molecole segnale e fattori di virulenza, e successivamente ne è stata verificata l'efficacia nel ridurre l'infezione da *P. aeruginosa* in un semplice modello animale. Infine è stato fatto un tentativo di individuare i possibili bersagli ed i meccanismi di azione dei farmaci identificati.

Risultati

1. Sviluppo di un biosensore per la rilevazione di 3OC$_{12}$-HSL. Una fusione trascrizionale fra l'operone reporter *lux* per la bioluminescenza, e il promotore del gene *rsaL*, dipendente dal sistema di QS *las*, è stata inserita in singola copia sul cromosoma in un ceppo di *P. aeruginosa* deleto del gene *lasI*, e quindi non in grado di produrre la molecola segnale 3OC$_{12}$-HSL. Di conseguenza, questo biosensore (denominato PA14-R3) è in grado di emettere luce solo in presenza di 3OC$_{12}$-HSL esogeno. La risposta di PA14-R3 al 3OC$_{12}$-HSL è stata verificata mediante incubazione del biosensore in presenza di concentrazioni crescenti di 3OC$_{12}$-HSL e misurazione dell'emissione luminosa nel tempo. Le curve dose-risposta ottenute hanno mostrato che PA14-R3 è in grado di rispondere al 3OC$_{12}$-HSL in un range di concentrazioni da 152 pM a 3 µM (Fig. 8 e 9). La specificità di PA14-R3 per la molecola segnale 3OC$_{12}$-HSL è stata positivamente verificata saggiando la risposta del biosensore in presenza di altre molecole segnale prodotte da *P. aeruginosa* o altri batteri patogeni presenti nel polmone FC (Tab. 1). L'ampio intervallo di risposta e la specificità di PA14-R3 hanno reso possibile l'impiego di tale biosensore per la rapida, economica e precisa quantificazione di 3OC$_{12}$-HSL in campioni biologici, mediante interpolazione da una curva dose/risposta ottenuta con concentrazioni note di 3OC$_{12}$-HSL. L'analisi di supernatanti di colture del ceppo parentale *P. aeruginosa* PAO1 e di due mutanti isogenici nel sistema di QS *las* ha dimostrato che PA14-R3 è in grado di misurare efficacemente la concentrazione di 3OC$_{12}$-HSL all'interno di colture di laboratorio (Fig. 10), e potrebbe quindi essere utilizzato nello screening di mutanti nel QS. L'analisi di 20 campioni di espettorato prelevato da pazienti FC ha rilevato la presenza di 3OC$_{12}$-HSL nel 40% dei casi (Tab. S2). Nonostante il limitato numero di campioni analizzato non renda possibile determinare una correlazione fra concentrazione di 3OC$_{12}$-HSL, colonizzazione da parte di *P. aeruginosa* e gravità dell'infezione cronica, questi risultati suggeriscono che PA14 potrebbe in futuro essere utilizzato come efficiente strumento per comprendere meglio il ruolo del QS nella progressione della FC.

Il biosensore PA14-R3 ha inoltre permesso lo sviluppo di un sistema di screening per inibitori del QS, basato sulla sua coltivazione insieme ad un ceppo selvaggio di *P. aeruginosa* PA14. In questo sistema il ceppo selvaggio fornisce il 3OC$_{12}$-HSL necessario per l'emissione luminosa, e l'aggiunta di una molecola in grado di inibire un qualsiasi processo connesso al QS determinerà una conseguente riduzione nell'emissione luminosa rispetto ad un controllo non trattato (Fig. 11). Questo sistema di screening basato sulla co-coltivazione di PA14-R3/PA14 è stato valutato

saggiandone la risposta al furanone C30 (FC30), noto inibitore del QS. L'aggiunta di FC30 (10 µM) alla co-coltura PA14-R3/PA14 determinava una riduzione di circa il 50% dell'emissione luminosa e della produzione di $3OC_{12}$-HSL (Fig. 13 e S4). Questi risultati dimostrano che il sistema di co-coltivazione PA14-R3/PA14 è adatto allo screening di QSI.

2. Screening di inibitori del QS (QSI). Il sistema di co-coltura PA14-R3/PA14 è stato utilizzato per lo screening di una libreria di composti chimici disponibile in commercio. Criteri per la selezione dei composti attivi sono stati la riduzione dell'emissione luminosa ≥50% e l'alterazione della crescita ≤20%. Di 7 composti inizialmente identificati (Tab. 2), il farmaco antiparassitario QSI-1 si è rivelato il più promettente, in virtù della sua bassa tossicità e alta attività inibitoria, ed è stato selezionato per ulteriori studi. La capacità di questo composto di inibire il sistema di QS *las* è stata ulteriormente verificata sia in una co-coltura PA14-R3/PA14 che nel singolo biosensore PA14-R3 con l'aggiunta di $3OC_{12}$-HSL dall'esterno. In entrambi i casi, QSI-1 ha causato una forte inibizione del QS, con IC_{50} al di sotto di quelle rilevate dal composto della libreria (Fig. 12). L'evidenza di un simile effetto inibitorio sia sulla co-cultura PA14-R3/PA14 che sul singolo PA14-R3 suggerisce che QSI-1 agisca a livello della recezione del segnale, piuttosto che sulla sintesi del segnale stesso. Essendo la sintesi del 3OC12-HSL sotto il controllo del QS, è stato studiata l'influenza di QSI-1 sulla produzione di $3OC_{12}$-HSL. Utilizzando il biosensore PA14-R3, è stato misurato il $3OC_{12}$-HSL presente nel supernatante di una coltura di PA14 trattata con varie concentrazioni di QSI-1. I risultati hanno mostrato che QSI-1 alla concentrazione di 5 µM è in grado di determinare una riduzione del 60% nella produzione di $3OC_{12}$-HSL (Fig. 15). Sono stati condotti ulteriori esperimenti volti a valutare un eventuale effetto di QSI-1 sugli altri due sistemi di QS, *las* e *pqs*. A tal fine sono stati impieganti due appositi biosensori nella quantizzazione delle rispettive molecole segnali, C_4-HSL e PQS, presenti nei supernatanti di colture di PA14 trattate con QSI-1. Mentre QSI-1 sembra non avere alcun effetto sulla produzione di PQS, i livelli di C_4-HSL risultano fortemente ridotti (Fig. 16). Inoltre, la riduzione di C_4-HSL in presenza di QSI-1 è stata osservata anche in mutante *lasI* non in grado di produrre $3OC_{12}$-HSL, suggerendo che l'effetto sulla produzione C_4-HSL non dipenda solamente dall'inibizione del sistema *las*.

E' stato anche esaminato l'effetto di QSI-1 sull'espressione di alcuni fattori di virulenza importanti nella patogenesi di *P. aeruginosa* e regolati, sia in modo diretto che indiretto, dal sistema di QS *las*. QSI-1 alla concentrazione di 10 µM si è dimostrato in grado di ridurre significamente la produzione di piocianina, elastasi LasB e ramnolipidi (Fig. 17), Infine, è stata valutata

l'efficacia di QSI-1 nel ridurre la patogenicità di *P. aeruginosa* in un semplice modello animale d'infezione . Il trattamento con 15 μM QSI-1 protegge quasi completamente le larve dell'insetto *Galleria mellonella* dall'infezione con una dose letale di *P. aeruginosa* (Fig. 18).

3. Screening di inibitori del "pyoverdine signaling" (PSI). Al fine di generare un biosensore in grado di rispondere al "pyoverdine signaling" è stata inserita nel cromosoma di *P. aeruginosa* PAO1 una fusione trascrizionale, in singola copia, contenente il promotore del gene *pvdE* (P*pvdE*), dipendente dal "pyoverdine signaling", a monte dell'operone reporter *lux* per la bioluminescenza (Fig. 19). Il ceppo ricombinante così ottenuto (PAO1 P*pvdE::lux*) è stato saggiato come biosensore in grado di rispondere al "pyoverdine signaling" (Fig. 20) per il successivo screening di PSI dalla libreria di composti chimici disponibile in commercio, precedentemente citata. Anche in questo caso i criteri per la selezione sono stati la riduzione dell'emissione luminosa ≥50% e l'alterazione della crescita ≤20%. Lo screening ha identificato un farmaco antimicotico (PSI-1) con forte effetto inibitorio sull'attività di P*pvdE* (IC$_{50}$ 2 μM) e sulla produzione di pioverdina (IC$_{50}$ 3 μM) (Fig. 21A). E' opportuno fare presente che differenti ceppi di *P. aeruginosa* sono in grado di produrre solo uno fra tre tipi di pioverdine diverse (tipo I, II e III), riconosciute ciascuna da un recettore specifico. Il simile effetto inibitorio di PSI-1 sull'attività di P*pvdE* e sulla produzione di pioverdina riscontrato in ceppi produttori di diverse pioverdine (Fig. 22) suggerisce che PSI-1 è efficace contro tutti i ceppi di *P. aeruginosa,* indipendentemente dal tipo di pioverdina prodotto. Nel tentativo di stabilire se PSI-1 agisse sul meccanismo di segnalazione abbiamo saggiato l'effetto di PSI-1 su una selezione di mutanti alterati in vari passaggi del signaling, in cui è stata inserita la fusione reporter P*pvdE::lux*. L'inibizione dovuta a PSI-1 riscontrata in tutti i mutanti (Fig. 23) induce ad escludere l'ipotesi di un effetto di PSI-1 a livello della cascata del signaling, suggerendo invece che PSI-1 è in grado di inibire la produzione di pioverdina mediante un meccanismo indipendente dal signaling. Esperimenti volti a valutare l'effetto di PSI-1 sull'espressione di PvdS, responsabile della trascrizione di tutti i geni biosintetici della pioverdina, oltre che di geni di virulenza, hanno dimostrato una forte riduzione della trascrizione di *pvdS* in presenza di PSI-1 (Fig. 24). Questi risultati, supportati da esperimenti successivi mirati a escludere un effetto generico di PSI-1 sul metabolismo del ferro, suggeriscono che PSI-1 agisca specificamente a livello della trascrizione di *pvdS*. Esperimenti mirati a valutare l'impatto di PSI-1 sull'espressione dei fenotipi di virulenza regolati da PvdS, hanno dimostrato che PSI-1 è in grado di ridurre fortememente

l'espressione di esotossina A e endoproteasi PrpL (Fig. 25).

Considerazioni conclusive

P. aeruginosa rappresenta un serio rischio infettivo in ambiente ospedaliero e, purtroppo, una ineludibile causa di mortalità per i pazienti FC. L'utilizzo di una strategia anti-*P. aeruginosa* basata sull'inibizione della virulenza piuttosto che della crescita potrebbe ridurre il danno all'ospite, facilitare l'azione del sistema immunitario e ridurre l'emergere di ceppi resistenti sotto la pressione selettiva di imposta dai trattamenti antibiotici tradizionali.

A questo proposito sono stati sviluppati e validati due sistemi di screening per inibitori del QS e del "pyoverdine signaling", in quanto meccanismi chiave per la regolazione dei geni coinvolti nella virulenza di *P. aeruginosa*. Il sistema di screening per inibitori del QS, basato sulla co-coltivazione PA14-R3/PA14, ha permesso di identificare composti con azione inibitoria a livello di (i) espressione/attività degli enzimi coinvolti nella sintesi della molecola segnale; (ii) espressione/attività dei recettori del segnale; (iii) importo/esporto delle molecole segnale. Questo rappresenta una novità rispetto ai precedenti sistemi di screening per inibitori del QS, il cui principale limite risiede nella possibilità di identificare solamente inibitori del recettore della molecola segnale (LasR).

Oltre al suo impiego nello screening della libreria di composti chimici, le caratteristiche di sensibilità e specificità per il $3OC_{12}$-HSL di PA14-R3 ne hanno permesso l'utilizzo per la quantificazione di $3OC_{12}$-HSL in campioni clinici ed in colture di laboratorio. L'utilizzo di biosensori per la quantizzazione di molecole segnale costituisce una alternativa veloce ed economica alle classiche procedure fisico-chimiche basate su LC-MS, GC-MS e LC-ESI.

Il farmaco QSI-1, identificato nel corso dello screening, ha attività anti-QS e si è dimostrato in grado di inibire la produzione di fattori di virulenza regolati dal QS. QSI-1 è anche in grado di contrastare l'infezione da *P. aeruginosa* in un semplice modello animale. Sono attualmente in corso esperimenti volti a valutare l'effetto di QSI-1 in un modello murino di infezione polmonare cronica.

Il sistema di screening per PSI basato sul biosensore P*pvdE::lux* rappresenta una novità in quanto per la prima volta viene valutata la possibilità di inibire il "pyoverdine signaling". Lo screening ha permesso l'identificazione di PSI-1, un farmaco antimicotico con attività inibitoria sulla produzione di pioverdina. Esperimenti volti a individuare il bersaglio di PSI-1 hanno proposto una suo possibile meccanismo d'azione basato sull'inibizione della trascrizione del gene *pvdS*. PSI-1 è in grado di ridurre la produzione di

esotossina A e dell'endoproteasi PrpL, due esoprodotti regolati da PvdS, importanti per la patogenesi delle infezioni sostenute da *P. aeruginosa*.

QSI-1 e PSI-1 sono entrambi farmaci commercializzati, già valutati ed approvati per l'uso clinico nell'uomo. L'efficacia dimostrata come inibitori della virulenza li rende ottimi candidati per ulteriori studi clinici mirati allo sviluppo di una terapia anti-*Pseudomonas* basata sull'inibizione della virulenza.

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SUMMARY

Pseudomonas aeruginosa is a major opportunistic pathogen in the hospital setting, and *P. aeruginosa* chronic lung infection is the main cause of morbidity and mortality in cystic fibrosis (CF) patients. *P. aeruginosa* infections are difficult to eradicate by conventional antibiotic therapy due to the inherent antimicrobial resistance of the bacterium and its propensity to acquire new resistances. The search for new drugs capable of inhibiting specific virulence-related traits of *P. aeruginosa* is a promising strategy to face with infections caused by multidrug-resistant *P. aeruginosa*. This approach offers the advantage of reducing the bacterial adaptability to the host environment, thus providing the host immune system with a better chance of clearing the infection without creating the selective pressure generally caused by conventional antibiotics. In *P. aeruginosa*, quorum sensing (QS) and pyoverdine signaling systems regulate a number of virulence phenotypes which are critical for *P. aeruginosa* pathogenicity, and therefore represent ideal targets for the development of novel anti-*Pseudomonas* drugs. QS is a cell-density dependent global regulatory system which relies in the production of a signal molecule that accumulates in the growth medium in a way that is proportional to the cell density. *P. aeruginosa* has three interconnected QS systems, namely *las*, *rhl* and *pqs*, based on the production of distinct signal molecules and organized in a hierarchical fashion with the *las* QS system on top. In the whole, these QS systems regulate ca. 10% of *P. aeruginosa* genes, including several virulence genes. Pyoverdine is a high-affinity iron chelator (siderophore) which binds Fe(III) in the extracellular milieu and delivers it to the cell through receptor-mediated active transport. Pyoverdine also acts as signal molecule, enhancing the expression of a number of *P. aeruginosa* virulence factors.

The aims of my PhD work were the development of novel high-throughput screening systems to be used for the detection of compounds able to inhibit QS and pyoverdine signaling and, subsequently, the characterization of the effects on *P. aeruginosa* virulence of the molecules identified by the screening experiments.

With regard to the screening system for QS inhibitors, a reporter system (PA14-R3) was developed, consisting in a *P. aeruginosa lasI* defective mutant, unable to produce the *N*-3-oxo-dodecanoyl-homoserin lactone ($3OC_{12}$-HSL) signal molecule and carrying the *lux* bioluminescence genes under the control of the *rsaL* promoter, inserted as a single copy in a neutral site of the *P. aeruginosa* chromosome. PA14-R3 was able to detect $3OC_{12}$-HSL concentrations in a 150 pM - 3 μM range, and showed high specificity towards

$3OC_{12}$-HSL, while not being significantly influenced by other acyl-HSLs produced by *P. aeruginosa* or by other bacterial pathogens that can coexist with *P. aeruginosa* in the lung of CF patients. The wide range of response and the high sensitivity and specificity of PA14-R3 made this biosensor a convenient tool for the direct micro-volumetric determination of $3OC_{12}$-HSL levels in *P. aeruginosa* laboratory cultures as well as in clinical samples, by non-linear interpolation from a standard curve obtained with purified $3OC_{12}$-HSL. The PA14-R3 biosensor was efficiently employed for direct measurements of the levels of $3OC_{12}$-HSL in culture supernatants of *P. aeruginosa* strains that differ in $3OC_{12}$-HSL production and for the quantification of $3OC_{12}$-HSL in sputa from CF patients with known history of colonization by *P. aeruginosa*.

A novel screening system for QS inhibitors (QSI) was developed, based on the co-cultivation of PA14-R3 with the *P. aeruginosa* wild-type strain PA14. In this system, the wild-type strain provides the PA14-R3 biosensor with the $3OC_{12}$-HSL required for luminescence emission. The addition of a molecule with inhibitory activity towards any process related to QS would reduce the emission of luminescence by the biosensor with respect to a control co-culture without any inhibitor added. This system would allow the detection of any inhibitory compound targeting one or more of the following steps: i) expression/activity of signal molecule biosynthesis enzymes; ii) expression/activity of signal receptors; iii) import/export of signal molecules. The PA14-R3/PA14 co-culture was validated as screening system for QSI compounds by testing the response of the system to furanone C30, a well-known QSI, and subsequently employed in a pilot screening for potential QSI in a commercial chemical library consisting of 1,120 FDA-approved drugs, selected for their high chemical and pharmacological diversity as well as for their known bioavailability and safety in humans. The screening led to the identification of a number of QS-inhibiting compounds, among which a teniacide drug (QSI-1), able to cause a decrease in the $3OC_{12}$-HSL production of about 50% and active at physiologically meaningful concentrations. The ability of QSI-1 to equally reduce the bioluminescence emission in both a PA14-R3/PA14 co-culture and a culture of PA14-R3 alone in presence of exogenously added $3OC_{12}$-HSL suggested that QSI-1 was likely to inhibit the sensing, rather than the synthesis or import/export of the signal molecule. In addition to $3OC_{12}$-HSL, QSI-1 also showed a relevant inhibitory effect on the production of the *rhl* QS signal molecule, namely C_4-HSL. The effect of QSI-1 on the production of several QS-dependent virulence determinants in *P. aeruginosa* was thus investigated. The presence of a 10 µM QSI-1 in the culture medium determined a

strong reduction of pyocyanine, rhamnolipids, and elastase production. Finally, the QSI-1 was tested for its ability to inhibit *P. aeruginosa* virulence in the *Galleria mellonella* (major wax moth) model of infection. A concentration of 15 μM QSI-1 almost completely abrogated the pathogenicity of *P. aeruginosa* in larvae inoculated with a lethal dose of *P. aeruginosa* PA14.

A screening system for pyoverdine signaling inhibitors (PSI) was developed, consisting in a *P. aeruginosa* PAO1 strain carrying the *lux* genes under the control of the signaling-responding *pvdE* promoter. In this system, the addition of a molecule with inhibitory activity towards pyoverdine signalling and/or pyoverdine synthesis would determine a decrease in the emission of luminescence by the biosensor and in pyoverdine-specific fluorescence. This system was used to screen a commercial chemical library for putative PSI, leading to the identification of an antimycotic drug (PSI-1) capable of reducing pyoverdine signaling and pyoverdine production in *P. aeruginosa* PAO1 by > 50%. In addition to the type I pyoverdine produced by *P. aeruginosa* PAO1, PSI-1 was also shown to reduce *pvdE* transcription and pyoverdine synthesis in type II and III pyoverdine-producing *P. aeruginosa* strains. A preliminary investigation was conducted to tentatively determine the molecular target(s) of PSI-1. PSI-1 was active against selected mutant strains impaired in different steps of the pyoverdine signaling pathway, suggesting that the inhibitory activity of PSI-1 does not depend on inhibition of pyoverdine signaling. Further experiments showed that PSI-1 was capable of reducing the transcription of the *pvdS* gene, encoding the alternative sigma factor PvdS, which is a central regulator essential not only for pyoverdine production but also for the expression of a number of *P. aeruginosa* virulence genes. PSI-1 at 10 μM concentration determined a relevant decrease in both transcription and expression of (i) pyoverdine synthesis genes, (ii) the *tox*A gene, which encodes the exotoxin A, a major determinant of *P. aeruginosa* virulence, and (iii) the *prp*L gene, encoding the extracellular protease PrpL. Further experiments are in progress to assess the effect of QSI-1 and PSI-1 in a mouse model of infection.

The evidence that QSI-1 and PSI-1 are effective in inhibiting crucial signaling processes for *P. aeruginosa* virulence and consequently reduce the expression of many virulence factors of *P. aeruginosa* sets the basis for further investigation focused on the characterization of the mode of action these drugs with the ultimate aim of using these compounds for the development of an anti-virulence strategy against *P. aeruginosa* infection.

1. Introduction

1.1. General features of *Pseudomonas aeruginosa*

Figure 1. A drawing of *Pseudomonas pyocyanea*, as it appeared in "*Die natürlichen Pflanzenfamilien*" (Migula 1895).

