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Quorum sensing in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients

Il quorum sensing in ceppi di *Pseudomonas aeruginosa* isolati da pazienti affetti da fibrosi cistica

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RIASSUNTO

Pseudomonas aeruginosa, appartenente al gruppo dei batteri patogeni definiti "ESKAPE" (Rice, 2008; Boucher *et al*., 2009;Pendleton *et al*., 2013), causa diversi tipi di infezione in pazienti ospedalizzati ed immunocompromessi. Questo batterio inoltre è causa di infezioni polmonari croniche che rappresentano la principale causa di morte nei pazienti affetti da Fibrosi Cistica (FC), una malattia genetica che colpisce circa 1/2500 nati nella popolazione caucasica (Welsh *et al*., 2001; Talbot *et al*., 2006; Driscoll *et al*., 2007; Döring *et al*., 2010; Stefani *et al*., 2017). Le infezioni da *P. aeruginosa* sono difficili da eradicare poiché questo microorganismo è dotato di meccanismi di resistenza innata agli antibiotici ed è in grado di acquisire facilmente nuove resistenze in ambiente ospedaliero (Latifi *et al*., 1995; Talbot *et al*., 2006; Driscoll *et al*., 2007).
La produzione di fattori di virulenza e la formazione di biofilm in *P. aeruginosa* sono finemente regolati dal quorum sensing (QS), un sistema comunicazione intercellulare che controlla l'espressione genica in funzione della densità cellulare (Smith and Iglewski, 2003; Lee *et al*., 2006; Williams and Càmara 2009; Papenfort and Bassler, 2016). *P. aeruginosa* ha quattro sistemi di QS interconnessi fra loro e gerarchicamente organizzati: in terreno ricco, il sistema *las* è al vertice della gerarchia poiché è necessario per la completa attivazione degli altri sistemi di QS, fra cui il sistema *pqs* (Williams and Càmara, 2009; Papenfort and Bassler, 2016). Il sistema *las* si basa sul recettore LasR (codificato dal gene *lasR*) e sulla sintasi LasI (codificata dall'omologo gene *lasI*), che produce la molecola segnale *N*-(3-oxododecanoil)omoserina lattone ($3OC_{12}$-HSL) (Schuster and Greenberg, 2006). Il sistema di QS *pqs* si basa su molecole segnale che appartengono alla classe degli alchil chinoloni (AQs). Gli enzimi codificati dall'operone *pqsABCDE* sono necessari per la sintesi della molecola segnale 2-eptil-4-chinolone (HHQ) che è convertita in 2-eptil-3-idrossi-4-chinolone (PQS) dalla monoossigenasi PqsH (codificata dal gene omologo, *pqsH*). PQS e HHQ legano e attivano il regolatore trascrizionale PqsR. Un altro alchil chinolone prodotto da *P. aeruginosa* a livello µM è il 4-idrossi-2-eptilchinolone-*N*-ossido (HQNO), per la cui produzione è necessaria la monoossigenasi PqsL (codificata dal gene *pqsL*). L'HQNO non attiva il recettore PqsR e non è considerato una molecola segnale del QS (Deziel *et al*., 2004; Rampioni *et al*., 2016), ma considerato un potente inibitore del complesso *bc1* del citocromo nei batteri Gram-positivi contribuendo così alla competizione di *P. aeruginosa* nell'ambiente (Heeb *et al*., 2011).
Sia il recettore LasR che il recettore PqsR, una volta attivati dalle rispettive molecole segnale, controllano positivamente l'espressione di numerosi di geni, compresi quelli per l'espressione di fattori di virulenza e la formazione di biofilm (Williams and Càmara, 2009; Papenfort and Bassler, 2016). Infatti, i mutanti di *P. aeruginosa* nel QS sono meno virulenti rispetto al wild type in modelli

animali e vegetali di infezione. Per questo motivo il QS è ritenuto un ottimo bersaglio per lo sviluppo di farmaci anti-virulenza (Rasko and Sperandio, 2010; Lasarre and Federle, 2013; Rampioni *et al*., 2014).

Il polmone è un ambiente eterogeneo ed ostile per i batteri, ad esempio il sistema immunitario causa stress ossidativo e nitrosativo, inoltre il muco viscoso tipicamente prodotto dai pazienti affetti da FC ostacola la *clearance* polmonare e causa stress osmotico. In aggiunta a questi fattori, anche le concentrazioni di antibiotico a dosi sub-MIC e la competizione con altri microorganismi rappresentano fattori di stress per il batterio. Dopo una serie di infezioni intermittenti, un ceppo di *P. aeruginosa* instaura un infezione cronica, che può durare anche decenni. Questa può essere tenuta sotto controllo con continui trattamenti antibiotici, ma non può essere eradicata. L'infiammazione polmonare causata dall'infezione cronica causa, nel tempo, il deterioramento della funzione respiratoria e porta alla morte del paziente. Durante gli anni di infezione cronica, la popolazione di *P. aeruginosa* va in contro ad un processo evolutivo che porta a variabilità genotipica e fenotipica, riscontrabile anche a livello del singolo paziente. Nel tempo, emergono dei ceppi di *P. aeruginosa* con fenotipi particolarmente adattati al polmone del paziente FC, tra cui antibiotico-resistenza, mancata produzione di fattori di virulenza, aumentata produzione di alginato o altri esopolisaccaridi e fattori associati alla formazione di biofilm (Folkesson *et al*., 2012; Winstanley *et al*., 2016). Sono stati anche isolati mutanti che esprimono un fenotipo litico, che si ipotizza sia causato dall'inattivazione del gene *pqsL*: D'Argenio *et al*., 2002), e mutanti nel gene *lasR* (Smith *et al*., 2006; Hoffman *et al*., 2008; Feltner *et al*., 2016). Quest'ultima osservazione ha aperto un dibattito sulla opportunità di utilizzare farmaci anti-virulenza mirati ad inibire il sistema di QS basato $3OC_{12}$-HSL nella terapia dei malati di FC (Feltner *et al*., 2016). Lo scopo generale di questo lavoro di tesi è stato quello di comprendere meglio il ruolo dei geni *pqsL* e *lasR* nel sistema di QS e nella fisiologia di *P. aeruginosa,* nel contesto dell'infezione polmonare cronica *in* FC.

In particolare, gli esperimenti descritti nel capitolo dimostrano che la mancata produzione di HQNO nel mutante di *P. aeruginosa* PAO1 *pqsL* causa l'accumulo di HHQ, tale accumulo induce la transizione del ciclo litico del profago Pf4 con conseguente la formazione di placche di lisi durante la crescita confluente su piastra di PAO1 *pqsL* (colony biofilm). Inoltre è stato analizzato il fenotipo di 101 ceppi di *P. aeruginosa* provenienti da pazienti FC, dei quali 34 esprimono il fenotipo litico. Inoltre, 26 su 34 (il 76%) ceppi FC litici sono mutati nel gene *pqsL*. Inoltre il fenotipo wild type (non litico) è stato ripristinato in 6 degli 8 ceppi FC litici in cui è stato possibile inserire un plasmide per l'espressione *in trans* di *pqsL*. In base alle nostre conoscenze, questo dato dimostra per la prima la correlazione genetica fra il fenotipo litico e la mutazione *pqsL* in ceppi clinici di *P. aeruginosa*.

Nel complesso i nostri risultati suggeriscono che la produzione di HQNO è importante non solo per la competizione ambientale con altri batteri, (Heeb *et al.*, 2011), ma anche per limitare l'accumulo di HHQ e la conseguente lisi cellulare causata dal rilascio di particelle di fago Pf4.

E' anche importante considerare che solitamente i pazienti FC vengono inizialmente infettati da *Staphylococcus aureus,* e successivamente l'infezione da *P. aeruginosa* prende il sopravvento (Filkins *et al.*, 2015; Nguyen *et al.*, 2016). Quindi è ragionevole ipotizzare che quando *P. aeruginosa* diviene la specie predominante (spesso l'unica) presente nel polmone, la pressione selettiva per la produzione di HQNO diminuisca e l'emergenza di una sotto-popolazione di mutanti nel gene *pqsL* possa costituire un vantaggio per l'intera popolazione batterica. Seguendo questa linea di pensiero, sono stati eseguiti esperimenti che dimostrano come il mutante di *P. aeruginosa* PAO1 *pqsL* forma biofilm più resistenti agli antibiotici rispetto al ceppo wild type. Questo dato, insieme ad altri studi che dimostrano una correlazione fra l'attivazione dei fagi, la lisi batterica e la resistenza del biofilm agli antibiotici (Webb *et al.*, 2004; Allesen-Holm *et al.*, 2006; Rice *et al.*, 2009; Chiang *et al.*, 2013) possono, almeno in parte, spiegare l'emergenza dei mutanti *pqsL* durante le infezioni nei pazienti FC.

Lo studio descritto nel capitolo 4 è stato eseguito per studiare la possibile efficacia di farmaci anti-virulenza che hanno come bersaglio il sistema di QS basato su $3OC_{12}$-HSL, nei riguardi di ceppi di *P. aeruginosa* isolati da pazienti FC. Tali farmaci sono considerati un ottima alternativa agli antibiotici e il sistema *las* è considerato il principale bersaglio per lo sviluppo di farmaci anti-virulenza attivi contro *P. aeruginosa* (Rasko and Sperandio, 2010; Rampioni *et al.*, 2014). Tuttavia il frequente isolamento di mutanti *lasR* fra i ceppi FC di *P. aeruginosa* mette in dubbio la possibile efficacia dei famaci anti-virulenza che agiscono sul sistema di QS *las* (Ciofu *et al.*, 2015; Winstanley *et al.*, 2016; Feltner *et al.*, 2016). A tal proposito è stata analizzata una collezione di 100 ceppi di *P. aeruginosa* isolati da pazienti FC infettati per la prima volta o con infezione cronica di diversa durata. Nello specifico tale collezione è stata analizzata per: *i)* suscettibilità agli antibiotici; *ii)* produzione di molecole segnale del QS; *iii)* suscettibilità alla niclosamide (NCL; Imperi *et al.*, 2013), un inibitore del QS che ha dimostrato di avere come bersaglio il sistema di QS *las* nel ceppo di laboratorio PA14. La nostra analisi dimostra che i ceppi di *P. aeruginosa* che sono isolati per la prima volta da pazienti affetti da FC o con infezione cronica non avanzata (fino a 7 anni di infezione cronica) sintetizzano $3OC_{12}$-HSL, quindi dovrebbero essere suscettibili a farmaci che hanno come bersaglio il sistema *las*. Tuttavia, i nostri risultati dimostrano che la NCL ha un effetto inibitorio molto variabile e in generale scarso, sulla produzione di $3OC_{12}$-HSL e di fattori di virulenza regolati dal $3OC_{12}$-HSL. Tale risultato è in accordo con uno studio simile, che ha utilizzato il furanone C-30 come modello di farmaco anti-QS (Garcìa-Contreras *et al.*, 2015) e

indica che i farmaci anti-virulenza che hanno come bersaglio il sistema di QS *las* dovrebbero essere valutati per la loro efficacia contro un numero significativo di ceppi FC prima di entrare in fasi di sviluppo più avanzate. Inoltre, comprendere il meccanismo responsabile della mancata efficacia di molecole con attività anti-QS nei confronti degli isolati FC di *P. aeruginosa* potrebbe guidare studi di "medicinal chemistry" volti ad aumentarne l'efficacia e lo spettro d'azione. Inoltre, prima della somministrazione di farmaci anti-virulenza sarà necessario effettuare dei test per verificare la loro efficacia dei nei confronti dello specifico ceppo isolato dal paziente FC, come si usa fare di norma per gli antibiotici. A tal scopo, sistemi semplici per una rapida identificazione delle molecole del QS, come quello descritto nel capitolo di libro allegato a questa tesi (Rampioni *et al.*, 2018, Supplementary information) potrebbero essere facilmente modificati e trasferiti dal laboratorio di ricerca al laboratorio di diagnosi.

Nel complesso questo lavoro di tesi ha chiarito alcuni aspetti del sistema di QS *pqs* che potrebbero essere importanti per lo sviluppo di nuove strategie volte a combattere le infezioni causate da *P. aeruginosa* nei pazienti FC. Inoltre sono stati messi in evidenza i limiti che dovranno essere superati per sviluppare farmaci anti-QS efficaci nella terapia dei pazienti FC.

SUMMARY

The ESKAPE pathogen *P. aeruginosa* causes a variety of severe infections in hospitalised and immunocompromised patients and chronic pulmonary infections which are the main cause of death in people affected by Cystic Fibrosis (CF), a genetic disease affecting about 1/2500 newborns in the Caucasian population (Welsh *et al*., 2001; Talbot *et al*., 2006; Driscoll *et al*., 2007; Döring *et al*., 2010; Stefani *et al*., 2017). *P. aeruginosa* infections are hard to eradicate, since this microorganism is intrinsically resistant to many antibacterials and it is particularly prone to acquire new resistances in the hospital environment by horizontal gene transfer (Latifi *et al*., 1995; Talbot *et al*., 2006; Driscoll *et al*., 2007). The production of virulence factors and biofilm formation in *P. aeruginosa* are strictly dependent on a global regulatory circuit known as quorum sensing (QS), a communication system that controls gene expression in response to cell density (Smith and Iglewski, 2003; Lee *et al*., 2006; Williams and Càmara 2009; Papenfort and Bassler, 2016). *P. aeruginosa* has four QS systems that are interconnected and hierarchically arranged: in rich medium, the *las* QS system is at the top of this hierarchy because it is required for full activation of the other QS systems, among which the *pqs* QS system (Williams and Càmara, 2009; Papenfort and Bassler, 2016). The *las* system is based on the QS signal receptor LasR (coded by the *lasR* gene), and on the QS signal synthase LasI (coded by the *lasI* gene), producing the QS signal molecule *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) (Schuster and Greenberg, 2006). The *pqs* QS system is based on signal molecules belongings to the chemical class of alkyl quinolones (AQs). The enzymes encoded by *pqsABCDE* operon are involved in the production of the signal molecule 2-heptyl-4-quinolone (HHQ), which is converted in 2-heptyl-3-hydroxy-4-quinolone (PQS) by the monooxygenase PqsH, encoded by the *pqsH* gene. PQS or HHQ binding activates the QS signal receptor PqsR. Another relevant AQ produced by *P. aeruginosa* at μM level is 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), synthesized by the PqsL monooxigenase (coded by *pqsL* gene). HQNO does not activate the PqsR receptor and cannot be considered a QS signal molecule (Deziel *et al*., 2004; Rampioni *et al*., 2016). Interestingly, HQNO is a potent inhibitor of the cytochrome *bc1* complex of Gram-positive bacteria, hence it contributes to competitiveness of *P. aeruginosa* in the environment (Heeb *et al*., 2011).