"Green. On slices of boiled potatoes I saw several times spots of a green color occur. The color was a dark, rich green, which began at the edge of the discs, spread eccentric and penetrated slightly into the nutrient substance… A more accurately known production of green dye by bacteria is instead found in the so-called green pus… The color in these cases was always the same verdigris green, tending somewhat to blue." With these words Joseph Schroeter described in 1872 the presence of water soluble, blue-green pigment on potatoes, previously reported in pus and surgical dressings. Although Schroeter neither microscopically observed nor isolated the organism responsible for the production of this pigment, he acknowledged the work of others who reported the presence of motile bacteria in green pus, and named it *Bacterium aeruginosum* (Schroeter 1872). A decade later, using the serial-dilution technique of Pasteur, Carle Gessard isolated in a pure culture the *"bacille pyocyanique"*, or bacillus of the 'pyocyanin', after the name given to the blue pigment extracted from pus (Fordos 1860; Gessard 1882a, b; Véron, 1965; Hugh & Lessel 1967), organism later described by Walter Migula as *Pseudomonas pyocyanea*: *"…it is the causative agent of blue pus. He has only one flagellum at one pole, which is about twice as long as the body… and creates a green fluorescent dye… Formerly, this species was considered harmless and believed to feed only on the pus produced by other organisms; but recently its pathogenic nature has been ascertained."* (Figure 1; Migula 1895). Not many years later Migula renamed this organism *Pseudomonas aeruginosa* (Migula 1900). At present the Bergey's manual of systematic bacteriology depicts *P. aeruginosa* as straight or slightly curved rods, 0.5-1.0 × 1.5-5.0 μm, Gram-negative, motile by means of a single polar flagellum, belonging to the class of γ-proteobacteria, optimum temperature 37° C, aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal

electron acceptor, although nitrate can be used as an alternate electron acceptor, allowing growth to also occur anaerobically (Palleroni 2005).

Endowed with terrific nutritional and ecological adaptability, *P. aeruginosa* is one of the most widespread bacterial species (Palleroni 2005). It is known to occupy a wide range of niches, from marshes, coastal marine habitats, sewages, as well as wet rocks and soil, to a variety of plant and animal tissues (Hardalo & Edberg 1997; Ahearn et al. 1999; Costerton et al. 1999). *P. aeruginosa* presents minimal nutritional requirements, and can use a very large selection of simple organic compounds as sole carbon and energy sources (Stanier et al. 1966). The ability to degrade toxic chemicals, including hydrocarbons, aromatic compounds and their derivatives of both biotic and xenobiotic origin, as well as to tolerate a broad variety of physical and abiotic challenges, further contributes to its competitive capabilities (Palleroni 2005).

The range of organisms *P. aeruginosa* can infect crosses all biological kingdoms, and this probably reflects its metabolic and physiological versatility. Indeed, *P. aeruginosa* is known to cause infection in protozoa, fungi, plants, nematodes, insects, and all the five classes of vertebrates (Walker et al. 2004). Given its wide distribution in the environment, it is remarkable that almost all diseases attributable to *P. aeruginosa* are associated with compromised host defense, while healthy individuals are highly unlikely to become infected. Consequently, this pathogen is recognized as opportunistic. *P. aeruginosa* is the most common Gram-negative bacterium responsible for hospital-acquired infections, and is a serious threat to immunocompromised individuals such as neutropenic, cancer, bone marrow transplant and AIDS patients. This bacterium is known to cause pneumonia, urinary tract, surgical wound, bloodstream infections, bacteremia in severe burn victims and keratitis in contact lenses users. Of great significance is the role of *P. aeruginosa* as the most common pathogen associated with morbidity and mortality in patients suffering from cystic fibrosis (CF), accounting for chronic lung infections which once established are impossible to eradicate and ultimately lead to pulmonary failure and death (van Delden & Iglewski 1998; Lyczak et al. 2000).

The capability of this organism to cause infection is attributable to an armamentarium of well-regulated virulence factors combined with a remarkable imperviousness to antibacterial agents. *P. aeruginosa* produces a plethora of both cell-associated and secreted virulence factors, a characteristic that reflects the various clinical diseases caused by this pathogen (van Delden 2004). Its resistance to antibacterial treatments and host defenses relies on a combination of traits, such as an intrinsic multidrug resistance, conferred by

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the outer membrane impermeability and, chromosomally-encoded multi-drug efflux pumps (Poole 2001), acquisition of resistance genes via horizontal gene transfer (Poole 2011), and the formation of biofilm (O'Toole et al. 2000). Furthermore, *P. aeruginosa* can metabolize a wide variety of organic substrates and has an intrinsic genetic and biochemical potential for drug modification and/or degradation (Stover et al. 2000).

The prodigious ability of *P. aeruginosa* to adapt to a wide variety of different hosts and environments and to find a suitable niche in almost all host tissues is determined by a complex and highly regulated genome. The *P. aeruginosa* genome is one of the largest among the sequenced bacterial genomes. With its 5,570 predicted open reading frames (ORFs), the genetic complexity of this bacterium comes close to that of the simple eukaryote *Saccharomyces cerevisiae*. Consistently with its acknowledged metabolic versatility, adaptability and ubiquity, the analysis of the fully sequenced *P. aeruginosa* PAO1 genome has revealed a considerable potential to transport, metabolize and grow on organic substances, several iron uptake systems, improved ability to export proteins and small molecules by numerous secretion and efflux systems, and four potential chemotaxis systems. This genetic complexity draws up alongside an unusually large regulatory capability, as inferred from the evidence that over 9% of the assigned ORFs encode known or putative transcriptional regulators and environmental sensors (Stover et al. 2000). Such a powerful regulatory potential would enable *P. aeruginosa* to modulate its diverse genetic and biochemical capabilities in response to changing environmental conditions.

1.2. Pathogenesis of *P. aeruginosa* infection

Although already recognized as a pathogen since the late 1800s (Charrin 1889), it is only after the introduction of sulfonamides and penicillin, and the subsequent availability of novel niches due to eradication of the sensible microorganisms that the incidence of *P. aeruginosa* infections actually arose, hand in hand with the growing concern for its resistance to treatments (Jacobs 1964).

The pathogenesis of *P. aeruginosa* is complex and multifactorial as it can be gathered from its large number of virulence factors (Figure 2) and the broad spectrum of diseases the bacterium can cause. As an opportunistic human pathogen, *P. aeruginosa* is only seldom recovered from the endogenous microbial flora in healthy individuals, although once the host defenses are compromised, tissues that cannot be infected are few. A breach or bypass in cutaneous or mucosal barriers (e.g. by mechanical ventilation, in-
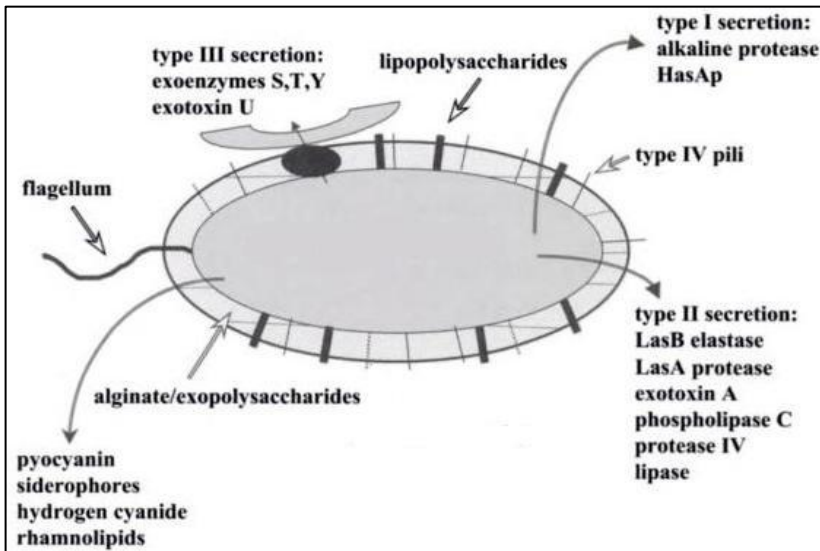
Figure 2. Virulence determinants in *P. aeruginosa*. *P. aeruginosa* has both cell-associated (flagellum, pili, lipopolysaccharide, alginate) and extracellular virulence factors (LasA protesase, LasB elastase, exotoxin A, phospholipase C, protease IV, PrpL protease, pyocyanin, siderophores, hydrogen cyanide, and rhamnolipids). The type III secretion apparatus is used to inject exotoxins S, T, Y, and U in the host cell (modified from van Delden 2004).

dwelling devices, surgery or severe burns), impaired immunity, alteration of the balance of normal mucosal flora by broad-spectrum antibiotics are usually required for colonization by *P. aeruginosa* (Bodey et al. 1983; Lister et al. 2009).

The initial critical step of tissue colonization requires *P. aeruginosa* to find and attach to a suitable site where bacterial replication could occur, while avoiding the host's immune defenses. At this stage, an important role is played by cell-associated factors. Flagella and pili are responsible for bacterial motility and adhesion to the epithelial surface, and lipopolysaccharide (LPS) is involved in both attachment and evasion from the host defenses.

The further development of the infection requires *P. aeruginosa* to grow at the initial site of infection, withstanding host defense mechanisms and damaging host cells and tissues. *P. aeruginosa* is able to directly inject toxins into the cytoplasm of the eukaryotic cells, *via* the syringe-like apparatus encoded by the type III secretion system, which is immediately activated upon contact with the host cells. Moreover, several other virulence factors, in-

cluding exotoxin A (ETA), pyocyanin, proteases, and hemolysins are secreted *via* the so-called type I and II secretion systems. The latter exoproducts are required for host tissues invasion as they destroy the protective glycocalix and expose epithelial ligands, and are also responsible for cytotoxicity and inactivation of the host defense mechanisms (Pier & Ramphal 2005; Kipnis et al. 2006).

P. aeruginosa copes with the scarce availability of nutrients, oxygen and other essential factors in the mammalian tissues by producing factors suited to acquire limited nutrients or by activating alternative metabolic pathways.

P. aeruginosa has the ability to colonize patients but can also cause serious infections and diseases. The balance between the two largely depends on its ability to respond to the host environment and to antimicrobial therapy. In acute infections there is first a growth at the site of infection followed by invasion, dissemination and tissue damage. However, in chronic infections, and particularly in CF patients, *P. aeruginosa* can also adapt genetically and phenotypically, while a persistent inflammatory state is sustained by extracellularly secreted virulence factors (Pier & Ramphal 2005; Kipnis et al. 2006).

Whether in acute or chronic infections, the fine tuning necessary for the precise expression of virulence genes is determined by a multiplicity of regulatory systems in response to host defenses and other environmental signals. Among these systems, a critical role is played by a mechanism that monitors bacterial cell density and allows cell-to-cell communication, known as quorum sensing (QS) (Smith & Iglewski 2003a; Kipnis et al. 2006), and by the low-iron dependent regulation of virulence gene expression, which mainly occurs through the signaling activity of the siderophore pyoverdine (Visca et al. 2007).

1.2.1. *P. aeruginosa* infection in cystic fibrosis

CF is a severe monogenic recessive disorder, with an incidence of 1 in 3000 live births and carrier frequency of 1 in 25, which results in a clinical syndrome characterized by chronic lung infection as well as gastrointestinal, nutritional, and other abnormalities. CF arises from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a protein functioning as a chloride ion channel and also as a regulator of other ion channels (Lyczak et al. 2002).

Whereas the gene defect results in several medical problems for the patient, the most severe impact is caused by the chronic lung infection with *P. aeruginosa*, which is responsible for damages to the epithelial surfaces and for

airway plugging, and leads to a decline in pulmonary function and respiratory failure, eventually causing the death of 80 to 95% of CF patients.

The abnormal composition of the airway secretions of the CF lung and the consequent failure of the host mechanical and immunological clearance mechanisms is hypothesized as the factor that predisposes CF patients to chronic colonization by several pathogens. Lungs of patients are typically composed of an increased mucus viscosity and an impared mucociliary and cough clearance which, in turn, are due to abnormal sodium absorption from the airway lumen and the failure to secrete chloride, resulting in isotonic salt concentrations (Williams et al. 2007a). Since early childhood lungs of CF patients are progressively colonized with different bacterial species, such as *Staphylococcus aureus* and *Haemophilus influenzae*, which are eventually and permanently replaced by *P. aeruginosa*. (Lyczak et al. 2002). Although the precise mechanisms behind the peculiar susceptibility of the CF lung to colonization by *P. aeruginosa* have not been fully characterized, various hypotheses have been proposed. Recent studies reported the ability of CFTR to bind to the conserved outer-core oligosaccharide of *P. aeruginosa* LPS, thus promoting the internalization of the organism by epithelial cells and the consequent clearance of the bacteria from the lung *via* cellular desquamation. Consequently, absent or nonfunctional CFTR might impair the internalization and clearance of *P. aeruginosa* from the lung (Pier 2000, 2002). Supporting studies showed that impaired binding to CFTR fails to activate the Src and Fyn tyrosine kinases, required for the internalization of *P. aeruginosa* (Esen et al. 2001). Another factor reported to promote the virulence of *P. aeruginosa* in the CF lung was its ability to shed the syndecan-1, a cell-surface heparin sulfate proteoglycan (Park et al. 2001). Finally, the increase in the levels of non-sialylated glycolipids and glycoproteins observed in CF cells has been associated to enhanced adherence of *P. aeruginosa* to the airways, perhaps due to the ability of this organism to bind to asialo GM1 ganglioside (Feldman et al. 1998; Hardie et al. 2009).

Under the selective pressure exerted by the unique environment represented by the CF lung, *P. aeruginosa* undergoes an adaptive evolution mediated by genetic variation, often leading to loss of its most immunogenic features and increased protection from the host defenses. The emergence of a hypermutator phenotype, resulting from defects in DNA repair and accounting for a rapid accumulation of genetic adaptations, has been observed in about 20% of isolates from CF patients (Oliver et al. 2000). Phenotypic and genotypic changes observed in CF isolates include increased alginate production and occurrence of mucoid variants, impaired QS mediated communication, loss

of motility, loss of effector proteins of the type III secretion system, alteration of the O-antigen components of the lipopolysaccharide, reduced virulence, and increased antibiotic resistance (Ciofu et al. 2009; Oliver et al. 2002). *P. aeruginosa* is able to effectively adapt and persist in the respiratory tracts of CF patients for several decades, overcoming host defense mechanisms and resisting intensive antibiotic therapy.

1.2.2. Cell associated virulence factors

Pili. *P. aeruginosa* produces type IV pili, composed of a single pilin subunit, PilA, and assembled into long polar surface appendages, with the receptor binding domain located at the tip of the pilus. Pili have been proposed to promote local adherence to host tissues by binding carbohydrate sequence βGalNAc(1-4)βGal found in asialo-GM1 and asialo-GM2 glycosphingolipids on the surface of epithelial cells (Sheth et al. 1994). Pili also mediate twitching motility of *P. aeruginosa*, a kind of mobility which is important for the formation of biofilms, and play an important role in natural DNA uptake, autoaggregation of cells, and development of microbial communities (Pier & Ramphal 2005).

Flagellum. *P. aeruginosa* has a single polar flagellum, a complex structure whose synthesis requires over 20 known genes involved in transcriptional regulation, and about 30 in production of the flagellar apparatus (Shapiro 1995). Bacterial flagella constitute important virulence determinants since the motility phenotype conferred by these organelles is often associated with the ability of to cause disease. Furthermore, *P. aeruginosa* flagella have been reported to bind to asialo GM1 and promote adherence to host tissues (Montie et al. 1987; Feldman et al. 1998)

Lipopolysaccharide is a large molecule found in the outer membrane of Gram-negative bacteria, consisting of a hydrophobic lipid A moiety inserted into the phospholipid bilayer, and a hydrophilic tail composed of the core polysaccharide and the O-specific polysaccharide. LPS functions as an endotoxin and can evoke strong immune responses, but also play a key role in resisting to the host innate defenses. Variants of the LPS O-side chains found in environmental and nosocomial strains prevent lysis by complement and affect resistance to antimicrobial agents (Schiller 1988; Conrad & Galanos 1989). Altered lipid A structures found in CF strains promotes resistance to cationic antimicrobial peptides (CAMPs) and other membrane active components of the innate immune system and generate increased inflammatory responses (Moskowitz & Ernst 2010). LPS has also been reported to play a role in binding to asialo-GM1, recognition of various Toll-like receptors or binding to the CFTR protein, which acts as a receptor for

epithelial cell internalization of *P. aeruginosa* on the airway surface (Hardie et al. 2009).

Catalases and superoxide dismutases. *P. aeruginosa* protects itself from the effects of reactive oxygen species produced by aerobic respiration and by host phagocytic cells by producing three catalases (KatA, KatB, and KatC) and two superoxide dismutases (SOD), Fe-SOD and Mn-SOD, containing iron and manganese as cofactors, respectively. SODs catalyze the dismutation of superoxide (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2), while catalases are required for the decomposition of H_2O_2 to water (H_2O) and O_2 (Hassett et al. 1999; Ochsner et al. 2000a).

1.2.3. Type I and II Secreted virulence factors

The type I secretion mechanism relies on three membrane proteins: an ATP binding cassette (ABC) protein, a membrane fusion protein (MFP) and an outer membrane protein (OMP) that form a contiguous channel across the inner and outer membranes of Gram-negative bacteria. Type I secretion system transports various molecules, from ions, drugs, to proteins of various sizes and is responsible for the secretion of alkaline protease (Duong et al. 2001).

The type II secretion pathway constitutes the main terminal branch of the general secretion pathway, which occurs *via* a two-step process. Exoproteins using this pathway are synthesized as a precursor with an N-terminal signal peptide that is cleaved during transport across the cytoplasmic membrane by the Sec machinery. In the periplasm these proteins fold into their final conformation and are then translocated across the outer membrane *via* the Xcp secretion apparatus encoded by 12 *xcp* genes (Filloux et al. 1998). Interestingly, this secretion pathway appears to be regulated in a QS-dependent manner (Chapon-Hervé et al. 1993). Virulence factors secreted by this pathway include LasA protease and LasB elastase, exotoxin A (ETA), lipases, phospholipase C and protease IV.

Proteases. The ability of *P. aeruginosa* to destroy components of human tissues is a major virulence determinant and proteases play a role during acute infection. *P. aeruginosa* produces several proteases including LasB elastase, LasA protease, alkaline protease and protease IV (also known as PrpL endoprotease)

LasB elastase is a zinc metalloprotease that degrades several tissue components including elastin, collagen and fibrin, also interfering with host defense mechanisms by inactivating immunoglobulins G and A (IgG, IgA), cytokines (interferon γ and tumor necrosis factor α), airway lysozyme and C1q and C3 complement components (van Delden & Igleski 1998).

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LasA is a serine protease known to enhance LasB elastolytic activity, and cause the shedding of the ectodomain of syndecan-1, a heparin sulfate proteoglycan abundant on airway epithelial cell surface which confers *P. aeruginosa* a disguise from host defenses (Park et al. 2001).

Protease IV is a serine protease that degrades C3, C1q, IgG, fibrinogen, plasmin, and plasminogen and capable of damaging the corneal epithelium. It was also reported to cleave casein, lactoferrin, transferrin, elastin and decorin, and contribute to *P. aeruginosa* virulence in a rat chronic pulmonary infection model (Wilderman et al. 2001). Protease IV contributes to the development and propagation of acute lung injury by degrading surfactant proteins and impairing host defenses (Malloy et al. 2005).

The metalloprotease alkaline protease can inactivate complement components C1q and C3, interferon γ and tumor necrosis factor α. This virulence factor is produced by *P. aeruginosa* in keratitis, bacteremia and CF lung infections (Caballero et al. 2001).

Exotoxin A (ETA) is an important virulence factor of *P. aeruginosa*. ETA is secreted into the extracellular milieu through the type II secretion system as a proenzyme; upon proteolytic cleavage it becomes an active ADP-ribosyl transferase, able to inhibit host protein synthesis by covalently modifying the elongation factor-2 (EF-2) in eucaryotic cells, acting similarly to diphtheria toxin (Vasil et al. 1986). ETA is involved in local tissue damage, bacterial invasion, and immunosuppression. The observation that purified ETA is highly lethal for mice supports its role as a major systemic virulence factor of *P. aeruginosa* (Woods & Iglewski 1983).

Hemolytic phospholipase C produced by *P. aeruginosa* hydrolyzes phospholipids containing quaternary ammonium groups, which are constituent of eukaryotic membranes and lung surfactant (e.g., phosphatidylcholine and sphingomyelin). Phospholipase C has been shown to induce vascular permeability, organ damage and death in mouse, rabbit and sheep models of infections (Terada et al. 1999)

Lipases are frequently produced by clinical *P. aeruginosa* strains isolated from CF and burn patients, and might play a role in pathogenesis by inhibiting monocyte chemotaxis and degrading lung surfactant lipids (König et al. 1996).