Both LasR and PqsR activated by the respective signal molecules positively control the expression of hundred of genes, including virulence genes and genes involved in biofilm formation (Williams and Càmara, 2009; Papenfort and Bassler, 2016). Accordingly, *P. aeruginosa* QS-deficient strains are less virulent than the wild type counterparts in animal and plant infection models, and QS is

considered a promising target for the development of new anti-virulence drugs (Rasko and Sperandio, 2010; Lasarre and Federle, 2013; Rampioni *et al*., 2014).

The lung of CF patients is an heterogeneous and stressful environment for invading bacteria: the immune response causes oxidative and nitrosative stresses, while the viscous mucus typically produced by these patients hampers bacterial clearance and causes osmotic stress. Further stressful factors are the sub-lethal concentrations of antibiotics and the competition with other microorganisms. CF patients experience in pediatric age a sequence of *P. aeruginosa* intermittent lung infections, followed by the establishment of a chronic infection which cannot be eradicated with antibiotics. The *P. aeruginosa* chronic infection can last for decades in the CF patient, causing severe lung inflammation which is the ultimate cause of decline of lung function and death (Folkesson *et al*., 2012; Winstanley *et al*., 2016).

During the long permanence in the CF lung, *P. aeruginosa* undergoes evolutionary changes and different sub-lineages coexist and interact within the lung. Overall, it is believed that clones particularly adapted to the CF lung emerge along years (Folkesson *et al*., 2012; Winstanley *et al*., 2016). Mutations conferring antibiotic resistance, lack of specific virulence factors production, overproduction of alginate and other factors associated to the biofilm matrix are frequently studied among *P. aeruginosa* CF isolates and can be correlated with the adaptation to the CF lung environment (Folkesson *et al*., 2012; Winstanley *et al*., 2016). The emergence of mutants expressing a lytic phenotype, postulated to be related to a mutation in the *pqsL* gene (D'Argenio *et al*., 2002), and the emergence of *lasR*-defective mutants (Smith *et al*., 2006; Hoffman *et al*., 2008; Feltner *et al*., 2016) in *P. aeruginosa* populations chronically infecting the CF lung has also being described. On these basis, the general aim of this thesis has been to increase our understanding about the *las* and *pqs* QS systems in *P. aeruginosa,* with special focus on the CF infection.

The research described in chapter 3 has been aimed at shedding new light on the role played by PqsL and HQNO synthesis in *P. aeruginosa* physiology. It has been shown that the abolition of HQNO synthesis in *P. aeruginosa* PAO1 *pqsL* causes accumulation of HHQ. This induces the activation of the lytic cycle in the Pf4 prophage, with consequent formation of lytic plaques in colony biofilms. Moreover, 101 strains isolated from CF patients have been screened for the lytic phenotype. Overall, 26 out of 34 (76%) CF strains expressing the lytic phenotype carry a deleted or mutated *pqsL* gene, and the wild type (non-lytic) phenotype is restored in 6 out of the 8 CF strains in which a functional *pqsL* could be expressed *in trans*. To the best of our knowledge, this is the first evidence of a clear genetic correlation between the expression of the lytic phenotype and the emergence of the *pqsL* mutation in clinical *P. aeruginosa* strains.

Overall, our results suggest that the production of HQNO confers an adaptive advantage to *P. aeruginosa* wild type not only by inhibiting the growth of competitors in polymicrobial communities (Heeb *et al.*, 2011) but also by limiting HHQ accumulation and consequent cellular lysis due to prophage(s) activation.

It should be highlighted that the first lung infections experienced by CF patients are usually caused by *Staphylococcus aureus,* which is subsequently overpowered by *P. aeruginosa* (Filkins *et al.*, 2015; Nguyen *et al.*, 2016). Hence it could be reasoned that when *P. aeruginosa* prevails upon earlier *S. aureus* in the CF lung infection and establishes a single-species infection, the selective pressure for the maintenance of HQNO synthesis could decrease and the emergence of a subpopulation of *pqsL* mutants could became advantageous in this challenging environmental niche. In this view, the increased antibiotic resistance of the *P. aeruginosa* PAO1 *pqsL* biofilm with respect to the wild type shown in this study, together with other studies suggesting the existence of a link between prophage activation, bacterial autolysis and biofilm resistance to antibiotics (Webb *et al.*, 2004; Allesen-Holm *et al.*, 2006; Rice *et al.*, 2009; Chiang *et al.*, 2013), could at least in part explain the emergence of *pqsL* mutants during the infection in antibiotic-treated CF patients.

The research described in chapter 4 has been specifically aimed at investigating the suitability of anti-virulence drugs targeting the *las* QS system in CF therapy. Anti-virulence compounds are promising as alternative or adjuvants of conventional antibiotics, and the *las* QS system is considered a good target for the development of anti-virulence drugs against *P. aeruginosa* (Rasko and Sperandio, 2010; Rampioni *et al.*, 2014). However, the frequent isolation of *P. aeruginosa lasR* mutants in CF patients has opened a debate about the suitability of drugs targeting the *las* QS system in CF therapy (Ciofu *et al.*, 2015; Winstanley *et al.*, 2016). Here, a collection of 100 *P. aeruginosa* isolates from CF patients infected for different years has been tested. In particular these strains have been characterized for: *i)* antibiotic susceptibility; *ii)* QS signal molecules production; *iii)* susceptibility to niclosamide (NCL; Imperi *et al.*, 2013), a strong QS inhibitor targeting the *las* QS system in the model strain PA14. Our analysis showed that the majority of *P. aeruginosa* strains isolated from CF patients for the first time or with chronic infection established up to 7 years are able to synthetize $3OC_{12}$-HSL, hence they should be considered susceptible to drugs targeting the *las* QS system. However, our results show that the production of $3OC_{12}$-HSL and of $3OC_{12}$-HSL-dependent virulence factors is overall scarcely affected in these CF isolates treated with the model QS inhibitor NCL. This result is in agreement with the results obtained with the QS inhibitor furanone C-30 (Garcìa-Contreras *et al.*, 2015) and indicates that future anti-virulence drugs targeting the *las* QS system could be successfully developed and used in CF therapy only if shown to be active against a large proportion of CF isolates. Understanding the mechanisms underlying the

lack of susceptibility of CF isolates to anti-QS compounds could drive further medicinal chemistry studies aimed at potentiating these drugs and at expanding their range of activity. It should also be highlighted that even if a QS inhibitor would reach the clinical use, the high variability of CF strains will require preliminary tests to ascertain the susceptibility of the specific *P. aeruginosa* strain(s) colonizing a patient toward the QS inhibitor, a practice routinely used before administering antibiotics. To this aim, methods for rapid and convenient detection of QS signal molecules have already been developed (Rampioni *et al*., 2018, Supplementary) and could be further improved and translated to diagnostic laboratories.

Overall the research carried out in this thesis has clarified some aspects of the *pqs* QS system, which could be relevant to understand and combat the lethal *P. aeruginosa* infections in CF patients, and has also highlighted the limitations that should be overcome for successfully translating anti-QS drugs to the CF therapies.