1.2.4. Type III Secretion systems

Bacterial type III secretion systems allow direct injection of bacterial toxins into the cytoplasm of eukaryotic cells. In *P. aeruginosa* four major effector proteins are known to be secreted by the type III secretion system: exoenzymes ExoS, ExoT, ExoY, and exotoxin ExoU. The apparatus for injection

is encoded by the chromosomal *pcrGVH-popBD*. ExoS and ExoT are bifunctional cytotoxins composed of an N-terminal Rho GTPase-activating-protein (RhoGAP) domain and a C-terminal ADP-ribosyltransferase domain. ExoS inactivates low-molecular-weight G proteins of the Ras family, leading to cytoskeletal changes, changes in cellular morphology and adherence, and inhibition of DNA synthesis, ultimately affecting cell viability. ExoS is also able to cause cellular apoptosis. ExoT interferes with internalization of *P. aeruginosa* by epithelial cells and macrophages by ADP-ribosylating target proteins of the eukaryotic cells involved in signal transduction and important for cellular adhesion and phagocytosis. The RhoGAP domain of ExoT inactivates proteins that regulate the cytoskeleton. ExoU, a potent cytotoxin, is a phospholipase able to readily lyse a variety of target cells. ExoY shows adenylate cyclase activity but its role in pathogenesis remains unclear (van Delden 2004; Pier & Ramphal 2005).

1.2.5. Other extracellular virulence factors

Rhamnolipids are rhamnose-containing glycolipidic surface-active molecules with hemolytic activity, synthesized by a rhamnosyltransferase complex encoded by the QS-controlled *rhlAB* genes. Rhamnolipids have a detergent-like structure which allows the solubilization of lung surfactants, thus facilitating their cleavage by phospholipase C, and contributing to the atelectasis associated with chronic and acute *P. aeruginosa* lung infection. Rhamnolipids were also reported to inhibit the mucociliary transport and ciliary function of human respiratory epithelium (van Delden & Iglewski 1998). They are the biosurfactants essential for swarming motility (Köhler et al. 2000) and play a crucial role in biofilm formation (Davey et al. 2003).

Hydrogen cyanide (HCN) is a secondary metabolite produced and secreted by *P. aeruginosa*, produced by an HCN synthase encoded by the biosynthetic genes *hcnABC*. It is a potent inhibitor of cytochrome c oxidase and other metalloenzymes, thereby suppressing aerobic respiration and causing severe poisoning and lethality (Blumer & Haas 2000). *P. aeruginosa* deals with the presence of HCN by using an alternative branch of the respiratory chain which involves a cyanide insensitive oxidase (Cunningham & Williams 1995) and by employing mechanisms to detoxify cyanide, such as the enzyme rhodanese (Cipollone et al. 2007). HCN has been found at relatively high concentrations in patients with freshly infected wounds (Goldfarb & Marggraf 1967) and was demonstrated to act as a virulence factor by inducing paralytic killing in the animal model of infection *Caenorhabditis elegans* (Gallagher & Manoil 2001) and contributing to lethality in a *Drosophila melanogaster* model (Broderick et al. 2008). Interestingly, Williams et

al. (2007a) revealed concentrations up to 200 μM of HCN in the sputum of CF fibrosis patients, and observed that the presence of HCN was associated with significantly poorer lung function (Ryall et al. 2008). Together with the knowledge that HCN is produced only under low-oxygen conditions, such as those represented by CF lungs, this finding supports its role in the exacerbation of this pathology.

Pyocyanin is a blue redox-active phenazine pigment, unique to *P. aeruginosa*, which generates reactive oxygen species due to intracellular redox cycling. After reduction by NAD(P)H pyocyanin reacts with O_2 to produce O_2^- and H_2O_2, thus exposing host cells to oxidative stress. This molecule is reported to damage host cells and tissues, disrupt nasal ciliary function, induce proinflammatory effects by augmenting IL-8 production, inactivate α_1 proteinase inhibitor, inhibit prostacyclin release, and induce apoptosis of human neutrophils (Britigan et al. 1992; van Delden 2004). Pyocyanin synthesis enzymes are encoded by two almost identical operons (*phzA1-G1* and *phzA2-G2*) with independent promoters and flanking regions (Li et al. 2011).

Alginate is a mucoid exopolysaccharide constituted as random copolymer of acetylated mannuronic acid and guluronic acid. Alginate is involved in the adhesion of *P. aeruginosa* to epithelial cell surfaces (May et al. 1991) and contributes significantly to the biofilm architecture (Ramsey & Wozniak 2005). In mucoid *P. aeruginosa* strains from CF patients, alginate has been shown to scavenge hypochlorite, reduce polymorphonuclear chemotaxis, inhibit activation of complement, and decrease phagocytosis of bacterial cells by neutrophils and macrophages (Leid et al. 2005).

1.2.6. Biofilm

Biofilm is a structured community of microbial cells attached on a surface and enclosed in self-produced matrix of hydrated extracellular polymeric substances (EPS) which protects bacterial cells in hostile environments (Figure 3). EPS typically consists of polysaccharides, extracellular DNA and proteins. In *P. aeruginosa*, at least three gene clusters have been identified that play a function in exopolysaccharide synthesis, including the *alg* biosynthetic genes, and the *psl* and *pel* operons (de Kievit 2009).

Biofilms are stable, synergistic microconsortia that rely on an intensified intercellular communication and whose complexity and metabolism are reminiscent of the complexity of tissues of higher organisms. They are composed of several layers which exhibit different patterns of gene expression, and many channels that allow nutrients to circulate and concentrate extracellular enzymes at the core of the matrix. Growth as a biofilm allows for a

greater resistance to antibiotic exposure and to the host immune responses (Costerton et al. 1999; Wingender & Flemming 2011).

Bacterial biofilms are involved in the pathogenesis of several bacteria including *P. aeruginosa* CF lung infection (Wagner & Iglewski 2008).
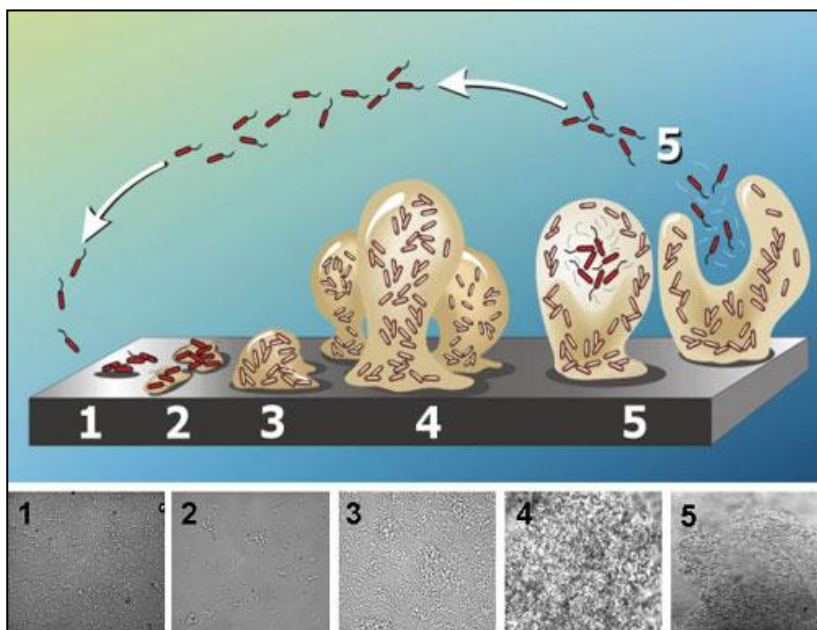

Figure 3. Five stages of biofilm development in *P. aeruginosa*: planktonic cells (1) attach onto a solid surface (2) and microcolonies are formed (3). Microcolonies proliferate forming stalk- and mushroom-like structures (4). At various points throughout biofilm maturation, cells can detach and resume the planktonic mode of growth (5). Each number in the graphic represents the number of the corresponding photomicrograph of an actual *P. aeruginosa biofilm*, pictured below (Graphic and photos taken by Peg Dirckx and David Davies © 2003 Center for Biofilm Engineering Montana State University).

1.2.7. Iron-uptake mechanisms

Iron is considered an essential micronutrient for almost all forms of life, due to its fundamental role in the biochemistry of all cells, including bacteria. Unfortunately, at physiological pH and in the presence of oxygen, ferrous iron (Fe^{2+}) is rapidly oxidized to the insoluble ferric form (Fe^{3+}), with concentrations of free $Fe^{3+} \leq 10^{-9}$ M, thus reducing its bioavailability to the mi-

croorganism. In mammals iron is mostly found in sequestered form: intracellularly it is bound to haem, iron-sulfur proteins and ferritins while in body fluids it is bound to high-affinity carrier proteins such as transferring and lactoferrin.

To meet its nutritional requirements, *P. aeruginosa* synthesizes two main siderophores, pyoverdine and pyochelin, which are low-molecular weight Fe^{3+}-chelating compounds. Both siderophores are synthesized in response to iron starvation (IS) and, once excreted in the medium, they can solubilize iron from insoluble precipitates, or extract it from complexing agents, delivering the metal to the cell through binding cognate receptor proteins located on the cell surface (Visca 2004).

Moreover, *P. aeruginosa* can acquire iron from a variety of exogenous chelators, including many heterologous siderophores, and utilize iron present in haem and haemoglobin, made available after lysis of erythrocytes, by means of the *phu* and *has* haem-uptake systems (Ochsner et al. 2000b).

Pyoverdine designates a structurally related group of diffusible green-fluorescent siderophores endowed with a very high affinity for Fe^{3+} which are produced by the so called "fluorescent" *Pseudomonas* species. For its additional role in regulating virulence, pyoverdine will be treated later in greater detail.

Pyochelin is a thiazolidine siderophore produced by *P. aeruginosa*, which binds iron with a 2:1 stoichiometry and relatively low affinity, but it can also form complexes with a variety of other transition metals (Visca 2004). Biosynthesis of pyochelin requires two iron-regulated operons, *pchDCBA* and *pchEFGHI*. Once pyochelin is secreted into the extracellular environment, it chelates iron, and is then transported back to the cytoplasm *via* a specific outer-membrane receptor, FptA, coupled with an inner-membrane permease, FptX. Iron-loaded pyochelin is required to activate the transcriptional regulator PchR which activates transcription of the *pchDCBA, pchEFGHI, fptABCX* and *pchR* genes, by binding to a conserved sequence motif (PchR-box) located at their promoter regions (Michel et al. 2005, 2007).

In addition to its role in iron acquisition, ferripyochelin has been reported to cause oxidative stress in host cells by catalyzing hydroxyl radical (HO•) formation (Dewitte et al. 2001).

Although essential, iron overload can result in toxicity, mainly due to the iron-catalyzed formation of hydroxyl radicals that strongly react with all kinds of biomolecules, including proteins, lipids and nucleotides (Andrews et al. 2003). As other Gram-negative bacteria, *P. aeruginosa* perceives intracellular iron levels through the ferric-uptake regulator (Fur), a homo-

dimeric protein composed of 17-kDa subunits. The Fur subunit contains two domains, a C-terminal domain, which binds Fe^{2+} and mediates dimerization, and an N-terminal transcriptional domain for binding to DNA. Under iron-rich conditions, Fur acts as a repressor by binding a 19-bp palindromic consensus sequence known as the 'Fur box' (GATAATGATAATCATTATC), which is generally present between the -35 and -10 sites in the promoters of Fur-repressed genes. Although the majority of these genes are directly implicated in iron transport and defense from oxidative stress, the Fur regulon also includes genes distantly related to iron metabolism and accounting for basic cellular functions (Visca, 2004).

1.3. Pyoverdine as an iron-carrier signal molecule

Pyoverdines are composed of a fluorescent dihydroxyquinoline chromophore, an acyl side chain bound to the C3 amino group of the chromophore, and a strain-specific peptide moiety linked *via* amide bond to the C1 carboxyl group of the chromophore. *P. aeruginosa* strains can produce one out of three structurally different pyovedines (type I, II, and III), each recognized by a specific outer membrane receptor (Figure 4).All pyoverdines are able to bind Fe(III) with extremely high affinity ($K_f \sim 10^{32}$ M^{-1}), generating a hexadentate complex involving the hydroxyl residues of the chromophore as well as hydroxamate, hydroxyl, and/or carbonyl groups of the peptidic moiety.

Pyoverdines chromophore and peptide moiety are synthesised from amino acid precursors that are assembled by non-ribosomal peptide synthases (NRPS). NRPS are large multimodular enzymes with each module governing the insertion of a single aminoacid into the peptide product (Visca et al. 2007). Several enzymes are involved in pyoverdine biosynthesis, and the corresponding encoding genes (*pvd*) are clustered in a single locus encompassing nearly 120 kb of the *P. aeruginosa* chromosome (Ravel & Cornelis 2003).

The pyoverdine precursor, ferribactin, is synthesized in the cytoplasm and transported by the ABC transporter PvdE into the periplasm, where further maturation occurs before secretion across the outer membrane by a still uncharacterized mechanism (Visca et al. 2007).

In the extracellular medium, pyoverdine binds iron and delivers it to the cell through a specific outer membrane receptor of 86 kDa, encoded by the *fpvA* gene.

The iron-pyoverdine complex dissociates into the periplasm, plausibly by reduction of Fe^{3+} to Fe^{2+}. Iron is supposed to enter the cytoplasm by means
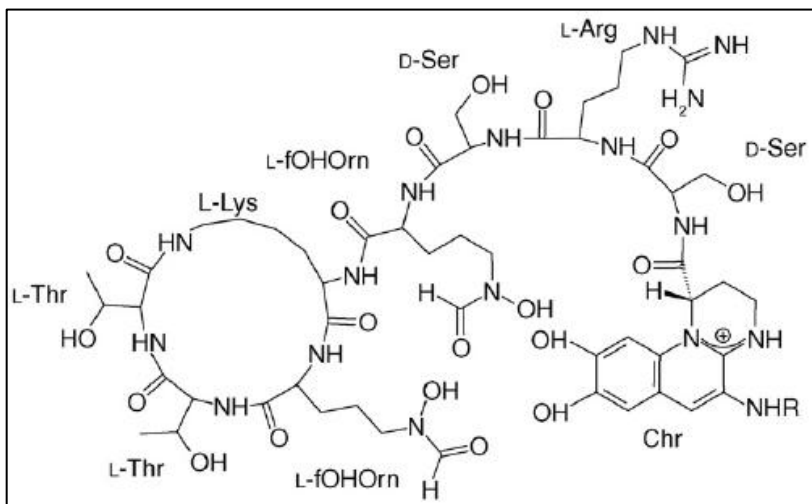
14

Figure 4. Pyoverdine of *P. aeruginosa* PAO1. Abbreviations: Chr, chromophore; fOHOrn, N5-formyl-N5-hydroxyornithine (modified from Visca et al. 2007).

of a periplasmic binding protein and an ABC importer, although the specific protein effectors have not been identified yet. After iron release, pyoverdine is recycled into the extracellular milieu by the tripartite PvdRT-OpmQ system, consisting of the ABC transporter PvdT, the periplasmic fusion protein PvdR, and the OM porin OpmQ (Imperi et al. 2009).

All pyoverdine synthesis and transport genes are regulated by two iron-dependent alternative sigma factors, PvdS and FpvI. Both sigmas are iron regulated at the transcriptional level and contain Fur boxes in their promoter regions. However, while FpvI is involved in the regulation of the sole FpvA receptor protein, PvdS is required to bind the promoters and initiate transcription of a large variety of genes, including pyoverdine biosynthetic genes as well as genes for different virulence factors.

PvdS-dependent promoters are characterized by the consensus TAAAT-N16/17-CGT, also known as the IS box, located approximately at position -33 relative to the transcription startpoint.

The activity of the two sigma factors PvdS and FpvI is modulated in response to pyoverdine binding to the surface receptor FpvA by signal transduction to the antisigma factor FpvR.

The Fur-regulated *fpv*R gene encodes a protein containing a membrane spanning domain, a C-terminal periplasmic extension proposed to interact
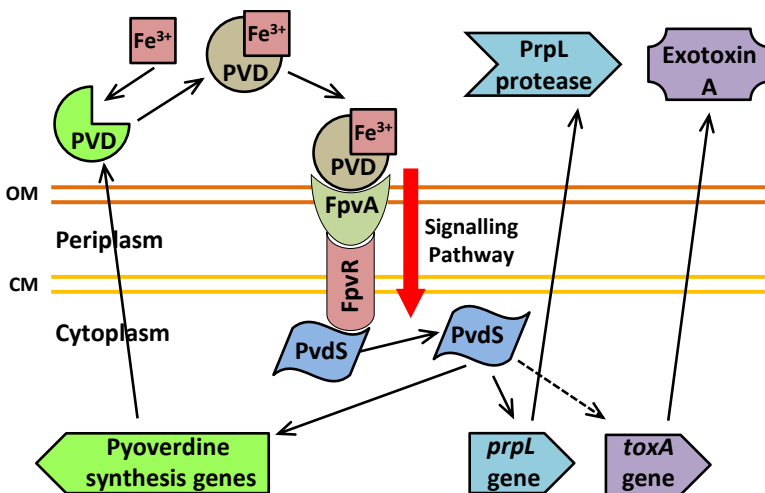
Figure 5. Model of the FpvA/FpvR/PvdS signaling pathway. Pyoverdine (PVD) is secreted and chelates extracellular iron [Fe(III)]. Iron-loaded pyoverdine complexes bind the FpvA receptor protein, transmitting a signal to the FpvR protein that otherwise suppresses the activity of PvdS. When the signal is transmitted through the FpvA/FpvR transmembrane system, PvdS is then released in the cytosol and binds the core fraction of RNA polymerase, forming the PvdS-Dependent RNA polymerase holoenzyme that drives the transcription of genes required for synthesis of pyoverdine, PrpL protease (*prpL*), and exotoxin A (*toxA*). OM: outer membrane; CM: cytoplasmic membrane (Modified from Lamont et al. 2002).

with FpvA N-terminus, and an N-terminal domain responsible for the modulation of both FpvI and PvdS activity. In the absence of pyoverdine, the activity of both PvdS and FpvI is suppressed by FpvR, while upon binding of ferri-pyoverdine to the FpvA receptor, a signal is transduced from the N-terminal domain of FpvA to FpvR, enabling PvdS and FpvI to become transcriptionally active (Figure 5).

Aside from its role in the regulation of the pyoverdine biosynthesis, there is evidence that pyoverdine signaling affects the expression of a relatively large subset of genes, including some involved in virulence, such as exotoxin A, alkaline protease, and PrpL endoproteinase genes. The meaning of the pyoverdine signaling in the regulation of apparently disparate activities may be explained by their role in iron release through destruction of host cells and cleavage of proteins that sequester iron (Visca 2004).

Pyoverdine-mediated iron uptake has been also demonstrated to be essential

for biofilm formation by *P. aeruginosa* under low-iron conditions (Banin et al. 2005; Yang et al. 2009).

Pyoverdine has been demonstrated to be required for optimal virulence in several animal and plant models of infection, including two distinct mice models (Meyer et al. 1996; Handfield et al. 2000; Mirleau et al. 2000; Takase et al. 2000; Taguchi et al. 2010). However, it has not been ascertained whether this is due to its crucial role in iron uptake or to its involvement in regulation of virulence gene expression.

1.4. Quorum Sensing

It is crucial for pathogenic microorganisms to be able to adapt and coordinate gene expression in response to host environmental cues. In a bacterial population, the behavior of individual cells can also be influenced by the population density *via* the production and secretion of small signal molecules. At low-cell densities, a basal level of signal molecules is constitutively produced. As the bacterial population increases, signal molecules accumulate in the extracellular milieu and, once a threshold concentration of the molecule is achieved, representing a specific population density (the "quorum"), a coordinated change in bacterial behavior is initiated. This type of cell-to-cell communication, termed quorum sensing (QS), allows a bacterial population to initiate a coordinated response within the whole bacterial population, which improves access to specific environmental niches, promotes collective defense against competitors or eukaryotic defense mechanisms, and facilitates survival through differentiation into morphological forms better able to combat environmental threats (Williams 2007b).

To date, three hierarchically arranged QS systems have been identified in *P. aeruginosa*, namely the *las* and *rhl* systems, employing N-acyl homoserine lactones (AHLs) as signaling molecule, and a quinolone-based system, *pqs* (Figure 6).

The *las* system consists of the transcriptional activator protein LasR, the LasI synthase and the negative regulator RsaL, encoded by the *lasR*, *lasI* and *rsaL* genes, respectively.

LasI directs the synthesis of the signal molecule, N-3-oxo-dodecanoyl-homoserine lactone ($3OC_{12}$-HSL) (Figure 7), which is actively secreted, likely through the MexAB-OmpD efflux pump, and accumulates in the medium until a critical threshold concentration is reached. At this concentration, $3OC_{12}$-HSL binds to its cognate receptor LasR which in turn becomes activated and stimulates or represses transcription of target genes. LasR expression is positively regulated by the global regulators Vfr (Albus et al.
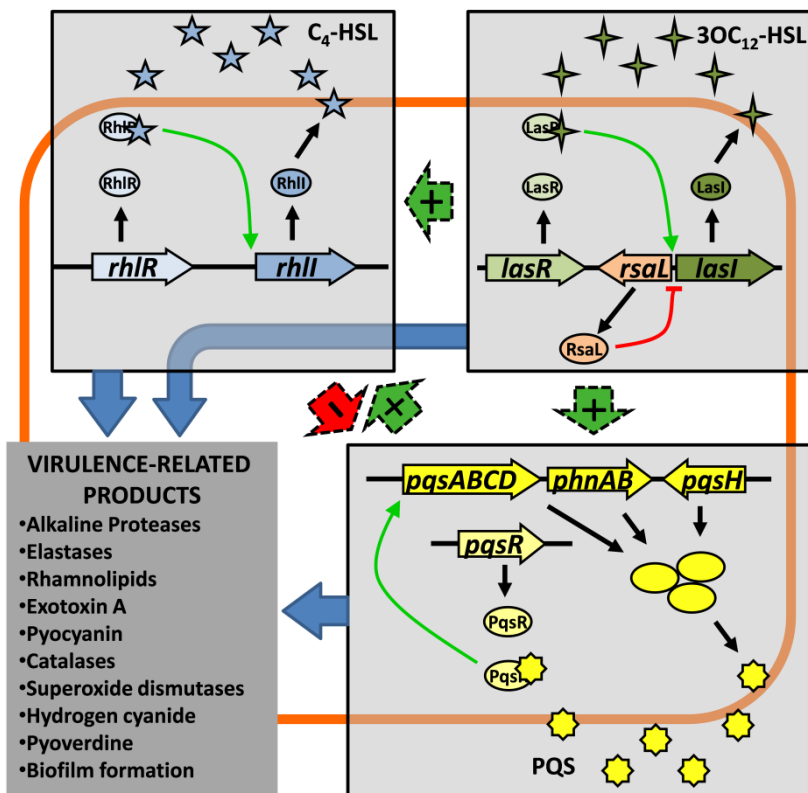
Figure 6. A schematic representation of the QS regulatory network in *P. aeruginosa*, with the *las*, *rhl*, and *pqs* systems. The QS network is organized in a hierarchical fashion, with the *las* system on top, positively regulating both *las* and *pqs* systems. In addition, *pqs* exterts a positive control on *rhl* system, which in turn negatively regulates the *pqs* system. The QS network is responsible for the expression of a number of virulence-related phenotypes.

1997) and GacA (Reimmann et al. 1997). Binding of LasR to a unique site located in the *rsaL-lasI* intergenic region activates the transcription of both *rsaL* and *lasI* divergent genes. The activation of the *lasI* gene generates a positive feedback loop, which leads to amplification of $3OC_{12}$-HSL production. On the other hand, the activation of the *rsaL* gene and the consequent binding of the RsaL protein to the *rsaL-lasI* divergent promoter prevent the LasR-dependent activation of both genes. Thus RsaL provides homeostasis

both by limiting $3OC_{12}$-HSL production to a physiological concentration, and by directly repressing its own expression, so that the levels of this negative regulator are maintained within a given range. The presence of this homeostatic system ensures that $3OC_{12}$-HSL concentration is kept at a steady level required for a coordinated population behavior, while allowing a rapid modulation of $3OC_{12}$-HSL levels in response to sudden changes in the environmental conditions (Rampioni et al. 2007). The *las* system has been shown to regulate the expression of several virulence factors, such as the LasB elastase and LasA protease (Toder et al. 1994; Anderson et al. 1999), the superoxide-dismutases Mn-SOD and Fe-SOD, the major catalase KatA (Hassett et al. 1999), alkaline protease and ETA (Gambello et al. 2003).

Interestingly, $3OC_{12}$-HSL molecule itself was shown to modulate the immune response by suppressing secretion of interleukin-12 and tumor necrosis factor α by LPS-stimulated macrophages, and also to inhibit T cell proliferation (Pritchard 2006).

LasR also regulates the *rhl* QS-system, consisting of the transcriptional activator protein RhlR and of the RhlI synthase, encoded by the *rhlR* and *rhlI* genes respectively, and based on the production of N-butyryl-homoserine lactone (C_4-HSL) as signal molecule (Figure 7). Similarly to the *las* system, C_4-HSL binds to LasR which in turn becomes activated and regulates transcription of target genes, including the *rhlI* synthase gene, thereby giving rise to an autoinduction loop.

Virulence factors whose expression depends on the *rhl* QS system include the RhlAB rhamnosyltransferase complex involved in the synthesis of rhamnolipids (Pearson et al. 1997), lipase, hydrogen cyanide (Reimmann et al. 1997) LasB elastase and pyocyanin (Brint & Ohman 1995). The *rhl* QS system was shown to activate the transcription of *rpoS* (Latifi et al. 1996), the stationary-phase sigma factor. RpoS is a master regulator of the bacterial stress response, and is important for adaptation to a variety of conditions including heat, osmotic and oxidative stress and involved in the expression of a number of virulence determinants (Schuster et al. 2004). It was also reported that both the *las* and *rhl* QS systems regulate the *xcpP* and *xcpR* genes of the type II secretory pathway, responsible for the secretion of *P. aeruginosa* exoenzymes and involved in the assembly of type 4 pili (Chapon-Hervé et al. 1993).

The third QS system in *P. aeruginosa, pqs*, differs from the *las* and *rhl* systems as it relies on the production of a chemically distinct class of signal molecule, the *Pseudomonas* quinolone signal 2-heptyl-3-hydroxy-4-quinolone (PQS (Figure 7), rather than acyl-HSLs. The *pqsABCD* and *phnAB* genes are required for the synthesis of the immediate PQS precursor,
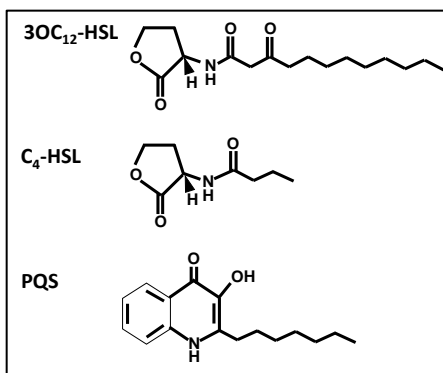
Figure 7. Structures of the QS signal molecules in *P. aeruginosa*: N-3-oxododecanoyl homoserine lactone (3OC$_{12}$-HSL), N-butanoyl-L-homoserine lactone (C$_4$-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS).

the 2-heptyl-4-quinolone (HHQ), which is then converted into PQS by the product of the *pqsH* gene, a putative mono-oxygenase.

PQS regulates its own production by activating the expression of *pqsABCDE* operon through a direct interaction with its cognate regulator PqsR (Diggle et al. 2006). The fifth gene of the *pqs* operon, *pqsE*, is not involved in PQS synthesis but encodes a putative zinc-dependent hydrolase which is considered a key mediator in the expression of a number of virulence and iron response genes, including the iron starvation sigma factor *pvdS* and genes involved in the synthesis of the siderophore pyochelin (Hazan et al. 2010).

The *pqs* system was shown to be directly involved in the regulation of pyocyanin and a number of virulence-related factors and toxic products, such as hydrogen cyanide (*hcnABC*), chitinase (*chiC*), lectins (*lecA and lecB*) (Deziel et al. 2005). Apart from its role as a signal molecule, PQS is also a potent immune modulator which inhibits human T cell proliferation (Pritchard 2006).

The *pqs* system interacts with the *las* and *rhl* systems; indeed, PqsR is involved in the positive regulation of the *rhlR* gene, and *pqsR* transcription is in turn under the positive control of LasR and under the negative control of RhlR (Figure 6; Wade et al. 2005).

Accordingly, a large overlap between the genes regulated by the *las*, *rhl* and *pqs* systems was reported (Deziel et al. 2005).

The intricate interweaving of these three systems adds further complexity to the QS network, and its hierarchical structure was proposed to guarantee a temporally ordered sequence of gene expression which is likely essential for the coordination of early and late events in the adaptation to the host environment (Schuster & Greenberg 2008).

The role of QS in the virulence of *P. aeruginosa* was tested in mouse models of infection including burn infection, acute pneumonia and chronic lung infection. In these models, strains mutated in QS genes were found to in-

duce less tissue destruction and cause reduced cases of bacteremia, pneumonia and mortality compared with wild-type *P. aeruginosa*, thus demonstrating that a functional QS system is required for an optimal pathogenesis in both acute and chronic infections (Smith & Iglewski 2003a). Additionally, the specific signal molecules of all three QS systems of *P. aeruginosa* were detected in the sputum of CF patients, thus suggesting that QS is functionally active in the CF chronic lung infections (Collier et al. 2002; Erickson et al. 2002).

A functional QS signaling was also found to be essential for creation of mature, differentiated biofilms. QS controls the production of the Pel polysaccharide and the release of extracellular DNA, two key components of the EPS matrix. Rhamnolipid, a QS-dependent product, are necessary for maintaining the open channel structures surrounding microcolonies and are involved in detachment of cells from the biofilm. Additionally, rhamnolipid-mediated swarming motility is required for the formation of microcolonies in early stages of biofilm establishment (de Kievit 2009). The importance of QS in the formation and maturation of biofilm is demonstrated by the increased susceptibility to antimicrobials and phagocytosis by polymorphonuclear leukocytes of QS mutant biofilms (Wagner & Iglewski 2008).

1.5. Resistance to antibiotics

P. aeruginosa is intrinsically resistant to many antimicrobial agents, and the emergence of multidrug resistant (MDR) strains during antimicrobial chemotherapy results in increased morbidity and mortality, length of hospital stay, and healthcare costs. Surveillance studies revealed the absence of a single drug which is active against all *P. aeruginosa* clinical isolates (Rossolini & Mantengoli, 2005).

The *P. aeruginosa* intrinsic resistance to antibiotic treatments relies on a combination of factors, including low permeability of outer membrane porins, active efflux, enzymatic degradation, target alteration and biofilm mode of growth. Additionally, *P. aeruginosa* resistance can increase through mutation in resistance-related genes or acquisition of additional resistance genes from other organisms *via* mobile genetic elements, such as plasmids and transposons, in which several resistance determinants are often clustered, so that an MDR phenotype can be acquired in a single step upon acquisition of the element. (Driscoll et al. 2007).

Porins. Gram-negative bacteria possess a semipermeable barrier, represented by the outer membrane, that largely accounts for the broad intrinsic resistance to antibiotics. Passage of nutrients into the periplasmic

space is accomplished through water-filled protein channels of porins, which however are also responsible for the entrance of certain hydrophilic antibiotics. A mutation causing reduced expression or loss of specific porin channels can decrease the susceptibility of *P. aeruginosa* to specific antibiotics, as it was shown in the case of OprD, a carbapenem-specific outer membrane porin (Rodríguez-Martínez et al. 2009).

Multidrug efflux pumps. The major multidrug efflux systems, responsible for intrinsic and acquired MDR in *P. aeruginosa*, utilize a drug-proton antiporter belonging to the resistance-nodulation-division (RND) family. RND pumps typically consist of a tripartite system including a periplasmic membrane fusion protein (MFP), an outer membrane factor (OMF), and a cytoplasmic membrane transporter, allowing drug extrusion through both membranes in a single step. In addition to this system, four efflux pumps, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM have been well characterized in *P. aeruginosa*. Substrates of these pumps include antibiotics, biocides, dyes, detergents, organic solvents, aromatic hydrocarbons, and homoserine lactones. Overexpression of a multidrug efflux pump by mutational events raises the minimal inhibitory concentration (MIC) of any drug secreted through the pump (Poole 2001; Schweizer 2003).

Enzymatic degradation. β-lactams inhibit the peptidoglycan-assembling transpeptidases located in the periplasm. β-lactamases are hydrolytic enzymes that disrupt the amide bond of the classical four-membered β-lactam ring thus rendering the antimicrobial ineffective. *P. aeruginosa* possesses two chromosomally-encoded β-lactamases, a cephalosporinase (AmpC) and an oxacillinase (PoxB). More recently, acquired β-lactamases have been described; typically encoded by plasmid- or transposon-borne genes, include the extended-spectrum β-lactamase (ESBL) enzymes able to hydrolyze a wider range of β-lactams, including cephalosporins, monobactams and carbapenemases.

Aminoglycosides inhibit protein synthesis by binding to the 30S subunit of the ribosome, and aminoglycoside modification leading to antibiotic inactivation typically involves their phosphorylation, acetylation, or adenylation. *P. aeruginosa* genes for aminoglycoside modification enzymes (AME) are typically found on integrons with other resistance genes (Poole 2011).

Target alteration. In Gram-negative bacteria, DNA gyrase is the preferred target of quinolones. In *P. aeruginosa*, gyrase mutations, typically occurring in the so-called "quinolone resistance determining region" (QRDR) of *gyrase* A subunit (GyrA), have been reported to confer resistance to fluoroquinolones. Jacoby 2005).

In the case of polymixins, while the mechanisms of resistance are unknown,

addition of aminoarabinose to the terminal phosphates of the lipid A moiety of LPS has been shown to contribute to polymyxin resistance in *P. aeruginosa* both in vitro and in CF isolates (Moskowitz & Ernst 2010).

Biofilms. An important and clinically relevant consequence of the biofilm mode of growth is its marked resistance to antimicrobial agents, due to the failure of antimicrobial agents to diffuse in depth through the biofilm, and the slow-growing or starved state of the cells in the biofilm, which makes them less susceptible to many antimicrobial agents (Costerton et al. 1999).

2. Aims of the thesis

P. aeruginosa represents a challenging target for the development of therapeutic strategies, as it is able to produce an arsenal of virulence factors and to co-regulate different resistance mechanisms. Current treatments, based on antibiotics that kill or inhibit the growth of the bacterium are nullified by the ability of *P. aeruginosa* to develop resistance to all known classes of antibacterial agents. The evolution of resistance is particularly problematic in CF, since chronic CF infections have to be controlled through repeated therapeutic treatments. Hence, there is a growing urgency to search for novel targets and new ways to control or suppress *P. aeruginosa* infections.

The development of compounds able to inhibit specific virulence determinants, as opposed to inhibitors of growth *per se*, represents a novel and potentially fruitful approach to the treatment of bacterial infections. Selective targeting of the pathogenic potential has the advantage to reduce the bacterial adaptability to the host environment thus providing the host immune system with a better chance of clearing the infection, without creating the selective pressure generally due to conventional antibiotics. An approach based on inhibition of virulence was validated in *Vibrio cholerae* with the discovery of virstatin, a small-molecule that selectively inhibits the transcriptional activator ToxT, required for the expression of the cholera toxin and the toxin coregulated pilus. Virstatin was found to be effective in protecting infant mice from intestinal colonization by *V. cholerae* (Hung et al. 2005).

In *P. aeruginosa*, QS and pyoverdine signaling systems regulate group behavior, enhancing its fitness, i.e. growth and virulence in the infected host. These regulatory networks are critical for virulence factor production and biofilm formation, and QS signal molecules and pyoverdine can be detected at biologically meaningful concentrations in sputum, bronchoalveolar and mucopurulent fluids from CF patients, indicating that QS and pyoverdine systems are active during infection in CF (Haas et al. 1991; Singh et al. 2000; Collier et al. 2002; Middleton et al. 2002).

Although it is not uncommon to isolate from CF sputa of patients chronically infected with *P. aeruginosa* clonal variants of the original wild-type strains carrying mutations in pyoverdine biosynthetic genes or in the $3OC_{12}$-HSL signal receptor gene (*lasR*), this apparent contradiction has been clarified by studies showing that QS and pyoverdine mutants are cheaters, i.e. clonal variants that emerge in the population, exploiting exoproducts released by proficient members of the community. However, if a too high proportion of the community fails to synthesize these molecules, iron up-

take and virulence potential would be affected in the whole population (De Vos et al. 2001; Smith et al. 2006; Buckling et al. 2007; D'Argenio et al. 2007; Diggle et al. 2007; Sandoz et al. 2007).

QS and pyoverdine signaling could represent preferential targets for the design of effective therapeutics against *P. aeruginosa* pathogenicity, as opposite to genes that contribute to pathogenicity in a limited subset of strains (Smith & Iglewski 2003b; Bjarnsholt & Givskov 2007).

Researches aimed at identifying inhibitors of the *P. aeruginosa* signaling systems started several years ago, but were mainly focused on searching for inhibitors of the *las* QS system of *P. aeruginosa*, by using screening systems conceived to search for antagonists of $3OC_{12}$-HSL, and thus having the signal receptor *LasR* as the only molecular target. In contrast, very few attempts have been made to identify inhibitors of other steps of the Las regulatory circuit, such as signal synthesis and transport (Persson et al. 2005).

Notably, several researches showed that the signal molecules are actively transported across the cell and that synthesis of these molecules is finely controlled also in response to environmental and metabolic stimuli, suggesting that other still-unidentified proteins could affect the *P. aeruginosa* signaling systems (Venturi 2006; Duan & Surette 2007; Gaines et al. 2007). These findings broaden the number of potential targets for inhibitors of *P. aeruginosa* signaling systems, making the development of new adequate screening systems an explicit goal.

The most known example of QS blockers is given by halogenated furanones. These molecules are able to interfere with acyl-HSL-based QS systems in different bacteria. Although natural furanones do not efficiently block QS in *P. aeruginosa*, the synthetic furanones C-30 and C-56 were shown to block the LasR-dependent QS system, reduce biofilm persistence, and promote the clearance of bacteria in a chronic pulmonary mouse infection model (Hentzer & Givskov 2003; Wu et al. 2004).

Concerning pyoverdine, no attempts have been made to investigate the possibility of interfering with the pyoverdine-mediated control of *P. aeruginosa* virulence. Antibiotic-siderophore conjugates have been proposed as a strategy to facilitate the entry of antibiotics into the bacterial cell, but the emergence of receptor mutants appears a major limitation of this "trojan horse" strategy (Budzikiewicz 2001). More recently, it has been reported that the transition metal gallium, disrupts *P. aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity by substituting for iron in iron-dependent enzymes, thus inhibiting iron-dependent cellular processes (Kaneko et al. 2007).

The main aim of my PhD studies was to develop novel high-throughput

screening systems able to detect inhibition of the *P. aeruginosa* QS and pyoverdine signaling processes at multiple levels. I have validated these screening systems by testing a library of synthetic compounds, with the final goal of identifying inhibitors of QS and pyoverdine signaling as new candidates for the treatment of *P. aeruginosa* infection.

In particular, pilot screening of a limited set of carefully chosen, structurally and pharmacologically diverse marketed drugs was performed. The rationale behind this approach is derived from the fact that, in addition to their main activity, drugs used in human therapy can show one or several pharmacological side effects, acting on targets often unrelated to the primary therapeutic activity of the compound. Although interaction with the "secondary" target might be less effective, a hit compound can be used as the starting point for a drug optimization program based on traditional, parallel or combinatorial chemistry, aimed at increasing the affinity for the new target while decreasing the affinity for the original one. This selective optimization of side activities (SOSA) approach (Wermuth 2006) offers the relevant advantage that for all these drugs, bioavailability and toxicity studies have been already performed thus reducing the time and cost required for clinical trials.

Among the inhibiting compounds identified upon screening, the most promising ones were selected, by virtue of their high activity against QS and/or pyoverdine signaling and low toxicity in humans at biologically active concentration. These compounds were subject to further investigation directed to ascertain their effect on the signaling mechanisms. The first step consisted in the assessment of their efficacy in reducing the production of signal molecules. Afterwards, their impact on the production of a number of QS- and pyoverdine signaling-dependent virulence factors was determined. Their ability to prevent *P. aeruginosa* pathogenesis was also evaluated in the animal model of *Galleria mellonella* infection. Further experiments in a mouse model of chronic lung infection are currently under way. Finally, an attempt has been made to characterize the effect of the selected compounds at a molecular level, with regard to their targets and mechanism(s) of action, by comparing their ability to affect signaling on selected mutant strains impaired in different steps of the signaling process.

The results reported in the first part of this PhD thesis, particularly those concerning the use of the PA14-R3 biosensor as a $3OC_{12}$-HSL quantification tool and the co-culture-based screening system, led to the registration of a patent:

Leoni L, Massai F, Imperi F, Zennaro E, Visca P. (2010) Title of inven-

tion: "Biosensore per la rilevazione di $3OC_{12}$-HSL, kit comprendenti il biosensore e usi di esso" - "Biosensor for the detection of $3OC_{12}$-HSL, kits including the biosensor and its uses" Patent RM2010A000541

Subsequently, this research was published:

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

Patent applications for the QS and pyoverdine signaling inhibitors identified upon screening are currently being generated. For this reason these compounds will be referred to as generic "Quorum Sensing Inhibitor 1" (QSI-1) and "Pyoverdine Signaling Inhibitor 1" (PSI-1). Patent applications will likely be finalized in the first quarter 2012 and the studies will be published thereafter.