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INTRODUCTION

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative highly adaptable opportunistic pathogen. This bacterium can colonize various environmental niches, including soil and marine habitats, plants, animals and humans. The ecological versatility of *P. aeruginosa* is reflected by its relatively large genome (6.3 Mbp) and genetic complexity (5570 open reading frames, ORFs), comparable to that of *Saccharomyces cerevisiae* (Stover *et al.*, 2000). Compared to the majority of known sequenced bacterial genomes, the genome of *P. aeruginosa* possesses an overall larger number of genes coding for outer membrane proteins, efflux systems and multiple chemotaxis systems, which may contribute to its pathogenesis. Moreover, up to 10% of the assigned ORFs are classified as transcriptional regulators, reflecting the ability of *P. aeruginosa* to respond and adapt to environmental fluctuations (Stover *et al.,* 2000; Goodman and Lory, 2004).

P. aeruginosa causes severe infections in hospitalised and immunocompromised patients and it is the main cause of death in people affected by Cystic Fibrosis (CF) (Talbot *et al*., 2006; Driscoll *et al*., 2007; Döring *et al*., 2010; Stefani *et al*., 2017). However, healthy people can also develop illnesses caused by *P. aeruginosa* infection. Ear infections, especially in children, and more generalized skin rashes may occur after exposure to inadequately chlorinated hot tubs or swimming pools. Eye infections have been reported in persons using extended-wear contact lenses (Centers for Disease Control and Prevention; https://www.cdc.gov/; Talbot *et al*., 2006; Driscoll *et al*., 2007).

The most common hospital-acquired *P. aeruginosa* infections are related to the use of medical devices, infections of wounds, burns and surgical sites. These infections are very frequent (11-13% of all nosocomial infections and 13.2-22.6% of infections in intensive care units), and associated with high morbidity and mortality rates when compared with infections caused by other bacterial pathogens (Osmon *et al*., 2004; Orsi *et al*., 2005; Driscoll *et al*., 2007). This is mainly due to the fact that *P. aeruginosa* infections are hard to eradicate, since this microorganism is intrinsically resistant to many antibacterials (including β-lactams, macrolides, tetracyclines, co-trimoxazole and most fluoroquinolones) and it is particularly prone to acquire new resistances in the hospital environment by horizontal gene transfer (Latifi *et al*., 1995; Talbot *et al*., 2006; Driscoll *et al*., 2007).

The capacity of *P. aeruginosa* to produce such diverse infections is due to a large number of virulence factors, such LasA and LasB elastases, exotoxin A, phospholipase C, protease IV, PrpL protease, pyocyanin, siderophores, hydrogen cyanide, and rhamnolipids, and also to the production of biofilm, that allows the colonization of host tissues and the protection of bacterial cells from the

immune system and antibiotics therapies. These factors are collectively capable of causing extensive tissue damage, bloodstream invasion and dissemination in humans and animals (Fig. 1; Smith and Iglewski, 2003). The production of virulence factors and the biofilm formation in *P. aeruginosa* are strictly dependent on a global regulatory circuit known as quorum sensing (QS), a communication system that controls gene expression in response to cell density (Smith and Iglewski, 2003; Lee *et al*., 2006; Williams and Càmara 2009; Papenfort and Bassler, 2016).

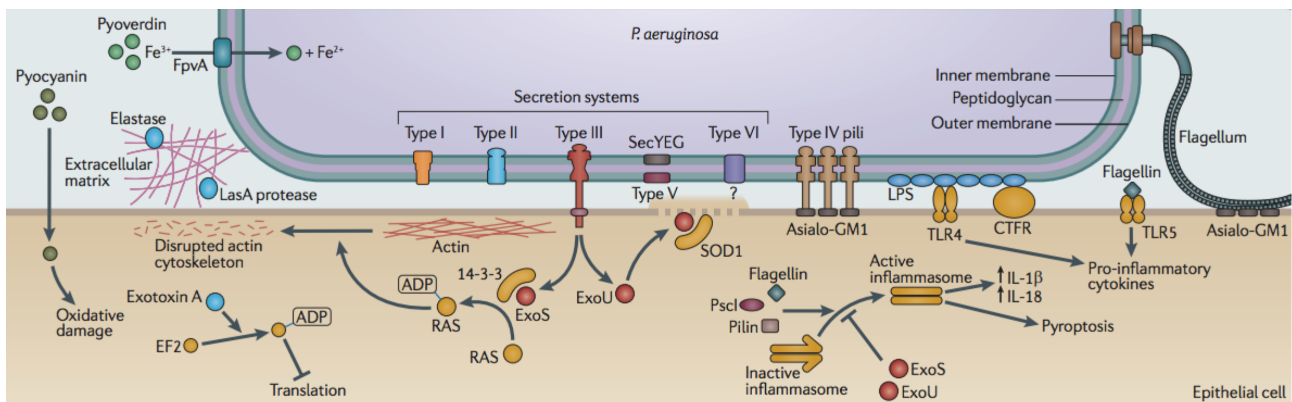

Figure 1. Overview of *P. aeruginosa* virulence determinants. *P. aeruginosa* has both cell-associated (e.g flagellum, pili, lipopolysaccharides, alginate) and extracellular virulence factors (e.g. elastase, proteases, pyocyanin, exotoxin A, siderophores). Hauser and Ozer, 2011.

1.2 Quorum sensing

Quorum sensing (QS) is a communication system by which a bacterial population co-ordinately reprograms gene expression in response to cell density, and it is based on the production, secretion and perception of signal molecules. QS is involved in the regulation of a wide variety of bacterial processes, including genetic competence, bioluminescence, antibiotic biosynthesis, motility, plasmid conjugal transfer, biofilm formation, and the production of bacterial virulence factors in plant, animal and human pathogens. QS communication systems rely on the production of different classes of signal molecules and on different mechanisms of signal response in Gram-positive and Gram-negative bacteria (Miller and Bassler, 2001; Williams and Cámara, 2009; Papenfort and Bassler, 2016).

In Gram-positive bacteria QS systems generally rely on genetically encoded peptides as signal molecules, often termed 'autoinducing peptides' (AIPs). AIPs are expressed as inactive pro-peptides via canonical ribosomal synthesis, and later processed and modified to generate the active QS signal. AIPs are not freely diffusible across membranes. AIP perception by the receiver cell is usually mediated by a sensor kinase, which transduces the signal from the membrane to the cognate

response regulator inside the cell via a phosphorylation cascade (Miller and Bassler, 2001; Atkinson and Williams, 2009).

The most intensively investigated QS systems in Gram-negative bacteria employ *N*-acyl-homoserine lactones (acyl-HSL) as signal molecules (Miller and Bassler, 2001; Atkinson and Williams, 2009; Papenfort and Bassler, 2016). The first QS system in Gram negative bacteria was described in the early 1970s in the marine bacterium *Vibrio fischeri*, in which it controls bioluminescence emission (Nealson *et al*., 1970). This QS system is based on the production of the signal molecule *N*-3-(oxohexanoyl)homoserine lactone ($3OC_6$-HSL). In *V. fischeri* the gene *luxI* encodes the synthase LuxI that, at low cell density, synthesizes $3OC_6$-HSL at a basal level; $3OC_6$-HSL accumulates in the extracellular environment proportionally to cell density of the bacterial culture, and when it reaches a certain threshold concentration, corresponding to the "quorum" cell density, it binds to and activates its cognate intracellular receptor LuxR, encoded by the gene *luxR*. The LuxR/$3OC_6$-HSL complex triggers the transcription of genes involved in bioluminescence production, and also of the gene *luxI* gene, generating a positive feedback loop which lead to a rapid increase in the concentration $3OC_6$-HSL (Fig. 2; Nealson *et al*., 1970; Fuqua *et al*., 1996).