3. Results

3.1. Development of a biosensor for 3OC12-HSL QS signal detection

3.1.1. Construction of reporter strains for the detection of $3OC_{12}$-HSL

A transcriptional fusion between the LasR-dependent *rsaL* promoter (P*rsaL*) and the *luxCDABE* operon was constructed and evaluated as reporter system for $3OC_{12}$-HSL quantification. P*rsaL* was selected because this promoter is directly activated by LasR and several hundred-fold inducible in the presence of $3OC_{12}$-HSL (Rampioni et al. 2007; Whiteley et al. 2001; Schuster et al. 2003), whereas the *luxCDABE* operon is a convenient reporter system

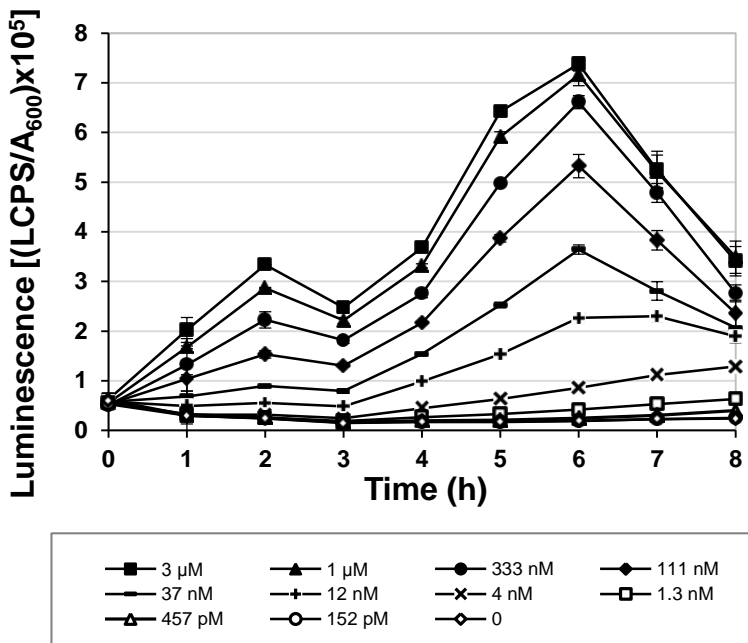**Figure 8.** Detection of $3OC_{12}$-HSL by PA14-R3 reporter strain. The chart shows the luminescence produced by the indicated reporter strains grown in LB in microtiter plates at 37 °C in the presence of increasing concentrations of exogenously-added $3OC_{12}$-HSL. $3OC_{12}$-HSL concentrations are given in the legend below the figure. Luminescence was measured every hour. Data are the mean (± SD) of three independent experiments.

that has no background in *P. aeruginosa* and provides direct signal detection without addition of exogenous substrate(s) (Meighen & Szittner 1992). The *P. aeruginosa* PA14 *lasI* mutant was chosen as the host strain for the P*rsaL*::*luxCDABE* fusion because it expresses LasR signal receptor protein but is impaired in the LasI synthase activity, and hence it does not produce $3OC_{12}$-HSL. If a P*rsaL*::*luxCDABE* fusion is introduced into PA14 *lasI*, this strain will produce luminescence only upon addition of the $3OC_{12}$-HSL signal molecule to the growth medium.

To generate reporter strains with different ranges of sensitivity to $3OC_{12}$-HSL, the P*rsaL*::*luxCDABE* fusion was introduced into PA14 *lasI* either as high-copy number construct in the pUCP18 plasmid (Schweizer 1991) or as low-copy number construct in the pMS402 plasmid (Duan et al. 2003) or chromosomally integrated as single copy at the *attB* neutral site (Hoang et al. 2000). The resulting strains were named PA14-R1, PA14-R2 and PA14-R3, respectively. In order to test the ability of the reporter strains to respond to $3OC_{12}$-HSL, the three strains were grown for 8 hours in 200 µl of LB medium in microtiter plates at 37 °C in the presence of increasing $3OC_{12}$-HSL concentrations, and luminescence was measured every hour (Figures 8 and S1). The data were fitted to a modified Hill equation model to generate nonlinear least-squares curves describing the dose-response relationships for each reporter strain at each hour of measurement (Figure S2).
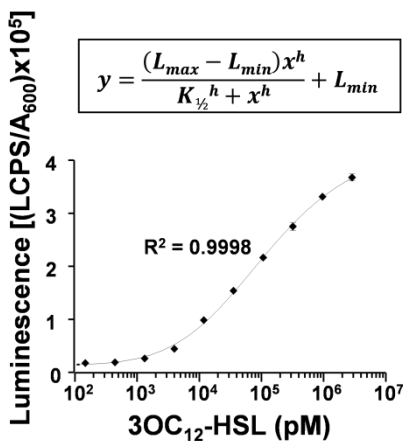
Figure 9. The $3OC_{12}$-HSL dependence of bioluminescence from PA14-R3 reporter strain. The line represents the nonlinear least-squares fit of the dose-response data shown in Figure 8, at the 4 hour time-point, to a modified Hill equation, where *y* is the bioluminescence emitted at a *x* concentration of $3OC_{12}$-HSL, *Lmax* is the maximal bioluminescence at the saturating concentration of $3OC_{12}$-HSL, *Lmin* is the basal bioluminescence in absence of $3OC_{12}$-HSL, *K½* is the concentration of $3OC_{12}$-HSL that provokes a response halfway between baseline and maximum, and *h* is the Hill slope or sigmoidicity coefficient that represents the steepness of the curve. Equation and R^2 value are shown. Data are the mean ($\pm$ SD) of three independent experiments.

$$y = \frac{(L_{max} - L_{min})x^h}{K_{\frac{1}{2}}^h + x^h} + L_{min}$$

$R^2 = 0.9998$

PA14-R1 and PA14-R2 gave optimum response at 5 hours of growth, with regard to goodness of fit and statistical dispersion, and showed a sensitivity range to $3OC_{12}$-HSL concentration from 457 pM to 3 µM (Figure S2). On the other hand, the PA14-R3 biosensor showed the best results at 4 hours of growth, within a sensitivity range to $3OC_{12}$-HSL concentration from 152 pM to 3 µM (Figures 9 and S2). Thus, PA14-R3 was able to measure $3OC_{12}$-HSL levels over a wider range of concentrations compared with PA14-R1 and PA14-R2, resulting more sensitive to low concentrations of signal molecule. The higher reproducibility of response data in the different experiments and the stability of the reporter system integrated into the chromosome are additional advantages of PA14-R3 with respect to the other two reporter strains. For these reasons, PA14-R3 was chosen for further studies.

3.1.2. Micro-volumetric determination of $3OC_{12}$-HSL levels in laboratory cultures and in clinical samples

The wide range of response and the high sensitivity of the PA14-R3 biosensor suggested that this engineered strain could be used for the direct micro-volumetric determination of $3OC_{12}$-HSL levels in *P. aeruginosa* laboratory cultures as well as in sputa of CF patients colonized by *P. aeruginosa*. To our knowledge, among the microorganisms colonizing the CF lung, only *P. aeruginosa* and members of the *Burkholderia cepacia* complex (Bcc) produce acyl-HSL signal molecules (Lyczak et al. 2002; Foweraker 2009), and both species can be isolated from the same CF sputum (Govan et al. 2007). Given that *P. aeruginosa* produces also C_4-HSL and Bcc members produce N-hexanoyl-HSL (C_6-HSL), N-octanoyl-HSL (C_8-HSL), and N-decanoyl-HSL (C_{10}-HSL) (Venturi et al. 2004; Table 1), it was necessary to rule out the possibility that these acyl-HSLs could influence PA14-R3 response to

Table 1. Sensitivity of PA14-R3 to *P. aeruginosa* and Bcc acyl-HSLs

	P. aeruginosa			Bcc	
Acyl-HSL	$3OC_{12}$-HSL	C_4-HSL	C_{10}-HSL	C_8-HSL	C_6-HSL
Detection limit[a]	10 pM	10 µM	100 µM	>100 µM	>100 µM

[a] The detection limit has been defined as the lowest concentration of acyl-HSL able to increase of $\geq 20\%$ the luminescence produced by the biosensor in the absence of any added molecule.

$3OC_{12}$-HSL. Therefore, we compared the sensitivity of PA14-R3 for all acyl-HSLs produced by *P. aeruginosa* and Bcc. PA14-R3 did not respond to C_6-HSL and C_8-HSL up to 100 μM concentration, while it responded to C_4-HSL and C_{10}-HSL, although at concentrations 6 and 7 orders of magnitude higher than the detection limit for $3OC_{12}$-HSL, respectively (Table 1).

According to previous studies, the highest concentration of acyl-HSLs in laboratory cultures and in CF sputa are ≤ 28 μM and ≤ 21 nM, respectively (Chugani et al. 2001; Erickson et al. 2002; Chambers et al. 2005).

On the other hand, the highest concentration of C_4-HSL in *P. aeruginosa* PAO1 laboratory culture is ≤ 12 μM (Whiteley et al. 2000), and the concentrations of C_4-, C_6-, C_8- and C_{10}-HSLs in CF sputa from patients colonized by both *P. aeruginosa* and *B. cepacia* are ≤ 5 nm, ≤ 88 nm, ≤ 22 nM and ≤ 66 nM, respectively (Erickson et al. 2002; Chambers et al. 2005). Considering that in our assays biological samples are 4-10 fold diluted (see Materials and Methods), the above results rule out any interference of C_4-, C_6-, C_8-
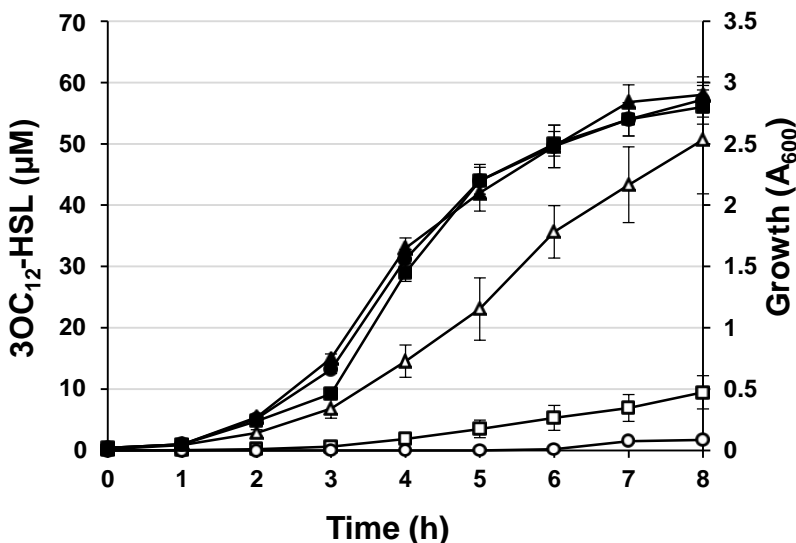
Figure 10. Micro-volumetric determination of $3OC_{12}$-HSL levels in laboratory cultures using the PA14-R3 biosensor. Cell density (A_{600}; filled symbols) and $3OC_{12}$-HSL level in culture supernatants (empty symbols) were measured along the growth curve in *P. aeruginosa* PAO1 (squares), PAO1 *rsaL* (triangles) and PAO1 *lasR* (circles). $3OC_{12}$-HSL concentrations were interpolated from dedicated calibration curves. Data are the mean ($\pm$ SD) of two independent experiments.

and C_{10}-HSLs with the response of PA14-R3 to $3OC_{12}$-HSL and suggest that the PA14-R3 biosensor could be suitable for determining the levels of $3OC_{12}$-HSL in both laboratory cultures and CF sputa.In order to check this hypothesis, the PA14-R3 biosensor was used to directly measure the levels of $3OC_{12}$-HSL in micro-volumes of culture supernatants of *P. aeruginosa* strains that differ in $3OC_{12}$-HSL production (Figure 10).

Considering the well-known role of the QS regulators LasR and RsaL as activator and repressor of *lasI* expression and $3OC_{12}$-HSL production, respectively, we used cultures of the wild-type strain PAO1 and its isogenic mutants PAO1 *rsaL* and PAOI *lasR* as source of the $3OC_{12}$-HSL inducer (Rampioni et al. 2007; Schuster & Greenberg 2006). Twenty µl of culture supernatants (or appropriate dilutions) were diluted in 200 µl (final volume) of a PA14-R3 culture, and luminescence was measured in microtiter plates after 4 hours of growth (see Materials and Methods for details). With respect to the wild-type strain, a dramatic reduction and a strong increase of $3OC_{12}$-HSL levels were observed in the *lasR* mutant and in the *rsaL* mutant, respectively (Figure 10), in good agreement with the opposite effects of these genes on *lasI* expression and $3OC_{12}$-HSL production (Rampioni et al. 2007; Schuster & Greenberg 2006).

These results confirmed that PA14-R3 can be used as a convenient and easy-to-handle tool to quantify $3OC_{12}$-HSL in *P. aeruginosa* culture supernatants. For this reason, this biosensor could be useful for large-scale studies aimed at investigating mutations and/or cultural conditions affecting $3OC_{12}$-HSL production in *P. aeruginosa*.

A preliminary investigation was also performed to test the possibility of using PA14-R3 for the quantification of the levels of $3OC_{12}$-HSL in micro-volumes of sputa from CF patients. As a preliminary test, we verified whether the CF sputum composition may influence the response of the PA14-R3 biosensor. To this aim, fluidified sputum samples (100 µl) of five CF patients not colonized by *P. aeruginosa* were added to an equal volume of the PA14-R3 culture in the absence or presence of known concentrations of $3OC_{12}$-HSL. No CF sputum activated the biosensor unless $3OC_{12}$-HSL was exogenously added, and the response of PA14-R3 to $3OC_{12}$-HSL in the presence of Sputasol alone or of the fluidified *Pseudomonas*-negative sputum samples was comparable (Figure S3), indicating that CF sputum composition does not affect $3OC_{12}$-HSL detection. Then, sputum samples were collected from 20 CF patients with known history of colonization by *P. aeruginosa*. Patients enrolled in this investigation were not treated with antibiotics in the three days preceding the sampling. Selective plate count confirmed the presence of *P. aeruginosa* cells in the sputum of 15 out of the 20

patients analysed, even if the bacterial load was highly variable among samples (Table S2). The PA14-R3 biosensor detected of $3OC_{12}$-HSL at measurable amounts (from 7 to 237 nM) in eight out of the 15 *P. aeruginosa* positive sputum samples, but no apparent correlation between the bacterial load and $3OC_{12}$-HSL levels was observed (Table S2).

The fact that $3OC_{12}$-HSL levels in CF sputum samples do not correlate well with the *P. aeruginosa* load is in line with the few studies in the field (Eriksson et al. 2002; Middleton et al. 2002; Chambers et al. 2005). Recent findings indicate that the number of QS-defective bacteria isolated from a single CF sputum sample is variable and seems to increase during years, suggesting that a reduction of $3OC_{12}$-HSL production could occur during late stages of the chronic infection (Hoffman et al. 2009; Winstanley & Fothergill 2009). It must be noted, however, that in all these studies only a limited number of CF sputum samples was analysed, likely due to the technical limits imposed by previous $3OC_{12}$-HSL measurement methods. To draw any conclusion about the relationship between $3OC_{12}$-HSL levels, *P. aeruginosa* colonization and the severity of the chronic infection, a larger number of samples should be analysed and the results correlated with the clinical history of the patient. Although out of the scope of my thesis, the above results suggest that PA14-R3 could be used in the future as a simple laboratory tool to address the still unexplored issue of the role of *P. aeruginosa* QS molecules in CF clinical progression. Finally, it must be pointed out that sputum samples collected from CF patients exposed to antibiotic treatment inhibited the growth of the biosensor strain (data not shown), indicating that the PA14-R3 biosensor can be used only for CF patients who had stopped antibiotic treatment at least three days before sampling, as in the case of the patients described above.

3.1.3. Development and validation of a co-cultivation method for the micro-volumetric, high-throughput screening of quorum sensing inhibitors (QSI)

One major aim of this study was the development of a novel QSI screening system based on the co-cultivation of PA14-R3 with wild-type *P. aeruginosa* PA14. In this system, the wild-type strain provides the biosensor with the $3OC_{12}$-HSL required for luminescence emission. The addition of a molecule with inhibitory activity towards any process related to QS would reduce the emission of luminescence by the biosensor with respect to the control co-culture without any added inhibitor (Figure 11). As expected, the co-culture of PA14-R3 with PA14 emitted significant luminescence levels, while the control co-culture of PA14-R3 with the PA14 *lasI* mutant, which
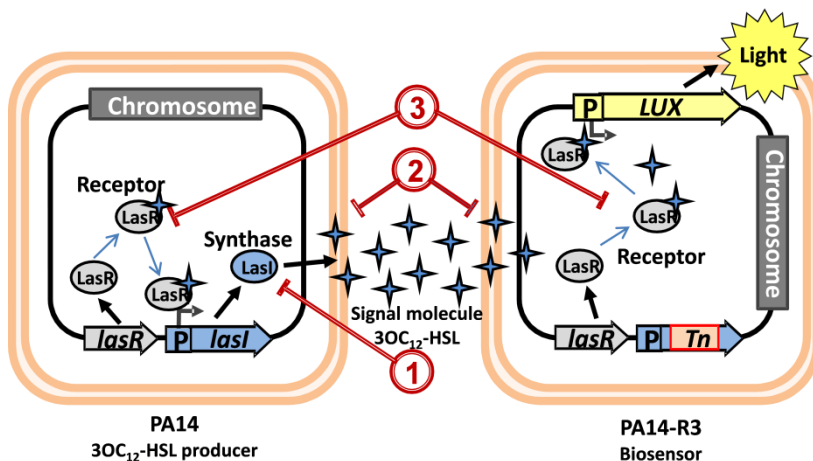
Figure 11. Schematic representation of the PA14/PA14-R3 co-cultivation screening system. The wild-type PA14 produces the $3OC_{12}$-HSL signal molecule that, in turn, activates PA14-R3 bioluminescence emission. Molecules interfering with (1) synthesis of the signal molecule, (2) transport of the signal 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. *Tn*, transposon MrT7 (Liberati et al. 2006).

is impaired in $3OC_{12}$-HSL production, did not (Figure 12). The addition of exogenous $3OC_{12}$-HSL (3 µM) to the PA14-R3/PA14 co-culture increased the luminescence levels during the first 4 hours of growth, after which the luminescence levels were comparable to those measured in the co-culture without addition of synthetic $3OC_{12}$-HSL (Figure 12). The anticipated response of the biosensor in the presence of exogenously-added $3OC_{12}$-HSL is a feature of QS-regulated systems and is due to the presence of high concentrations of signal molecule at low cell densities. This experiment shows that, under our experimental conditions, the PA14-R3/PA14 co- culture did not reach a saturation response to $3OC_{12}$-HSL for the first 5 hours of growth, suggesting that during this period any decrease in $3OC_{12}$-HSL synthesis and/or sensing can be detected by the system. Since at 4 hours of growth the system disclosed a good response and was far below the saturation level, we chose 4 hours as the optimal time point for the high-throughput screening of potential QSI.

To validate the efficacy of using the PA14-R3/PA14 co-culture as screening system for the identification of QSI compounds, we tested the response of
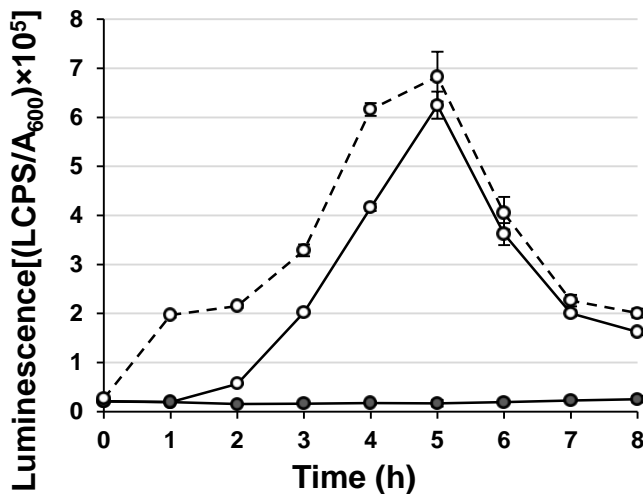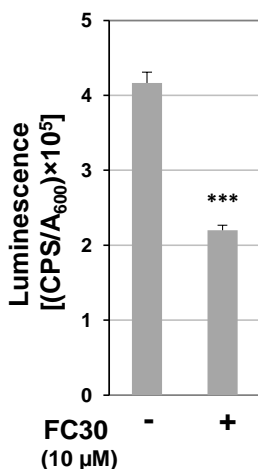
Figure 12. Development of the PA14-R3/PA14 co-cultivation system for the screening as QSIs. Luminescence emitted by PA14-R3 co-cultured with *P. aeruginosa* PA14 (empty circles) in the absence (solid line) or in the presence of 3 μM exogenously-added 3OC$_{12}$-HSL (dashed line). As control, PA14-R3 was co-cultivated with *P. aeruginosa* PA14 *lasI* (filled circles). The co-cultures were grown in microtiter plates at 37 °C, and cell density and bioluminescence were measured every hour. Data are the mean (± SD) of three independent experiments.

the system to FC30, a well-known QSI (Hentzer et al. 2003). The mechanism of action of FC30 remains poorly understood, although preliminary studies suggest that this synthetic derivative of natural furanones interferes with LasR signaling in *P. aeruginosa* (Kjelleberg et al. 2008). Accordingly, transcriptomic studies showed that FC30 reduces the transcription of many 3OC$_{12}$-HSL-regulated genes, affects biofilm structure, and promotes the clearance of *P. aeruginosa* in a mouse model of pulmonary infection (Hentzer et al. 2003).