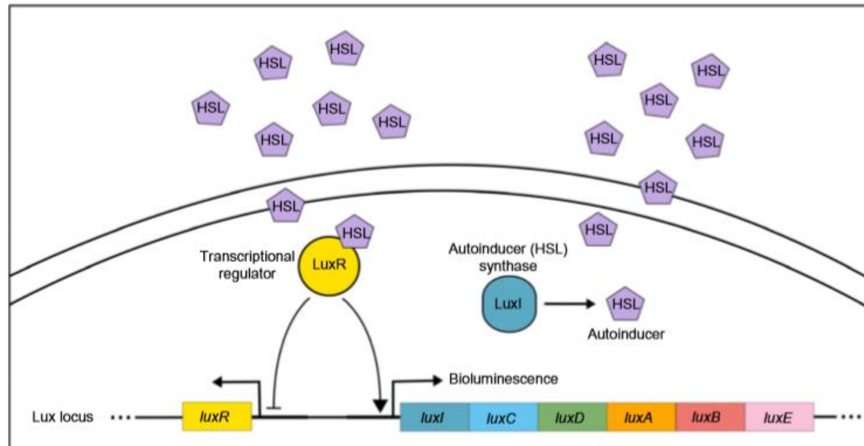

Figure 2. Schematic representation of the QS system in *V. fischeri*. The signal molecule (HSL) produced by the LuxI synthase binds and activates the LuxR receptor, inducing transcription of *luxI* gene for increasing HSL biosynthesis and of the *luxCDABE* genes required for light emission. Reuter *et al*., 2015.

QS systems homolog to the *lux* system of *V. fischeri* are widespread among Gram negative bacteria. Acyl-HSL biosynthesis is typically catalysed by LuxI-family synthases, which transfer an acyl group from an acylated acyl carrier protein (acyl-ACP) to the methionyl amine of S-adenosyl-L-methionine (SAM), after which cyclization of the methionyl moiety to homoserine lactone occurs.

The length of the acyl side chain (usually from 4 to 18 carbons), saturation and oxidation state at position 3, determine the resulting acyl-HSL structure, and thus signal-specificity. Short-chain acyl-HSL generally freely diffuse across membranes, while there is some evidence for active efflux of acyl-HSLs with longer acyl side-chains. acyl-HSLs generally function by binding to a cognate intracellular receptor protein belonging to the LuxR-family. In most cases, the LuxR receptor/acyl-HSL complex binds to target promoters, activating gene expression (Miller and Bassler, 2001; Atkinson and Williams, 2009; Papenfort and Bassler, 2016).

1.3 Quorum sensing in *Pseudomonas aeruginosa*

P. aeruginosa has four QS systems that are interconnected and hierarchically arranged: in rich medium, the *las* QS system is at the top of this hierarchy, because it is required for full activation of the other three QS systems, the *rhl*, the *pqs*, and the recently characterized IQS systems (Fig. 3; Williams and Càmara, 2009; Papenfort and Bassler, 2016).

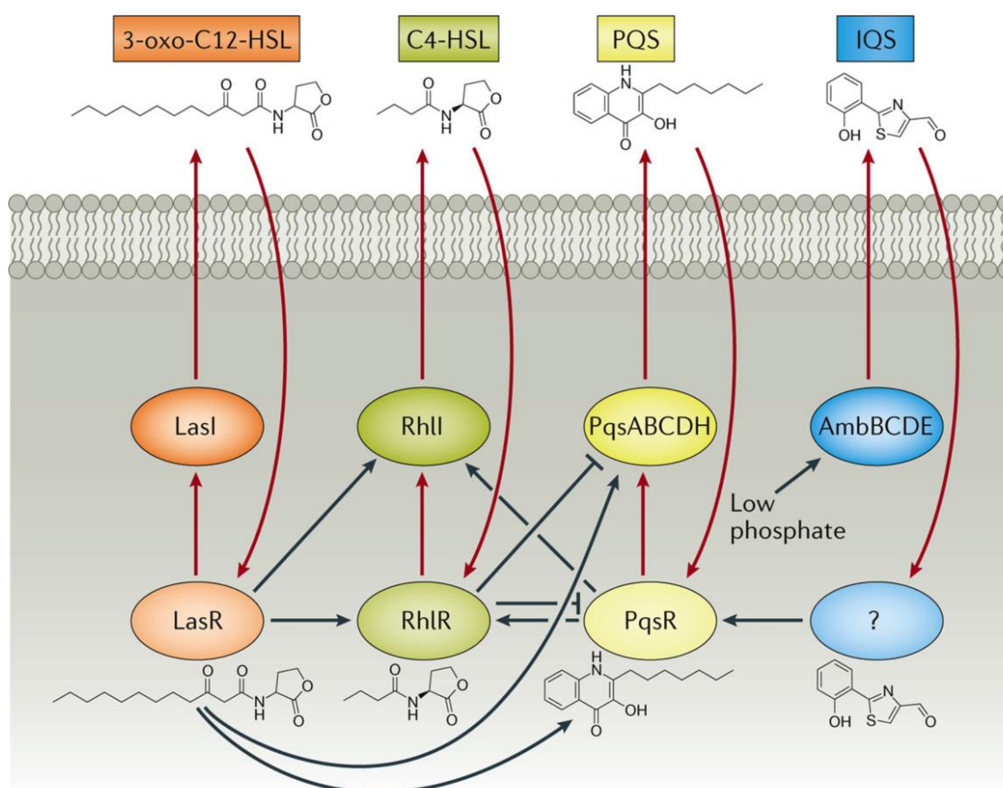

Figure 3. The four interconnected QS systems of *P. aeruginosa.* Papenfort and Bassler, 2016.

The *las* system consists of the LuxR-like transcriptional regulator LasR (encoded by the *lasR* gene), and of the LuxI-like acyl-HSL synthase LasI (encoded by the *lasI* gene), that directs the synthesis of the QS signal molecule *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) (Fig. 4; Schuster and Greenberg, 2006). Similarly to the *V. fischeri lux* QS system previously described, at low-cell density the $3OC_{12}$-HSL molecule is synthesized by LasI at a basal level, and is secreted into the surrounding medium. With increasing cell density, the signal molecule accumulates until its concentration reaches the threshold level; at this critical concentration, $3OC_{12}$-HSL binds its cognate receptor, the QS-activator LasR. The LasR/$3OC_{12}$-HSL complex triggers *lasI* transcription, generating a positive feedback loop that leads to the amplification of $3OC_{12}$-HSL production. As a consequence, the whole QS-system of *P. aeruginosa* becomes active and *P. aeruginosa* transcriptome is drastically reprogrammed. Indeed, the LasR/$3OC_{12}$-HSL complex triggers the expression of hundreds of genes, including virulence genes and genes of the *rhl*, *pqs* and IQS QS systems (Schuster *et al*., 2003; Schuster and Greenberg, 2006; Williams and Càmara, 2009; Lee *et al*., 2013; Papenfort and Bassler, 2016). Therefore the regulative cascade leading to the expression of virulence phenotypes in *P. aeruginosa* strongly relies on the *las* QS system (Lee and Zhang, 2015). As a whole, the QS circuit regulates about 7% of all the *P. aeruginosa* genes, and has a key role in the infection processes being required for the production of many virulence factors and for biofilm formation (Kirisits and Parsek, 2006; Schuster and Greenberg, 2006).

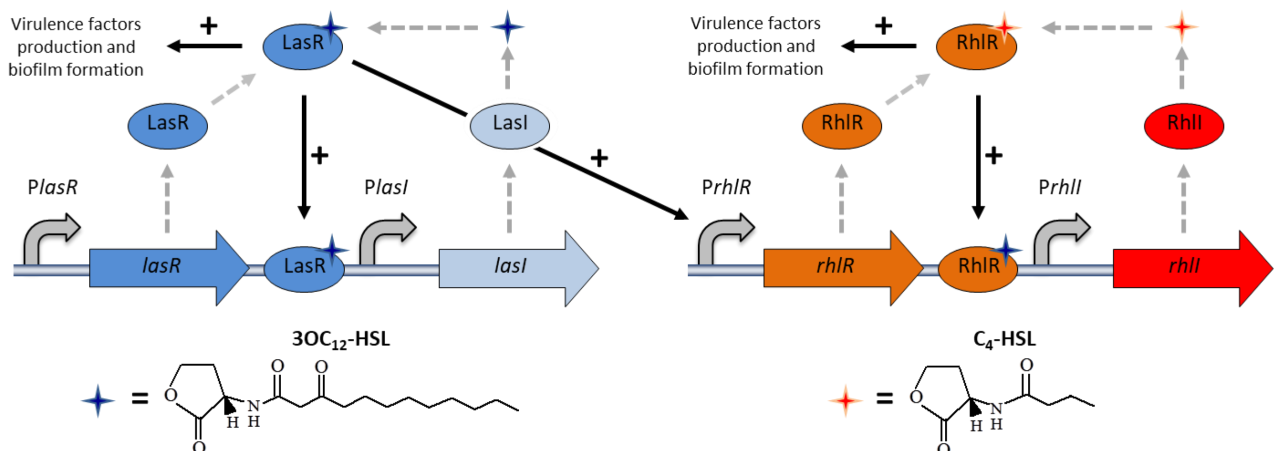

Figure 4. Interactions between the two acyl-HSL QS systems in *P. aeruginosa*. LasI synthase produces $3OC_{12}$-HSL signal molecule, which binds and activates LasR. The LasR/$3OC_{12}$-HSL complex increases *lasI* expression, with a resulting autoinduction mechanism. LasR/$3OC_{12}$-HSL also activates the expression of genes related to biofilm development and virulence factors production. Moreover, LasR/$3OC_{12}$-HSL complex is required for the full expression of RhlR, the C_4-HSL receptor which promotes the activation of the *rhl* QS system. Image modified from Williams and Càmara, 2009.

Also the *rhl* QS system relies on the production of an acyl-homoserine lactone as signal molecule: the *N*-butanoylhomoserine lactone (C_4-HSL). C_4-HSL is synthesised by the LuxI-like enzyme RhlI, and it is released into the extracellular environment; as the bacterial population grows, C_4-HSL binds to its LuxR-like cognate receptor RhlR, and the RhlR/C_4-HSL complex regulates the expression of target genes (Fig. 4).

The *pqs* QS system (Fig. 5) is based on signal molecules belongings to the chemical class of alkyl quinolones (AQs). The enzymes encoded by *pqsABCDE* operon are involved in the production of the signal molecule 2-heptyl-4-quinolone (HHQ), which is converted in 2-heptyl-3-hydroxy-4-quinolone (PQS) by the monooxygenase PqsH, encoded by the *pqsH* gene (Deziel *et al*., 2004). In addition to HHQ and PQS, *P. aeruginosa* produces another AQ at µM level, namely 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) (Lépine *et al*., 2004). The biosynthesis of this molecule requires the monooxygenase PqsL in addition to the enzymatic complex involved in HHQ production. Actually, *P. aeruginosa* synthesizes more than 50 molecules belonging to the chemical class of AQs (AQs congeners), differing from HHQ, PQS or HQNO in length and/or saturation of the aliphatic chain. The AQ congeners are produced at much lower levels (nm level) with respect to these molecules HHQ, PQS or HQNO and their biological function is generally considered not relevant and seldom taken into consideration in studies concerning the *pqs* QS system (Lépine *et al*., 2003; Déziel *et al*., 2004). Also in this study, the acronym AQs will be used to refer to HHQ, PQS and HQNO, unless otherwise noted.

As represented in figure 6, the first step of AQs biosynthesis is performed by PqsA, an anthranilate-coenzyme A ligase (Gallagher *et al*., 2002; Deziel *et al*., 2004; Coleman *et al*., 2008), which activates anthranilate (AA) to form anthraniloyl-coenzyme A (AA-CoA). PqsD synthesizes 2-aminobenzoylacetate-CoA (2-ABA-CoA) from AA-CoA. 2-ABA-CoA is converted into 2-aminobenzoylacetate (2-ABA) by PqsE. *P. aeruginosa* enzymes other than PqsE can perform the latter catalytic step, since a *pqsE* mutant is still able to produce AQs. 2-ABA undergoes decarboxylation and reacts with octanoate *via* a reaction catalyzed by PqsC and PqsB, thus resulting in the production of HHQ. HHQ is converted into PQS by the PqsH monooxygenase. (Gallagher *et al*., 2002; Deziel *et al*., 2004; Dubern and Diggle, 2008; Schertzer *et al*., 2009). 2-ABA can also be hydroxylated by the PqsL monooxygenase and then and reacts with octanoate *via* a reaction catalyzed by PqsC and PqsB, this resulting in the production of HQNO. Moreover, 2-ABA can also spontaneously convert in 2,4-dihydroxyquinoline (DHQ) and in 2-aminoacetophenone (2-AA) (Dulcey *et al*., 2013; Fig. 6).

PQS or HHQ binding activates the transcriptional regulator PqsR, triggering the transcription of the *pqsABCDE* operon and generating a positive feedback loop that amplifies AQs