However, to our knowledge, any direct effect of FC30 on 3OC$_{12}$-HSL production by *P. aeruginosa* has not yet been demonstrated. The level of 3OC$_{12}$-HSL produced by *P. aeruginosa* PAO1 was decreased by 50% in the presence of 10 μM FC30, demonstrating that FC30 negatively affects 3OC$_{12}$-HSL production (Figure S4). Coherently, the addition of 10 μM FC30 to the PA14-R3/PA14 co-culture determined a comparable reduction

(ca. 50%) of the luminescence levels with respect to the control co-culture without any added compound (Figure 13), indicating that the inhibitory effect of FC30 on $3OC_{12}$-HSL production by the wild-type strain is faithfully recorded by the PA14-R3 biosensor. Overall, these results indicated that the PA14-R3/PA14 co-cultivation system is suitable for the screening of QSI.

Figure 13. Validation of the PA14-R3/PA14 co-cultivation system for the screening as QSIs. Luminescence emitted by the PA14-R3/PA14 co-culture at 4 hours of growth in the absence or in the presence of 10 μM FC30. Data are the mean (± SD) of three independent experiments. *** $P<0.001$ (ANOVA).

3.2. Screening for QSI

3.2.1. Identification of a promising QSI

We used the PA14/PA14-R3 co-cultivation system to screen a commercial library of marketed drugs. The library contained 1,120 chemical compounds with known biological activities, selected for their high chemical and pharmacological diversity, as well as for their known bioavailability and safety in humans. Each drug was tested at three different concentrations (100, 10 and 1 μg/ml) in duplicate, as described in detail in Materials and Methods. Criteria used for the selection of hit compounds were: (i) ≥ 50% inhibition of bioluminescence emission and (ii) ≤ 20% alteration of growth with respect to the untreated controls. The latter criterion was aimed at avoiding any unspecific effect of impaired growth on QS expression.

The screening assay allowed the identification of seven putative QSIs, which reproducibly inhibited the QS response of the PA14/PA14-R3 co-cultivation system without affecting bacterial growth, at least at the highest concentration tested. These compounds were further assayed in triplicate at 100, 80, 60, 40, 20, 10, 5 and 2.5 μg/ml final concentrations. Overall, the identified compounds showed a half maximal inhibitory concentration (IC_{50}) ranging from 10 to 150 μM (Table 2). Four of the identified compounds are antibiotics, further confirming the well-known negative effect of sub-inhibitory concentrations of antibiotics on the *P. aeruginosa* QS re-

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Table 2. Hit compounds identified by screening the commercial chemical library containing 1120 FDA-approved compounds with the PA14/PA14-R3 QSI screening system.

No.	IC$_{50}$ (µM)[a]	Properties
1	10	Anthelmintic
2	20	Aminoglycoside antibiotic
3	150	Antineoplastic agent
4	50	Antibiotic of the rifamycin group
5	50	Macrolide glycopeptide antibiotic
6	60	Quaternary ammonium salt
7	10	Antibiotic of the rifamycin group

[a] The IC$_{50}$ values have been determined using the PA14/PA14-R3 co-culture grown for 4 hours at 37°C in the presence of 100, 80, 60, 40, 20, 10, 5 and 2.5 µg/ml of each compound, and then expressed as µM concentrations

sponse (Hoffmann et al. 2007, Babić et al. 2010). The remaining three compounds include a quaternary ammonium salt, an anti-cancer drug and a teniacide for the treatment of tapeworm infections (Table 2). Among non-antibiotic drugs, the anthelmintic drug (QSI-1) showed the highest anti-QS activity, expressed as the lowest IC$_{50}$ (Table 2), and was therefore selected for further studies.

3.2.2. QSI-1 inhibits the 3OC$_{12}$-HSL-dependent QS system of *P. aeruginosa*

To verify the result of the screening assay, QSI-1 was purchased from an alternative supplier (Sigma-Aldrich) and retested using the PA14/PA14-R3 co-cultivation system. As expected, a strong inhibition of the 3OC$_{12}$-HSL-dependent QS response was observed, with an IC$_{50}$ even lower than that calculated for the library compound (Figure 14A). Notably, QSI-1 was also active against the PA14-R3 biosensor alone, i.e. PA14-R3 grown in the presence of exogenously added synthetic 3OC$_{12}$-HSL (3 µM final concentration) without the signal-producing strain PA14 (Figure 14B). This result suggests that the QS inhibitory activity of QSI-1 plausibly relies on its ability to hamper the response of *P. aeruginosa* to the signal molecule rather than to inhibit its synthesis. On the other hand, QSI-1 had no effect on the bioluminescence emitted by a *P. aeruginosa* strain in which the bioluminescence reporter genes were under the control of the promoter of the QS-independent gene *cysB* (data not shown), which encodes a transcriptional
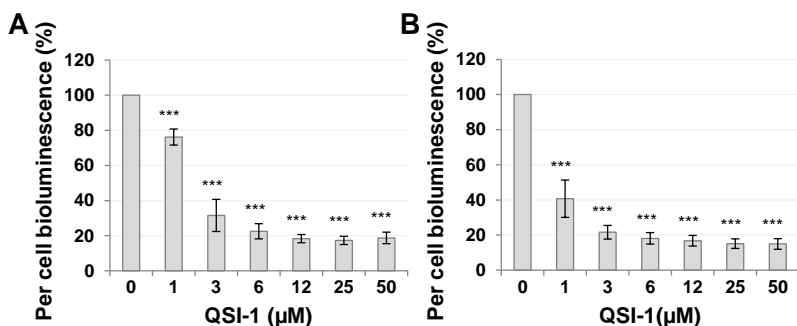
Figure 14. Effect of QSI-1 on the 3OC12-HSL-dependent QS of P. aeruginosa. Response of (A) the PA14/PA14-R3 co-cultivation system and (B) of the PA14-R3 biosensor in the presence of 3 μM exogenously-provided $3OC_{12}$-HSL to increasing concentrations (0-50 μM) of QSI-1. Bioluminescence emission was normalized to the cell density of the bacterial culture, and expressed as percentage relative to the untreated control. Values are the mean (± SD) of at least three independent experiments. *** P<0.001 (ANOVA).

Figure 15. Effect of QSI-1 on $3OC_{12}$-HSL production. $3OC_{12}$-HSL levels in culture supernatants of *P. aeruginosa* PA14 increasing concentrations (0-50 μM) of QSI-1. QSI-1 showed no significant effect on *P. aeruginosa* PA14 growth (data not shown). Values are the mean (± SD) of at least three independent experiments. *** P<0.001 (ANOVA).
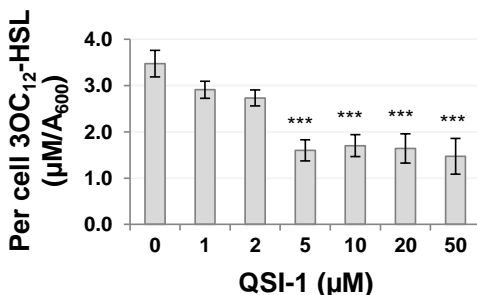
regulator involved in cysteine metabolism and iron uptake (Imperi et al. 2010). This result ruled out the possibility that the observed QS-inhibitory activity of QSI-1 could be due to an unspecific inhibition of either bioluminescence-generating enzymes or bacterial transcription in general. Given that the synthesis of the signal molecule is itself regulated by the QS response, the effect of QSI-1 on the production of $3OC_{12}$-HSL was also investigated. To this aim, *P. aeruginosa* PA14 was grown in the absence or in the presence of different QSI-1 concentrations (0-50 μM), and $3OC_{12}$-HSL levels in culture supernatants were quantitatively determined at 8 hours of growth using the PA14-R3 biosensor. QSI-1 caused a significant reduction

of $3OC_{12}$-HSL production with the maximum inhibitory effect on $3OC_{12}$-HSL production (about 60%) at 5 μM concentration. Higher QSI-1 concentrations did not further reduce $3OC_{12}$-HSL production (Figure 15). The reduction of $3OC_{12}$-HSL levels by QSI-1 was also confirmed by semi-quantitative determination of $3OC_{12}$-HSL extracted and separated by thin-layer chromatography (TLC) (Figure S5).

A pilot assay was performed in order to assess the effect of QSI-1 on the production of the other QS signal molecules of *P. aeruginosa*, namely C_4-HSL and PQS. To this aim, C_4-HSL and PQS levels in *P. aeruginosa* PA14 cultures treated or not with 20 μM QSI-1 were determined at 8 hours of growth using specific biosensors (see Figure S6 and Materials and Methods for details). While QSI-1 did not significantly affect PQS levels, C_4-HSL levels were significantly lower in QSI-1-treated with respect to QSI-1-untreated cultures (Figure 16).

Interestingly, the QSI-1-induced reduction in C_4-HSL production was also evident in a $3OC_{12}$-HSL-defective mutant strain (deficient in the gene for the $3OC_{12}$-HSL synthase LasI) (data not shown), suggesting that the effect of QSI-1 on C_4-HSL levels is not only due to its inhibitory activity of the *las* QS system.

A

B
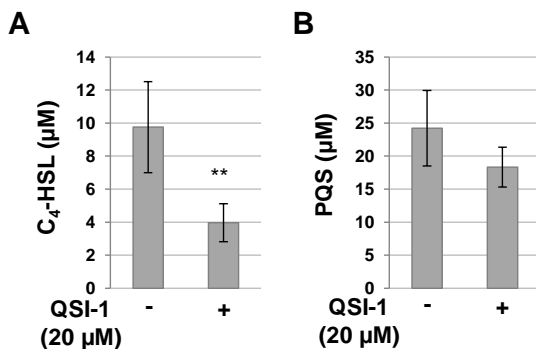
Figure 16. Effect of QSI-1 on C_4-HSL and PQS production. (A) C_4-HSL levels and (B) PQS levels in culture supernatants of *P. aeruginosa* PA14 grown for 8 h in the presence of or in the absence of 20 μM QSI-1. Values are the mean (± SD) of three independent experiments. ** $P<0.01$ (ANOVA).

3.2.3. QSI-1 strongly reduces the virulence potential of *P. aeruginosa in vitro*

As described above, the $3OC_{12}$-HSL-dependent QS system of *P. aeruginosa* controls, directly or indirectly, a great number of virulence traits which have been demonstrated to play a role in *P. aeruginosa* pathogenicity (Girard & Bloemberg 2008, Winstanley & Fothergill 2009). In order to verify the pos-

sibility of using QSI-1 as an anti-virulence drug against *P. aeruginosa*, we assessed the effect of this compound on the expression of a set of QS-regulated virulence phenotypes. In particular, we focused on production of:

(i) the LasB elastase, which is directly regulated by the $3OC_{12}$-HSL receptor LasR at the transcriptional level (Anderson et al. 1999), (ii) pyocyanin and rhamnolipids, which are regulated by both $3OC_{12}$-HSL-dependent QS and a number of different regulatory pathways and extracellular signals (Lau et al. 2004, Reis et al. 2011).

Overall, QSI-1 had a clear inhibitory effect on the levels of QS-regulated secreted virulence factors of *P. aeruginosa* PA14 (Figure 17). Both pyocyanin and elastase production was almost completely inhibited (85-90% reduction) by 5-10 µM QSI-1. Also rhamnolipid production was significantly affected by the QSI-1 treatment of 10 µM, which reduced the amounts of rhamnolipids in culture supernatants to about 25% of the control (QSI-1-untreated) sample (Figure 17 and S7).
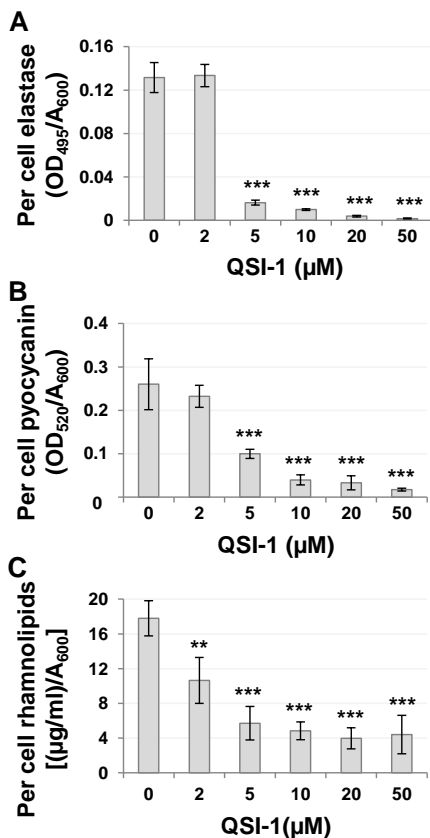
Figure 17. Effect of QSI-1 on the production of QS-regulated extracellular virulence factors. (A) LasB elastase, (B) pyocyanin and (C) rhamnolipids levels in culture supernatants of *P. aeruginosa* PA14 grown for 10 hours (in the case of A and B) and for 24 hours (in the case of C) in the presence of increasing concentrations (0-50 µM) of QSI-1. Values were normalized to the cell density of the bacterial culture, and are the mean (± SD) of four independent experiments. ** *P*<0.01; *** *P*<0.001 (ANOVA).

3.2.4. QSI-1 prevents *P. aeruginosa* pathogenicity in an animal model

In order to explore the possibility of using QSI-1 as an anti-virulence drug in *P. aeruginosa* infection, we assess the ability of this compound to inhibit the pathogenicity of *P. aeruginosa* in the *G. mellonella* insect model of infection (Jander et al. 2000).

Larvae of the greater wax moth *G. mellonella* are quite sensitive to *P. aeruginosa* injected into the hemolymph, and the PA14 strain was found to be highly virulent in this model, with an LD_{50} of 1-10 bacterial cells (Jander et al. 2000). In our study, *G. mellonella* larvae were inoculated with 10 µl of saline solution containing a lethal dose of *P. aeruginosa* PA14 (10 ± 4 exponentially-growing cells) and containing or not 750 µM QSI-1, and then incubated at 28°C for up to one week. Considering that the average weight of *G. mellonella* larvae was about 500 mg (see Material and Methods), and arbitrarily assuming 500 µl as the hemolymph volume of the larva, the final concentration of QSI-1 in each larva was estimated to be approximately 15 µM. We have just shown that, in our *in vitro* assays, such QSI-1 concentration inhibited $3OC_{12}$-HSL production and expression of $3OC_{12}$-HSL-dependent virulence factors (Figures 15, 17 and S7), without affecting bacterial growth (Figure S9). While 100% of the larvae untreated with QSI-1
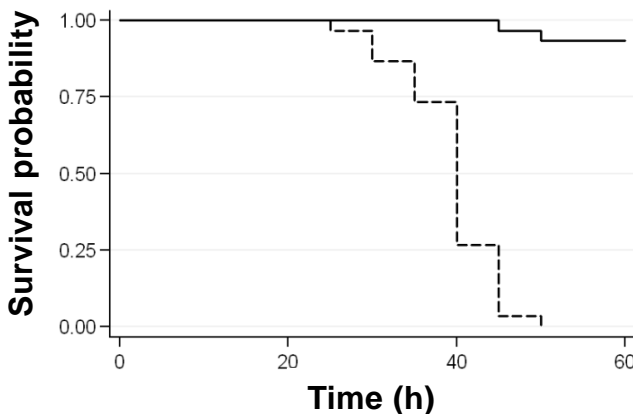
Figure 18. Efficacy of QSI-1 on the protection of *G. mellonella* larvae from *P. aeruginosa* killing. Kaplan-Meier plot showing the survival of *G. mellonella* larvae inoculated with a lethal dose of *P. aeruginosa* PA14 (10 ± 4 exponentially-growing cells) in 10 µl of saline containing supplemented or not with 750 µM QSI-1, and then incubated at 28°C. While 100% of the larvae untreated with QSI-1 died within 60 hours post-infection, QSI-1 almost completely protected *G. mellonella* larvae from *P. aeruginosa* infection. $\chi^2_{(1)} = 61.07$; $P = 0.0000$ (Log-rank).

died within 60 hours post-infection, QSI-1 almost completely protected *G. mellonella* larvae from the lethal dose of *P. aeruginosa* (Figure 18), even if they were left for a week at 28°C (data not shown). To monitor the presence of PA14 in QSI-1-treated and -untreated larvae, at 60 h post-infection five larvae per group were homogenated in saline solution and serial dilutions of the resulting homogenates were plated on *Pseudomonas* isolation agar. Dead larvae contained about 7 ($\pm$ 5) $\times$ 10^8 *P. aeruginosa* cells per larva, while live bacterial cells were undetectable in QSI-1-treated larvae, indicating that QSI-1 allowed the innate immune response of *G. mellonella* to efficiently counteract the infection.

3.3. Screening for pyoverdine signaling inhibitors (PSI)

3.3.1. Identification of pyoverdine signaling inhibitors
In order to develop a screening system for inhibitors of pyoverdine signaling, we generated a reporter strain in which a transcriptional fusion between the PvdS-dependent *pvdE* promoter (P*pvdE*) and the *luxCDABE* operon was inserted into the *attB* neutral site of the *P. aeruginosa* PAO1 chromosome (Figure 19). The same fusion was also inserted into the chromosome of the pyoverdine-defective mutant PAO1 *pvdA*. The resulting strains were grown under iron-depleted conditions (TSBD medium) in microtiter plates and the emission of bioluminescence was monitored along the growth curve. Although the bioluminescence emitted by the two strains was comparable during the early exponential phase, the pyoverdine proficient strain PAO1 emitted significantly higher levels of bioluminescence than the pyoverdine-defective strain PAO1 *pvdA* from the mid-exponential to the stationary phase of growth (Figure 20). In particular, a steady level of bioluminescence for the two strains was observed between 12 and 20 hours of growth, during which the activity of the *pvdE* promoter was double in the wild type than in the *pvdA* mutant. This is because in the wild type strain pyoverdine is produced and acts as an autoinduction signal (Figure 20). Therefore, the PAO1 P*pvdE::lux* strain effectively responds to pyoverdine signaling, and the response is stable and reproducible enough to allow the use of this reporter to perform a screening for pyoverdine signaling inhibitors.

The PAO1 P*pvdE::lux* reporter was thus employed in the screening of the afore-mentioned commercial chemical library. PAO1 P*pvdE::lux* was cultured in microtiter plates in the absence or in the presence of two different concentrations of each compound (50 and 5 µg/ml) and cell density and bioluminescence were assessed at 14 hours of growth. In each plate, the pyoverdine-defective strain PAO1Δ*pvdA* P*pvdE::lux* was used as control to

Figure 19. Schematic representation of the biosensor employed in the screening for pyoverdine inhibitors. In the presence of an active signaling, PvdS activates bioluminescence emission by the biosensor. Molecules interfering with pyoverdine synthesis (1) and export (2), FpvA/FpvR-mediated signalling (3), and PvdS activity or expression (4) will cause a reduction of luminescence relative to the untreated control.

determine the pyoverdine signaling-independent background of P*pvdE* activity.

The criteria used for the selection of hit compounds were: (i) $\geq$ 50% inhibition of pyoverdine-signaling dependent bioluminescence emission (calculated as PAO1 P*pvdE::lux* bioluminescence minus PAO1 *pvdA* P*pvdE::lux* bioluminescence), and (ii) $\leq$ 20% growth variation with respect to the untreated controls. The latter criterion was aimed at avoiding any unspecific effect of impaired growth on pyoverdine signaling and/or gene expression.

The screening led to the identification of four compounds which reproducibly reduced the bioluminescence emitted by the reporter in three independent assays (data not shown). However, when we measured the effect of these compounds on the amount of pyoverdine released in culture supernatants, only one compound (PSI-1) showed a significant inhibitory activity on pyoverdine production (data not shown). Thus, PSI-1 was selected for further analysis.
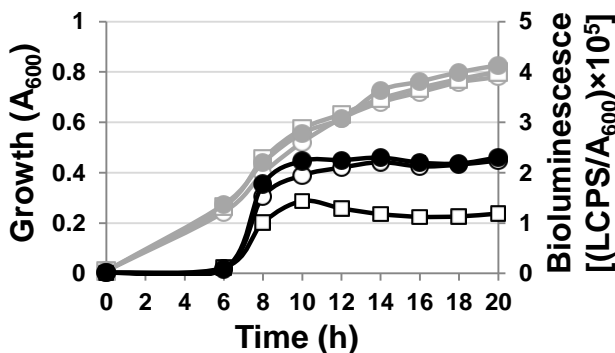
Figure 20. Response of the P*pvdE::lux* reporter construct to the pyoverdine signaling. Bacterial growth (grey lines, left y axis) and bioluminescence emission (black lines, right y axis) of PAO1 P*pvdE::lux* (filled circles), PAO1 *pvdA* P*pvdE::lux* (open squares) and PAO1 *pvdA* P*pvdE::lux* in the presence of 10 µM exogenously-added pyoverdine, cultured in TSBD medium at 37°C in microtiter plates. Values represent the mean of five independent experiments (SD are < 10% of the indicated values).
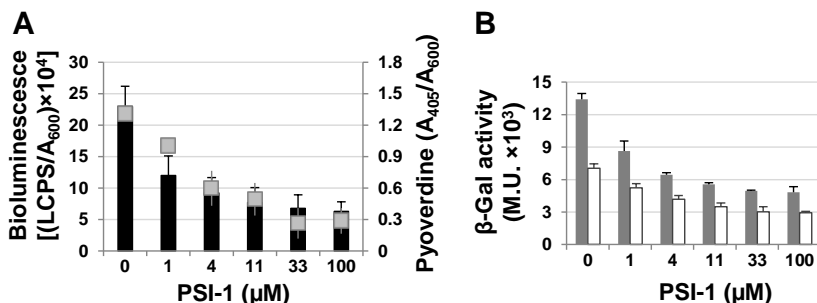

Figure 21. PSI-1 inhibits transcription of *pvd* genes and pyoverdine production. (A) Dose-response curves for bioluminescence emission (black bars, left y axis) and pyoverdine production (grey squares, right y axis) by the PAO1 P*pvdE::lux* reporter strain at 14 h of growth in the presence of increasing PSI-1 concentrations (0-100 µM). Values are normalized to the cell density of the bacterial cultures, and represent the mean (± SD) of four independent experiments. (B) Dose-response curves for β-galactosidase expression by PAO1 P*pvdA::lacZ* (grey bars) and PAO1 P*pvdD::lacZ* (white bars) at 14 h of growth in the presence of increasing PSI-1 concentrations (0-100 µM). Values represent the mean (± SD) of three independent experiments.

In order to confirm the anti-pyoverdine activity of PSI-1, this compound was purchased from a different supplier (Sigma-Aldrich) and tested against the PAO1 P*pvdE::lux* reporter strain. As shown in Figure 21A, PSI-1 had a very high inhibitory activity against both P*pvdE*-dependent bioluminescence and pyoverdine synthesis, with half-maximal inhibitory concentration (IC_{50}) values of 2 and 3 μM for P*pvdE* activity and pyoverdine production, respectively. PSI-1 also showed a similar inhibitory effect on the transcription of other pyoverdine synthesis genes, namely *pvdA* and *pvdD* (Figure 21B), suggesting that PSI-1 negatively affects the expression of the entire pyoverdine biosynthesis machinery. Moreover, given that the *pvdA* and *pvdD* promoters were fused to the β-galactosidase reporter gene *lacZ* (Figure 21B), this result ruled out that the effect of PSI-1 on the PAO1 P*pvdE::lux* reporter strain used for the screening assay could be due to direct and/or indirect inhibition of bioluminescence production.

3.3.2. PSI-1 is active against all pyoverdine types

As anticipated in Introduction, *P. aeruginosa* strains can produce only one out of three different pyoverdine types (I, II or III), which differ for the amino acid composition of their peptide chain (Visca et al. 2007). Each pyoverdine type is recognized and internalized by specific variants of the outer membrane pyoverdine receptor FpvA, which are named FpvAI, II or
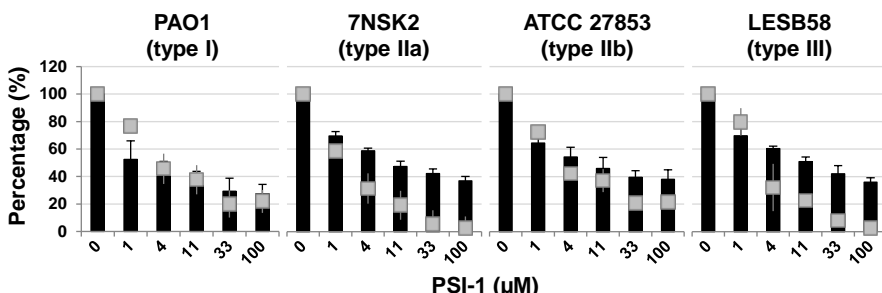

Figure 22. PSI-1 is active against *P. aeruginosa* strains producing different pyoverdine types. Dose-response curves for bioluminescence emission (black bars, left y axis) and pyoverdine production (grey squares, right y axis) by *P. aeruginosa* strains producing different types of pyoverdine (I, IIa, IIb and III), and carrying the genomic P*pvdE*::*lux* reporter fusion, at 14 h of growth in the presence of increasing PSI-1 concentrations (0-100 μM). Values are normalized to the cell density of the bacterial cultures, and expressed as percentage of the corresponding untreated control values. Each value represents the mean (± SD) of three independent experiments. The specific pyoverdine receptor type expressed by the different strains is shown for each panel.

III on the basis of the cognate pyoverdine type (Bodilis et al. 2009). Moreover, two distinct FpvAII sub-variants have been shown to bind type II pyoverdine, and have therefore been named FpvAIIa and FpvAIIb (Bodilis et al. 2009). Apart from structural variation of the receptor, the signaling pathway is conserved in all *P. aeruginosa* strains, irrespective of the type of pyoverdine they produce (Smith et al. 2005).

With the aim of assessing the activity of PSI-1 as anti-pyoverdine compound against any strain of *P. aeruginosa*, the P*pvdE::lux* reporter construct was inserted in prototypic *P. aeruginosa* strains producing different types of pyoverdine, and pyoverdine production and P*pvdE* activity were monitored as described above. As shown in Figure 22, the inhibitory effect of PSI-1 on both pyoverdine production and *pvdE* transcription was overall comparable among *P. aeruginosa* strains producing type I, II or III pyoverdine. This result indicates that the anti-pyoverdine activity of PSI-1 is not affected by either the chemical nature of the pyoverdine molecule or the type of the FpvA receptor.

3.3.3. The inhibitory activity of PSI-1 is independent of the pyoverdine signaling cascade

In order to assess whether PSI-1 can act as inhibitor of pyoverdine signaling, we tested the anti-pyoverdine activity of PSI-1 on a set of *P. aeruginosa* PAO1 mutant strains which are impaired in different steps of the pyoverdine signaling cascade (Figure 23). To this aim, the P*pvdE::lux* reporter construct was inserted into mutants unable to produce the outer membrane receptor FpvA (pyoverdine proficient, signaling-off), the anti-sigma factor FpvR (signaling constitutively activated) or the pyoverdine biosynthesis enzyme PvdA (pyoverdine deficient, signaling-off). In fact, the FpvA- and PvdA-deficient strains are defective in ferri-pyoverdine uptake and pyoverdine synthesis respectively, and are therefore unable to promote pyoverdine-mediated activation of the alternative sigma factor PvdS (signaling-off mutants). On the other hand, the FpvR-deficient mutant is unable to exert negative control on PvdS activity in the absence of ferri-pyoverdine-mediated signaling, thus resulting in signaling-insensitive full expression of PvdS-dependent genes (constitutive signaling-on mutant) (Figure 23). PSI-1 strongly inhibited pyoverdine production and *pvdE* gene expression in all mutants tested (Figure 23), ruling out the possibility that the pyoverdine signaling cascade could be the target of the anti-pyoverdine activity of PSI-1. However, although the inhibitory activity of PSI-1 was comparable between the wild type and signaling-off mutants (*pvdA* and *fpvA*), PSI-1 appeared to be slightly less active against the constitutive signaling-on *fpvR*
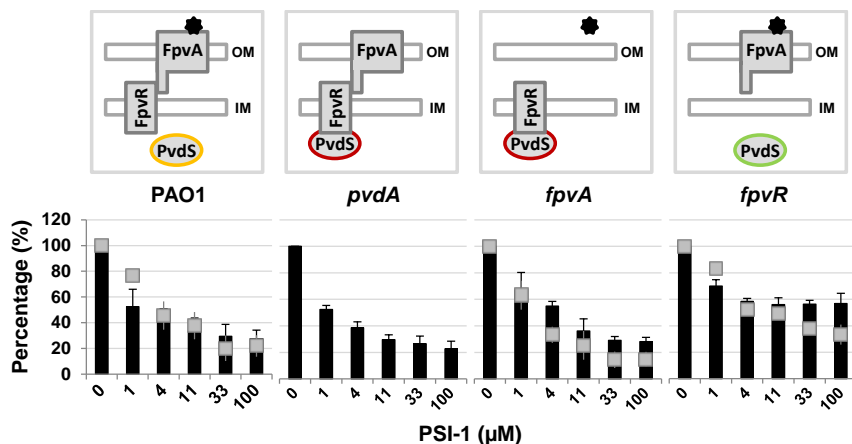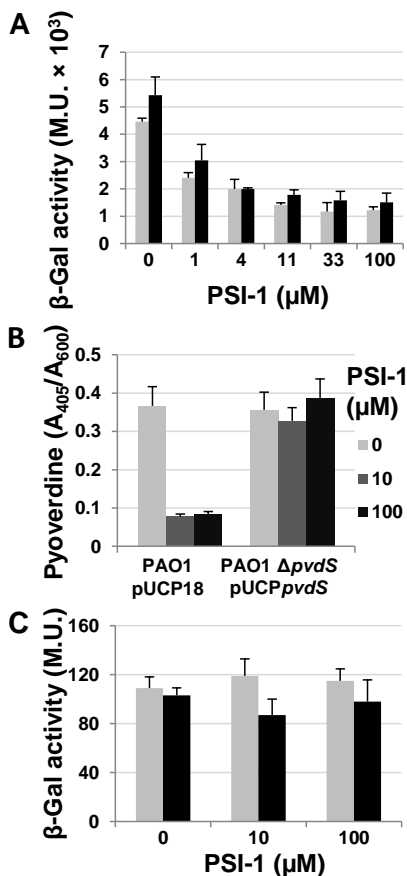
Figure 23. The inhibitory activity of PSI-1 does not depend on pyoverdine signaling. Dose-response curves for bioluminescence emission (black bars, left y axis) and pyoverdine production (grey squares, right y axis) by *P. aeruginosa* PAO1 and isogenic mutants defective in different steps of the pyoverdine signaling cascade, at 14 h of growth in the presence of increasing PSI-1 concentrations (0-100 µM). Values are normalized to the cell density of the bacterial cultures, and expressed as percentage of the corresponding untreated control values. Each value is the mean (± SD) of three independent experiments. The different behavior of mutants with respect to pyoverdine signaling is illustrated in the upper panels (the black star represents the pyoverdine molecule). The orange, red and green outlines of PvdS indicate the active, inactive and constitutively-active states of the pyoverdine signaling, respectively.

mutant (Figure 23). As reported above, in the absence of FpvR the pyoverdine signaling is in a constitutively active state, which corresponds to maximal activation of the PvdS intracellular pool. Thus, the lower activity of PSI-1 in the *fpvR* mutant could be a consequence of the higher activation of PvdS, suggesting that the intracellular levels and/or activity of PvdS could be critical for the pyoverdine inhibitory activity of PSI-1.

3.3.4. PSI-1 specifically inhibits transcription of the *pvdS* gene

As discussed in the previous paragraphs, PSI-1 inhibits transcription of several pyoverdine genes through a mechanism that appears independent of pyoverdine signaling. Given that transcription of all pyoverdine biosynthesis genes are directly controlled by the alternative sigma factor PvdS, we investigated the effect of PSI-1 on the expression of the *pvdS* gene. A plasmid harboring a transcriptional fusion between the *pvdS* promoter region and the β-galactosidase reporter gene was inserted into *P. aeruginosa*

A

β-Gal activity (M.U. × 10³)

PSI-1 (µM)

B

Pyoverdine (A_{405}/A_{600})

PSI-1 (µM)

- 0
- 10
- 100

PAO1 pUCP18   PAO1 ΔpvdS pUCPpvdS

C

β-Gal activity (M.U.)

PSI-1 (µM)

PAO1, and promoter activity was assessed at 8 and 18 hours of growth under iron-depleted conditions (TSBD medium), corresponding to mid-exponential and stationary phase of growth, respectively (Figure S8).

PSI-1 showed a dose-dependent inhibitory activity on *pvdS* transcription, and at concentrations ≥ 10 µM reduced the activity of the P*pvdS* promoter to about 25-30% of the untreated control during both exponential and stationary growth phases (Figure 24A). This strongly suggests that PSI-1 acts by inhibiting transcription of the *pvdS* gene, thus reducing the PvdS intracellular levels and, as a result, the expression of pyoverdine genes and pyoverdine production. To corroborate this hypothesis, a *pvdS*-deficient PAO1 mutant was transformed with a plasmid harboring the *pvdS* coding sequence under the control of a constitutive promoter (see Material & Methods for details), and the effect of PSI-1 on pyoverdine production by this strain was as-

Figure 24. PSI-1 specifically inhibits *pvdS* transcription. (A) Dose-response curves for β-galactosidase expression by PAO1 P*pvdS*::*lacZ* during exponential (grey bars) and stationary phase of growth (black bars) in the presence of increasing PSI-1 concentrations (0-100 µM). (B) Effect of PSI-1 (0-100 µM) on pyoverdine production by *P. aeruginosa* PAO1 carrying the empty vector pUCP18 and by its isogenic *pvdS* mutant carrying the pUCP*pvdS* construct at 8 h of growth. (C) Effect of PSI-1 (0-100 µM) on β-galactosidase expression by PAO1 P*pchR*::*lacZ* during exponential (grey bars) and stationary phase of growth (black bars). Values represent the mean (± SD) of at least three independent experiments.

sessed during exponential growth. As shown in Figure 24B, although PSI-1 strongly reduced pyoverdine production by the PAO1 wild-type strain carrying the empty plasmid, it had no effect on pyoverdine production by the *pvdS* mutant strain which constitutively expresses PvdS, confirming that PSI-1 (directly or indirectly) exerts its negative effect on the activity of the *pvdS* promoter.

This result led us to investigate whether the effect of PSI-1 on *pvdS* transcription is specific or derives from a general alteration of intracellular iron homeostasis. To this aim, the transcription of the iron-regulated *pchR* gene, encoding a Fur-dependent transcriptional regulator which controls the expression of genes involved in the synthesis of the second siderophore pyochelin, was monitored in the absence or in the presence of PSI-1 by means of a reporter plasmid containing a P*pchR::lacZ* fusion. Notably, PSI-1 had no effect on the activity of the *pchR* promoter in the PAO1 wild type, either in exponential or stationary phase (Figure 24C).

Overall, these results indicate that PSI-1 can specifically inhibit transcription arising from the *pvdS* promoter, plausibly by interfering with a regulator (or a regulatory pathway) which is involved in the control of *pvdS* but not *pchR* expression. Experiments are in progress to determine the molecular target(s) and the mode of action of PSI-1 in *P. aeruginosa*.

3.3.5. PSI-1 down-regulates the expression of PvdS-dependent virulence genes

The finding that PSI-1 acts by inhibiting *pvdS* gene expression could imply that this compound is also endowed with an effect on the expression of PvdS-regulated virulence factors other than pyoverdine. To verify this hypothesis, we investigated the effect of PSI-1 on the expression of two major virulence factors of *P. aeruginosa*, the endoprotease PrpL and exotoxin A, which are directly and/or indirectly regulated by PvdS (Ochsner et al. 2002).

The expression of PrpL and exotoxin A was monitored at both the transcriptional level, by using *PprpL::lacZ* and *PtoxA::lacZ* transcriptional fusions, and the post-translational level, using anti-exotoxin A Western blot analysis and a specific enzymatic assay to measure PrpL activity (Imperi et al. 2010). Interestingly, the expression of both *toxA* and *prpL* was strongly impaired at the transcriptional level in PSI-1-treated cultures with the respect to untreated controls, during both exponential and stationary phase (Figure 25). Conversely, exotoxin A and PrpL levels in culture supernatants were markedly affected by the PSI-1 treatment only in exponentially growing cultures, while they were similar between PSI-1-treated and -untreated cul-

tures during the stationary phase of growth (Figure 25). Although the apparent inconsistency between transcriptional and post-translational results can reasonably be explained by a different sensitivity of the two assays, it cannot be excluded that the PSI-1 treatment can also have an effect on exotoxin A and PrpL production at a translational and/or post-translational level, resulting in either enhanced translation rate or increased stability of the two proteins.
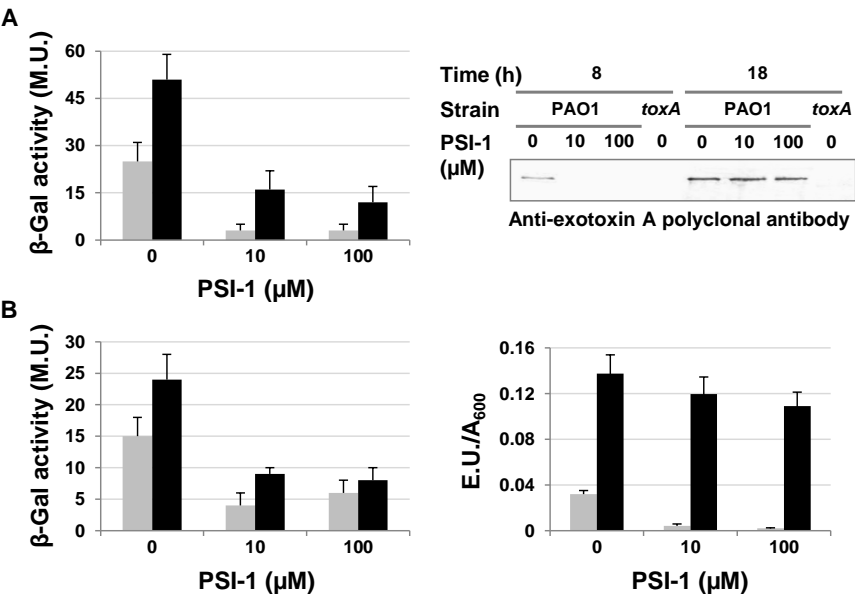
A

B

Figure 25. PSI-1 down-regulates PvdS-dependent virulence gene expression. (A) Effect of PSI-1 (0-100 μM) on β-galactosidase expression by PAO1 PtoxA::lacZ (left panel) and exotoxin A levels in PAO1 culture supernatants (right panel) during exponential (grey bars or 8 h) and stationary phase of growth (black bars or 18 h). (B) Effect of PSI-1 (0-100 μM) on β-galactosidase expression by PAO1 PprpL::lacZ (left panel) and PrpL enzymatic activity in PAO1 culture supernatants (right panel) during exponential (grey bars or 8 h) and stationary phase of growth (black bars or 18 h). Values represent the mean (± SD) of three independent assays. The Western blot is representative of three independent experiments giving similar results.

4. Discussion

P. aeruginosa still represents a huge public health issue, as it remains one of the leading nosocomial pathogens and a major threat to patients with CF. Its adaptability to many environmental condition and resist to antibiotic treatments presents a serious clinical and therapeutic challenge (van Delden & Iglewski 1998; Lyczak et al. 2002; Driscoll et al. 2007; Lister et al. 2009).

Under a clinical perspective, the emergence and spread of bacteria resistant to all antimicrobial agents calls for the development of alternative treatments. A novel and promising approach is the search for compounds able to inhibit specific virulence determinants, as opposed to inhibitors of bacterial growth *per se*. The use of these new-generation anti-virulence drugs could reduce the damage to the host, provide the immune system with a better chance of clearing the infection, and circumvent the problem of the emergence of drug-resistant strains. Furthermore, this approach is not expected to affect the community of beneficial bacteria present as the normal commensal flora of host (Williams 2002; Hentzer & Givskov 2003; Cegelski et al. 2008).

Although bacteria are capable of living as individual cells, their survival within the infected host depends, among other factors, on the development of complex communities that have the ability to coordinate their behavior in a cell density-dependent manner. The QS and pyoverdine signaling regulatory systems control the group-behavior of *P. aeruginosa* and the expression of several virulence factors, in addition to biofilm formation and antibiotic resistance, ultimately enhancing the fitness of *P. aeruginosa* in the host environment (Smith & Iglewski 2003a; Diggle et al. 2006; Rasmussen & Givskov 2006; Rampioni et al. 2007; Visca et al. 2007). Therefore QS and pyoverdine signaling are regarded as ideal targets for the development of an anti-virulence therapy against *P. aeruginosa*.

The aim of my PhD research was the setting-up, validation and employment of novel high-throughput screening systems to detect molecules suppressing *P. aeruginosa* QS and pyoverdine signaling. For this purpose, two screening systems were developed, based on either the PA14/PA14-R3 co-cultivation system or the PAO1 P*pvdE::lux* reporter strain, which allow to detect changes in the activation of QS and pyoverdine signaling caused by compounds endowed with inhibitory activity against any process related to QS and pyoverdine signaling activity (Figures 11 and 19).

Besides its suitability to be employed in the screening for QS inhibitors, the PA14-R3 biosensor showed additional features which could be exploited. This biosensor showed a very high sensitivity and specificity for the main

QS signal molecule $3OC_{12}$-HSL, suggesting that it could represent a convenient and easy-to-handle tool to quantify the $3OC_{12}$-HSL over a wide range of concentrations in micro-volumes of laboratory or clinical samples. Commonly used methods for detecting AHLs relies either on physical-chemical techniques or bacterial cell-based reporter systems. Due to the absence of chromogenic or spectrophotometrically unique feature in AHL molecules, the application of conventional physical-chemical analytical methods for their quantification is usually based on liquid or gas chromatography procedures coupled with mass spectrometry or electrospray ionization, such as LC–MS, GC–MS and LC-ESI (Shaw et al. 1997; Morin et al. 2003; Cataldi et al. 2007, 2008). These procedures are expensive, labor-intensive, and require sophisticated laboratory equipments. Although some of the latest techniques may allow a direct analysis of the sample (Cataldi et al. 2008), in most cases the AHL has to be extracted from the culture or clinical sample by means of a low-efficiency (ca. 75%) partitioning with an organic solvent (Ravn et al. 2001; Shaw et al. 1997), and then concentrated several-fold, ultimately requiring high volumes (minimum 500 μl) of starting samples. While such procedures are still required for a more refined separation and chemical identification of different AHLs, biosensors constitute an alternative method for fast, sensitive, and cost-effective quantification assays. (D'Souza 2001; Steindler & Venturi 2007). Indeed, the PA14-R3 biosensor allows the direct measurement of $3OC_{12}$-HSL in microvolumes and the fast analysis of a large number of samples simultaneously.