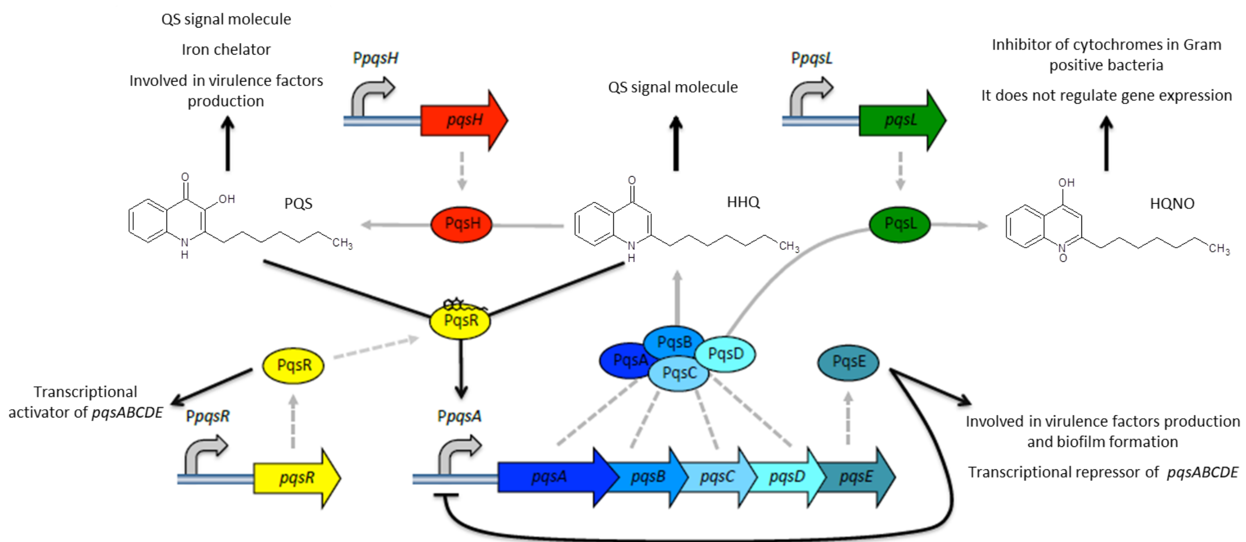

Figure 5. Schematic representation of *P. aeruginosa pqs* QS system. *pqsABCD* genes codify for enzymatic complex involved in HHQ synthesis. This enzymatic complex, in addition to the monooxygenase *pqsL*, are required for HQNO synthesis. HHQ is converted in PQS by the monooxygenase PqsH. Both HHQ and PQS bind to and activate PqsR. By binding P*pqsA* promoter, PqsR enhances the transcription of *pqsABCDE* operon. Both HHQ and PQS act as QS signal molecules. Moreover, PQS is an iron chelator and controls expression of gene involved in iron starvation. HQNO is an inhibitor of cytochromes in Gram-positive bacteria but, differently from HHQ and PQS, it is not a QS signal molecule. PqsE inhibits *pqsABCDE* transcription and is the effector of *pqs* QS system because it activates expression of genes involved in virulence factors production and in biofilm formation.

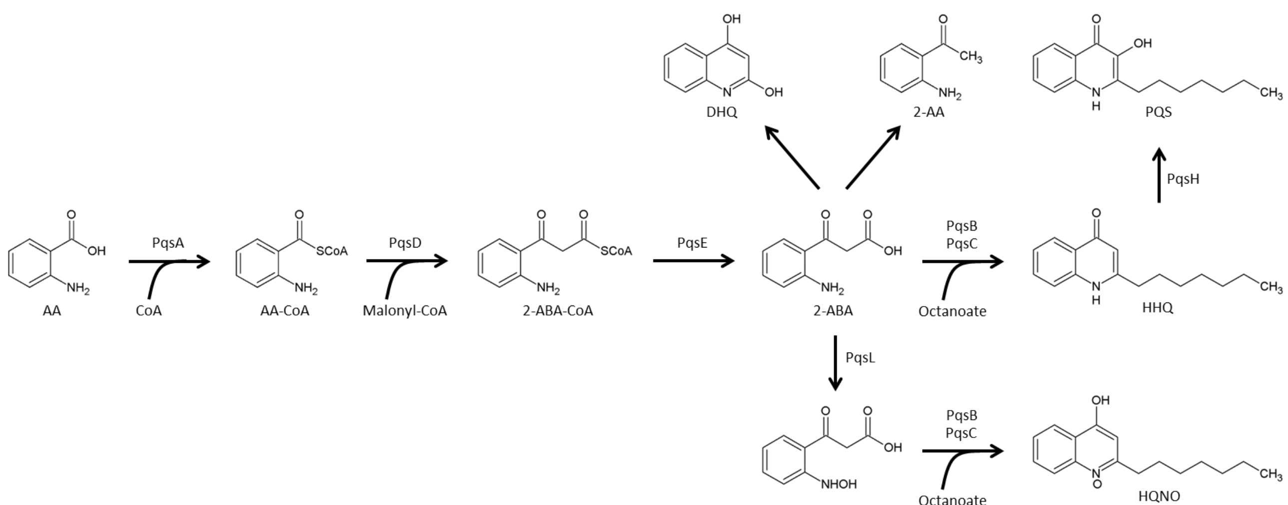

Figure 6. Synthetic representation of AQs biosynthesis in *P. aeruginosa*. Figure modified from Dulcey *et al*., 2013.

production (Fig. 5; Heeb *et al*., 2011). A recent study from our group showed that PqsR does not control the transcription of other genes besides *pqsABCDE.* In fact, the main effector of this QS system is the PqsE protein, which is responsible for the expression of hundreds of genes by a mechanism still not well understood, including genes encoding virulence factors (Rampioni *et al*., 2016). On the contrary, HQNO is not a QS signal molecule because does not affect genes transcription in *P. aeruginosa* (Deziel *et al*., 2004; Rampioni *et al*., 2016). However, it is a potent inhibitor of the cytochrome *bc1* complex of Gram-positive bacteria, hence it could contribute to competitiveness of *P. aeruginosa* in the environment (Heeb *et al*., 2011).
The IQS system has been characterized very recently, and it is based on the production of the signal molecule 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS); the genes involved in IQS synthesis are a non-ribosomal peptide synthase genes of the *ambBCDE* operon, and the receptor for IQS is currently unknown. IQS production is activated by phosphate starvation, a stressful condition that usually occurs during bacterial infections (Fig. 3; Lee *et al*., 2013).

1.4 Quorum sensing as target of anti-virulence drugs

In last years the massive (and often improper) use of antibiotics in settings like hospitals and intensive animal farming has dramatically accelerated the emergence of antibiotic-resistant strains. Today we are witnessing an alarming increase of multi-drug, or even pan-drug resistant pathogens (World Health Organization, 2014). This problem especially concerns a group of pathogens particularly able to avoid antibiotics therapies, which has been named ESKAPE from the initials of the bacterial species belonging to this group: *Enterooccus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* spp.(Rice, 2008; Boucher *et al*., 2009;Pendleton *et al*., 2013).
In the last decades researchers started proposing new therapeutic approaches, different from antibiotics, especially focusing in limiting the emergence of resistant phenotypes. Progress in understanding the mechanisms that govern infective processes has led researchers to consider bacterial virulence factors as potential targets for the development of anti-virulence drugs (Rasko and Sperandio, 2010; Rampioni *et al*., 2014). Anti-virulence drugs disarm bacteria without affecting their growth, hence their use is not expected to confer a selective advantage to resistant strains (Rasko and Sperandio 2010; Allen *et al*., 2014; Rampioni *et al*., 2017).

Since QS in *P. aeruginosa* plays a key role in virulence, it is considered a promising target for the development of new anti-virulence drugs (Rasko and Sperandio, 2010; Lasarre and Federle, 2013; Rampioni *et al*., 2014). Indeed, *P. aeruginosa* QS-deficient strains are less virulent than the wild type counterparts in all the animal and plant infection models tested so far: mice, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, and *Arabidopsis thaliana* (Smith and Iglewski, 2003, Juhas *et al*., 2005; Diggle *et al*., 2006; Rampioni *et al*., 2010).

As stated in a recent review by our group, when considering QS inhibition as an anti-infective strategy, the appearance of strains carrying mutations in key QS genes in clinical samples should not be overlooked (Rampioni *et al*., 2014). For example, *P. aeruginosa lasR* and *S. aureus agr* defective mutants are frequently isolated especially in chronic infections (Smith *et al*., 2006; Paulander *et al*., 2013). This phenomenon raises concerns about the importance of QS systems in infection establishment and progression. However, a number of studies have provided convincing evidence that QS mutants behave as “social cheaters” that exploit the extracellular factors produced by the QS-proficient population, without the cost of contributing to the production of these “public goods”. Notably, as the proportion of cheaters increases in a population, their relative fitness decreases, as there are fewer QS-proficient bacteria to exploit. As a consequence, the percentage of QS mutant strains in the population cannot exceed a certain limit (West *et al*., 2006; Diggle *et al*., 2007; Sandoz *et al.*, 2007). These findings suggest that a quorum sensing inhibitor should decrease the fitness of the whole bacterial population in infected sites, despite the presence of QS-defective bacteria. This hypothesis is supported by *in vivo* experiments showing that co-infection with *P. aeruginosa lasR* mutants reduces mortality caused by *P. aeruginosa* wild type in a mouse burn model of infection (Rumbaugh *et al*., 2009).