Up to date, several biosensors have been developed for AHL detection. These biosensors usually involve an AHL synthase mutant containing an AHL-inducible promoter which positively regulates the transcription of a reporter gene. Examples of AHL biosensors employ different reporter systems, such as β-galactosidase (Pearson et al. 1997), ice nucleation protein (*inaZ*) (De Angelis et al. 2007), fluorescent proteins (Riedel et al. 2001; Burmølle et al. 2003), violacein pigment (Blosser & Gray, McClean et al. 1997) and *lux* bioluminescence (Boettcher & Ruby 1995, Bernier et al. 2008; Kumari et al. 2006, Yan et al. 2007). With respect to other reporter system, the *luxCDABE* operon appears more convenient in so far as it shows no background noise in *P. aeruginosa* and provides direct signal detection without addition of exogenous substrate (Meighen & Szittner 1992). Many of these biosensors have been developed to detect a wide range of AHLs, at the cost of a reduced specificity for single AHLs. The impossibility to infer the contribution of each AHL to the overall biosensor response limits their efficiency in the quantification of AHLs in biological samples. Other biosensors show increased specificity for a single AHL but lack of the

sensitivity required for a possible use in a quantitative analysis of microvolume samples. Conversely, PA14-R3 displays both the wide range of sensitivity (Figures 8 and 9) and the high specificity for 3OC12-HSL (Table 1), which opens the possibility of a broad range usage of this new tool for quantitative measurements of 3OC12-HSL.

Indeed, results reported in this thesis confirm that the PA14-R3 biosensor can be used as a $3OC_{12}$-HSL quantification tool in studies aimed at investigating mutations and/or cultural conditions affecting $3OC_{12}$-HSL production in *P. aeruginosa*. Although limited to the analysis of CF patients who are not undergoing antibiotic treatment, it was also demonstrated that PA14-R3 can be efficiently employed to measure $3OC_{12}$-HSL levels in CF sputum samples (Table S2). At present, very few studies investigated the role of QS during *P. aeruginosa* colonization of the CF lung (Erickson et al. 2002; Middleton et al. 2002; Ward et al. 2003), and the majority of these studies suffered from severe limitations, including the small number of samples analysed, primarily due to the cost and complexity of the available QS signal quantization methods. These limitations can now be overcome by PA14-R3, which represents a cost-effective, user friendly tool for clinical studies aimed at determining the relationship between $3OC_{12}$-HSL levels, *P. aeruginosa* colonization, and the severity of the chronic infection.

With regard to the screening system based on PA14/PA14-R3, it must be pointed out that the innovative approach relying on the co-cultivation of a signal reporter strain with its signal-producing wild type strain warrants a number of useful features compared to previously-developed screening systems for QSI.

Inhibition of QS circuits can be achieved in a number of ways, such as blockage of signal production and transport, inactivation or entrapment of the signaling molecules, interference with QS receptors, and by inhibition of transcription of QS-related genes. So far, the most prominent examples of QSI compounds are the halogenated furanones from the red alga *Delisea pulchra*, able to interfere with acyl-HSL-based QS systems in different bacteria. Although natural furanones do not efficiently inhibit QS in *P. aeruginosa*, synthetic furanone C-30 was reported to block the LasR-dependent QS cascade, reduce biofilm persistence and promote the clearance of bacteria in a mouse model of chronic lung infection (Hentzer & Givskov 2003; Wu et al. 2004). The mechanism of action of FC30 remains poorly understood, although it is proposed to stimulate degradation of acyl-HSL receptor proteins by cellular proteases (Manefield et al. 2002). Sadly, halogenated furanones are generally very reactive and highly toxic, and several derivatives showed mutagenic activity (Ramos et al. 2000), which prevents the

use of this group of compounds in medicine (Hentzer & Givskov 2003).

A number of screening systems for QS inhibitors in *P. aeruginosa* have been developed. Smith et al. (2003a, b) identified some strong inhibitors of the QS systems of *P. aeruginosa* by employing a reporter system, dependent on the *lasI* promoter, for the screening of a library of synthetic $3OC_{12}$-HSL analogues. Müh et al. (2006) developed an ultra-high-throughput cell-based assay to screen a library of ca. 200,000 compounds for the presence of inhibitors of LasR-dependent gene expression. The *rsaL* promoter was chosen to drive the reporter-screen, as *rsaL* is one of the most sensitive LasR-controlled genes. Despite the large number and broad range of chemical structure of the compounds tested, this screening failed to identify classes of inhibitors other than $3OC_{12}$-HSL analogues. Although these inhibitors were reported to reduce the production of QS-controlled extracellular virulence factors, some AHL signal molecules function also as virulence factors *per se*, as they possess strong immune-modulatory activity and were reported to affect vascular smooth muscle contractions, thus preventing the therapeutic use of AHL-based antagonists (Smith et al. 2002; Hentzer & Givskov 2003; Pritchard 2006; Lawrence et al. 1999; Gardiner et al. 2001). Other interesting screening systems for QS inhibitors were expressly designed to circumvent the problem of false positives emerging due to growth (and hence QS) inhibition in the screening process (Rasmussen et al. 2005a, b). These systems employ bacteria (either *Escherichia coli* or a *lasI rhlI* mutant of *P. aeruginosa*) which do not produce any signaling molecule by itself and carry a QS-controlled killer gene encoding a toxic gene product. In the presence of AHLs, the killer gene is expressed and bacterial growth is arrested. Conversely, in the presence of a non-toxic QSI compound the killer gene is not activated and the bacteria are able to grow. These systems were employed in the screening of libraries of several extracts from plants and fungi, as well as pure chemical compounds, and an inhibitory effect was identified for patulin, penicillic acid (PA), garlic extract, and 4-nitro-pyridine-N-oxide (4-NPO). Transcriptomic (GeneChip) analysis showed that these compounds exert a strong effect on gene expression, especially on virulence genes regulated by QS. Furthermore, these compounds were found to be able to cause significant reduction of infections in mice and to increase *P. aeruginosa* susceptibility to tobramycin. Unfortunately, both patulin and PA are mycotoxins and therefore not suitable for treatment of human patients. The quantity of garlic extract required to determine significant QS inhibition would correspond to a dose of 50 garlic bulbs per day in a human adult, amount which probably would have severe side effects (Rasmussen & Givskov 2006; Rasmussen 2005a,b). Finally, 4-NPO is a toxic

pesticide (Hazardous Substances Data Bank, http://toxnet.nlm.nih.gov/cgi-bin/sis/search/r?dbs+hsdb:@rn+1124-33-0).

At the best of our knowledge, the majority of the screening systems so far developed for the identification of inhibitors of *P. aeruginosa* signaling systems involves the exogenous addition of signal molecule. This restricts the range of potential inhibitors which can be identified to compounds exerting an inhibitory activity at the level of signal recognition, as highlighted by the identification of many AHL-based antagonists targeting the $3OC_{12}$-HSL signal receptor LasR. In contrast, very few attempts have been made to identify inhibitors of other steps of the Las regulatory circuit (*e.g.* signal synthesis and transport) as well as of other QS systems (Persson *et al.*, 2005). Notably, several researches showed that while C_4-HSL is able to diffuse passively across bacterial membranes, $3OC_{12}$-HSL molecules are instead actively transported across the cell by several nonspecific multidrug efflux pumps, including MexAB-OprM, MexEF-OprN, and MexGHI-OpmD (Pearson et al. 1999; Köhler et al. 2001; Aendekerk et al. 2002), and that the synthesis of these molecules is finely controlled also in response to environmental and metabolic stimuli. These findings suggest that other still-unidentified proteins could affect the *P. aeruginosa* signaling systems and broaden the number of potential targets for inhibitors (Venturi 2006; Duan & Surette 2007; Gaines et al. 2007).

In this perspective, we developed an alternative screening strategy based on the PA14/PA14-R3 co-cultivation system, which does not need exogenously added synthetic $3OC_{12}$-HSL. Indeed, the signal molecule is provided by the *P. aeruginosa* producer strain (PA14) during co-cultivation with the biosensor (PA14-R3). Besides being novel, handy and cost-effective, this approach has the advantage of allowing the identification of compounds targeting not only LasR activity, but any cellular process critical for QS response. In particular, this system would allow the detection of any inhibitory compound targeting one or more of the following steps: i) expression/activity of signal molecule biosynthesis enzymes; ii) expression/activity of signal receptors; iii) import/export of signal molecules (Figure 11). This could represent a further asset in the search for novel QSI-based drugs active against *P. aeruginosa* infection.

Up to date, no studies have been conducted to verify the possibility of blocking the pyoverdine signaling system. In 2007, Kaneko and colleagues investigated the possibility to use gallium nitrate as inhibitor of bacterial iron metabolism. Ga(III) has an ionic radius nearly identical to that of Fe(III), and can substitute for Fe in many biologic systems. However, unlike Fe^{3+}, Ga^{3+} is cannot be reduced under physiological conditions and unable

to take part in oxidation and reduction processes, thus inhibiting Fe-dependent processes. Ga(III) was found able to inhibit *Pseudomonas aeruginosa* growth and biofilm formation, and was effective in reducing mortality in two murine models of acute and chronic lung infection. Nevertheless, gallium is a strong growth inhibitor in *P. aeruginosa*, and as for antibiotics, resistances might emerge under the selective pressure of the treatment.

More recently, a small-scale screening has been performed to find inhibitors of PvdQ, a periplasmic acylase essential for pyoverdine maturation, which also acts as a quorum quencher due to its ability to degrade long-chain AHLs (Drake & Gulick 2011). Two compounds able to inhibit the activity of purified PvdQ in vitro were identified. However, the effect of these molecules on pyoverdine production and iron uptake by *P. aeruginosa* cells was not determined. Further studies are mandatory to assess the suitability of these compounds as anti-*P. aeruginosa* drugs as well as their toxicity to humans.

Differently from QS, many crucial steps of the pyoverdine regulative cascade occur on (or through) the bacterial cell envelope, and potential molecular target(s) should be more easily accessible to active compound(s) that do not have to cope with *P. aeruginosa* cell wall impermeability and constitutive expression of efflux pumps (Mesaros et al. 2007). Thus pyoverdine signaling represents an ideal target for the development of inhibitors against *P. aeruginosa* virulence. However this does not exclude that inhibitors targeting processes taking place at different levels of the cell might be identified. Inhibition of pyoverdine signaling can be achieved (i) on the outer membrane surface, either by blocking the binding of pyoverdine to its receptor Fpva or by preventing the pyoverdine-dependent activation of FpvA; (ii) in the periplasmatic space, by blocking the signal transmission between FpvA and the antisigma factor FpvR; (iii) at the level of the cytoplasmic membrane, by affecting the interaction between FpvR and the alternative sigma factor PvdS; (iv) in the cytoplasm, either by impeding the interaction of PvdS with the core RNA polymerase, or by repressing the transcription of *pvdS* or other genes involved in pyoverdine biosynthesis (Figure 19).

As documented by the previous screening attempts, not all of the inhibitor molecules identified upon screening could be used practically; toxicity analysis and *in vivo* efficacy evaluation might significantly narrow the repertoire of potential candidates. In order to maximize the number of positive hits potentially applicable to human therapy the PA14-R3/PA14 co-culture system and the PAO1 P*pvdE::lux* reporter were employed in a pilot screening for potential QSI and pyoverdine inhibitors based on the selective optimization of side activity (SOSA) approach (Wermuth 2006). This approach

relies on the possibility that drugs already used in medicine can also possess pharmacological side effect(s), which could act on molecular target(s) often unrelated to the primary therapeutic activity of the compound. The identified side activity could be either improved through a specific drug optimization program or, alternatively, directly tested in clinical trials, strongly reducing the time and costs usually required for the discovery and clinical transfer of new drugs.

In agreement with the SOSA approach, we screened a chemical library consisting of 1,120 FDA-approved drugs, selected for their high chemical and pharmacological diversity as well as for their known bioavailability and safety in humans, for potential QS and/or pyoverdine signaling inhibition. The two screening approaches were able to identify compounds by virtue of their capacity to reduce the activation of reporter strains under a given threshold, in either the QS or the pyoverdine signaling process. For each screening system, a small number of hit compounds were identified, and the most promising of them - in terms of safety, efficacy, active concentration, and ineffectiveness on bacterial growth - were chosen for further investigation.

The anti-QS compound QSI-1 showed anti-QS activity at physiologically relevant concentrations (Figure 14), and was able to strongly inhibit many $3OC_{12}$-HSL-dependent virulence phenotypes (Figure 17A, B, C). QSI-1 was found to be effective in protecting *G. mellonella* larvae from *P. aeruginosa* acute infection (Figure 18), and experiments are now in progress in collaboration with A. Bragonzi (Infections and Cystic Fibrosis Unit, San Raffaele Scientific Institute, Milan, Italy) to assess the efficacy of QSI-1 in treating and/or eradicating chronic lung infection by *P. aeruginosa* in a mouse model (Bragonzi et al. 2009). QSI-1 is an anthelmintic drug, effective against cestodes such as dwarf tapeworm (*Hymenolepis nana*), beef tapeworm (*Taenia saginata*) and fish tapeworm (*Diphyllobothrium latum*). QSI-1 was shown to inhibit oxidative phosphorylation in parasitic cestodes. In adults, QSI-1 is administered orally, at the dose of 2g/day for up to one week, corresponding to approximately 8-10μM, concentration that we observed sufficient to determine QS inhibition. In mice, an $LD_{50} > 1,500$ mg/kg (b.w.) was observed, and no carcinogenic, teratogenic or mutagenic effect was reported. These pharmacological properties support a possible use of QSI-1 in humans.

The anti-pyoverdine compound PSI-1 showed high inhibitory activity on pyoverdine production (Figure 21). Our analyses highlighted that PSI-1 exerts its anti-pyoverdine activity by reducing the transcription of the *pvdS* gene. This gene encodes an alternative sigma factor responsible for the expression not only of pyoverdine biosynthesis and transport genes, but also

other genes which encode important virulence factors, such as exotoxin A and endoprotease PrpL (Ochsner et al. 2002). Accordingly, PSI-1 was effective in reducing expression of exotoxin A and PrpL, although this effect was stronger in exponentially-growing than in stationary-phase cultures (Figure 25). Interestingly, PSI-1 was found to be effective against any *P. aeruginosa* strain tested, irrespective of the specific type of pyoverdine produced (Figure 22), strongly suggesting that PSI-1 could potentially be used against all strains of *P. aeruginosa*. This is consistent with the notion that all *P. aeruginosa* strains sequenced so far, as well as other fluorescent *Pseudomonas* species, harbour the *pvdS* gene (or an orthologue of it).

PSI-1 is an antimycotic drug. Inside the target microorganism, the drug is converted into a series of intermediates. Two metabolites are responsible of antifungal activity, one by hampering RNA synthesis and consequently inhibiting transcription of specific proteins, the other by impairing DNA synthesis. The recommended daily dose of 50 to 150 mg/kg of bodyweight orally, for up to 7 days, corresponds to approximately 1 mM, concentration far superior than that required to inhibit pyoverdine signaling in our experimental setting. Also these pharmacological properties support a possible use of PSI-1 in humans.

Although out of the scope of this thesis, it is tempting to briefly speculate on the possible molecular targets of QSI-1 and PSI-1. With regard to QSI-1, the ability of this compound to equally reduce the bioluminescence emitted by the PA14-R3/PA14 co-culture and the PA14-R3 alone in the presence of exogenously-added $3OC_{12}$-HSL suggests that QSI-1 inhibits a component of the QS network involved in signal sensing, rather than the synthesis of the signal molecule. Moreover, the analysis of $3OC_{12}$-HSL production in the presence of different QSI-1 concentrations revealed that 5 μM QSI-1 is able to cause maximal reduction (about 50%) of $3OC_{12}$-HSL levels, while higher QSI-1 concentrations had no further effect on $3OC_{12}$-HSL production (Figure 15). This evidence suggests that QSI-1 could act on a protein or a regulatory system which modulates the *las* response, rather than on a crucial QS regulator such as LasR. Notably, QSI-1 belongs to a class of molecules which have been reported as potential inhibitors of two-component systems in bacteria (Macielag et al., 2002). Given that some two-component systems have been shown to play a role in the *P. aeruginosa* QS response (Filloux et al. 1988; Reimmann et al. 1997; Dong et al. 2005), it would be interesting in the future to verify if these systems are possible targets of QSI-1.

Concerning PSI-1, we clearly demonstrated that this compound acts at the level of *pvdS* transcription. Notably, PSI-1 had no effect on the expression of another iron-regulated and Fur-dependent gene, *pchR,* which is involved

in the production of the second siderophore pyochelin (Heinrichs & Poole 1996; Crosa et al. 2002). This finding rules out the hypothesis that PSI-1 could have a generic effect on the *P. aeruginosa* iron response, while it suggests that the target of PSI-1 could be a regulator of *pvdS* transcription. In the last few years, some studies have proposed or experimentally-demonstrated the existence of further regulators of *pvdS* gene expression in addition to the Fur repressor protein (Imperi et al. 2010; Funken et al. 2011). Whether PSI-1 inhibits one of these ancillary regulators or a still-unidentified one remains to be determined, and experiments are in progress to figure out the mechanism of action and the molecular details of PSI-1-dependent inhibition on *pvdS* transcription.

The results just discussed lead to the conclusion that QSI-1 and PSI-1 are effective in inhibiting QS- and pyoverdine-dependent virulence in vitro, and offer the opportunity of further development of these compounds for an anti-*P. aeruginosa* therapy. A better characterization of the molecular mechanisms involved in the inhibitory action of QSI-1 and PSI-1 is certainly needed to make possible their utilization as the starting point for a drug optimization program aimed at increasing their affinity for the new target, while decreasing the affinity for the original one. Although a therapy based on the inhibition of virulence is not effective as a stand-alone treatment, its combination with conventional antibiotic therapies might facilitate the role of the immune system in clearing the infection and increase the efficacy of antimicrobial treatments, thus providing better chances to effectively eradicate the pathogen. Overall, this work resulted in the construction and validation of two distinct screening systems for virulence inhibitors against *P. aeruginosa*, and allowed the identification of two promising inhibitors of *P. aeruginosa* pathogenicity. These findings represent an important step towards the development of an anti-virulence therapy for treating *P. aeruginosa* infections.

Acknowledgements

List of abbreviations

3OC$_{12}$-HSL	N-3-oxo-dodecanoyl homoserine lactone
4NPO	4-NitroPyridine 1-Oxide
ABC	ATP Binding Cassette
AHL	Acyl-Homoserine-Lactone
AME	Aminoglycoside Modification Enzymes
Asialo GM	Asialoganglioside Gangliotetraosylceramide
Bcc	*Burkholderia cepacia* complex
bp	Base pair
C10	N-decanoyl- homoserine lactone
C4-HSL	N-butyryl homoserine lactone
C6	N-hexanoyl- homoserine lactone
C8	N-octanoyl- homoserine lactone
CAMP	Cationic Antimicrobial Peptide
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CM	Cytoplasmic Membrane
EPS	Extracellular Polymeric Substance
ESBL	Extended-Spectrum β-Lactamase
ESI	ElectroSpray Ionization
ETA	Exotoxin A
EU	Enzymatic Units
FC	Fibrosi Cistica
FC30	Furanone C30
FDA	Food and Drug Administration
GC	Gac Chromatography
HCN	Hydrogen Cyanide
HHQ	2-heptyl-4-quinolone
HSL	Homoserine Lactone
IC50	half maximal inhibitory concentration
Ig	Immunoglobulin
IL-8	Interleukin-8
IS	Iron Starvation
LB	Luria-Bertani Broth
LC	Liquid Chromatography
LCPS	Light Counts Per Second
LD50	Median Lethal Dose
LPS	Lipopolysaccharide

MDR	Multi Drug Resistance
MFP	Membrane Fusion Protein
MIC	Minimal Inhibiting Concentration
MS	Mass Spectrometry
MU	Miller Units
NRPS	Non-Ribosomal Peptide Synthases
OM	Outer Membrane
OMP	Outer Membrane Protein
ORF	Open Reading Frame
PA	Penicillic Acid
PQS	Pseudomonas Quinolone Signal (2-heptyl-3-hydroxy-4-quinolone)
PSI	Pyoverdine Signaling Inhibitor
QRDR	quinolone resistance determining region
QS	Quorum Sensing
QSI	Quorum Sensing Inhibitor
RhoGAP	Rho GTPase-activating-protein
RNAP	RNA Polymerase
RND	Resistance-Nodulation-Division
SOD	Super Oxide Dismutase
SOSA	Selective Optimization of Side Effects
TLC	Thin-Layer Chromatography
TSBD	Dyalized Trypitcase Soy Broth

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