The issue of social cheating is closely associated with the possible emergence of resistance to QS inhibitors. Wood and collaborators reported the selection of *P. aeruginosa* mutants resistant to the QS inhibitor furanone C-30 in a growth medium containing adenosine as sole carbon source. Importantly, adenosine is intracellularly degraded by a QS-controlled enzyme; therefore, in this case, the QS inhibitor directly inhibited bacterial growth, similarly to an antibiotic (Maeda *et al*., 2012). A subsequent study reported that the enrichment of QS inhibitor-resistant strains in a bacterial population is an unlikely event when QS controls “public goods” (e.g., extracellular enzymes used by the whole population) rather than “private goods” (e.g., intracellular enzymes used by individual cells). This is because the potential benefit conferred from a small proportion QS inhibitor-resistant cells to the bacterial population is diluted with the higher proportion of QS inhibitors-sensitive cells (Mellbye and Schuster, 2011). These results may be clinically relevant for those pathogens, including *P. aeruginosa*, in which virulence mostly relies on secreted extracellular

virulence factors. However, these studies highlight the importance of validating the activity of QS inhibitors against fresh clinical isolates rather than laboratory reference strains alone.
The spectre of bacterial pathogens totally resistant to all classes of conventional antibiotics and the inability to treat simple infections is only now beginning to challenge the dogma that effective antibacterials must be broad spectrum and growth inhibitory. Consequently, anti-virulence drugs including QS inhibitors require careful consideration and thorough evaluation (Rampioni *et al.*, 2014).

1.5 *Pseudomonas aeruginosa* cystic fibrosis clinical isolates

Cystic fibrosis (CF) is inherited as an autosomal recessive disease and affects 70.000 persons in the world (Cystic Fibrosis Foundation; https://www.cff.org/). The defective gene is the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and it is carried by one out of 25 persons (among Caucasians). Since CFTR encodes for a chloride channel of the epithelial cell surface, CF patients manifest a variety of multi-organ problems due to the alteration of sodium and chloride secretion across cell membranes and the subsequent luminal dehydration (Lyczak *et al.*, 2000). The impairment of mucociliary clearance, which should remove all the microbes entering the airways, leads to the production of a thick and dehydrated mucus in the CF lung which promote the onset of chronic bacterial colonization (Anderson, 2012). The microbiology of CF respiratory tract is peculiar: in the early stage of life, it is characterized by the prevalence of the Gram-positive bacterium *S. aureus* while, in early adolescence, the lung of CF patients typically becomes chronically infected with Gram-negative non-fermenting bacteria. Among these, *P. aeruginosa* is the most relevant and recurring, so that 30% of children and up to 80% of adult (25 years old and older) CF patients are chronically colonized with this pathogen (Gibson *et al.*, 2003; Stuart *et al.*, 2010). Thanks to current therapeutic treatments, life expectancy for CF patients has consistently grown, reaching a median life of 40 years (MacKenzie *et al.*, 2014).
P. aeruginosa is acquired by CF patients from the environment and, in some cases, from other infected persons through Flügge droplets or contaminated hands (Döring, 2010). The persistency of *P. aeruginosa* high cell densities in CF patient respiratory tract for decades causes a severe inflammation and, consequently, leads to fatal lung deterioration (Murray *et al.*, 2007). In fact from 80% to 95% of CF patients die for respiratory failure due to chronic *P. aeruginosa* infection and concomitant airway inflammation. Up to 97% of CF patients are infected with *P. aeruginosa* by the age of 3 years (Lyczak *et al.*, 2002; Murray *et al.*, 2007). Early aggressive antibiotic treatment can increase life expectancy in CF patients and delay the onset of the chronic infection (Fig. 7;).

Continuous antibiotic therapy can keep the chronic infection under control but it it is apparently unable to eradicate it. In fact, the chronic colonization by *P. aeruginosa* in CF patients is associated with a poor prognosis and a significant increase of morbidity and mortality (Frederiksen *et al*., 1997; Moreau-Marquis *et al*., 2008; Folkesson *et al*., 2012). The continuous presence of *P. aeruginosa* in the chronically infected lung leads to immune complex-mediated inflammation which together to the damage that is actively caused by the bacteria, is the major cause of lung damage and lung function decrease (Folkesson *et al*., 2012).

Overall, it is recognized that the extraordinary ability of *P. aeruginosa* to adapt to challenging and ever-changing environments could explain why *P. aeruginosa* chronic infections have such an high prevalence in CF patients. After the first isolation of *P. aeruginosa* in the lung of a CF patients a period of intermittent colonization of the airway begins. During this phase, which can last from birth until twenties/thirties year old, the recurrent infections can be fought with aggressive antibiotics therapies. Despite during the intermittent infection period the patient can be re-infected with *P. aeruginosa* strains of different genotype, in approximately 25% of cases re-colonization occurs with the same genotype. It has been shown that a strain previously eradicated from the lung, can persist in the paranasal sinuses and re-colonize the lung even after years (Fig. 7; Folkesson *et al*., 2012).

Figure 7. Schematic representation of the development of *P. aeruginosa* CF infection in an hypothetical patient. Folkesson *et al*., 2012.

During years of infection *P. aeruginosa* strains colonizing the CF lung grow in an ever-changing environment. The CF lung is an heterogeneous, hostile and stressful environment for invading bacteria: the immune response causes oxidative and nitrosative stresses, while the viscous mucus is cause of osmotic stress. Moreover, the bacteria itself, releasing virulence factors, enhance the lung inflammation that contributes to modify the physiological structure of airways. Further stressful factors are the sub-lethal concentrations of antibiotics and the competitions with other

microorganisms. In this way, different regions of lung tissue are likely to vary along a range of environmental axis, including variations of mixtures of nutrients, concentration of penetrating antibiotics, other microorganisms or factors such as oxygen availability, potentially leading to spatially variable selection for different ecotypes (Folkesson *et al*., 2012; Winstanley *et al*., 2016).

All these factors act as driving forces that make a heavy selective pressure on the *P. aeruginosa* population colonizing the lung of CF patients. In response to these various selective forces, *P. aeruginosa* undergoes evolutionary changes during the infection process and different sub-lineages coexist and interact within the lung. In fact, the various mutations could be present not only in strains from different patients but also in members of the *P. aeruginosa* population within the individual patients. Overall, it is believed that clones particularly adapted to the CF lung emerge along years (Folkesson *et al*., 2012; Winstanley *et al*., 2016).

Specific phenotypes that commonly emerge in chronic CF strains are for example: mucoid phenotype, small colony variants (SCVs), hypermutator phenotype, antibiotics resistance, loss of virulence factors, loss of QS regulation, lytic phenotype. Many of these phenotypic changes are observed repeatedly in isolates sampled from different patients and clinical settings (Fig. 8).

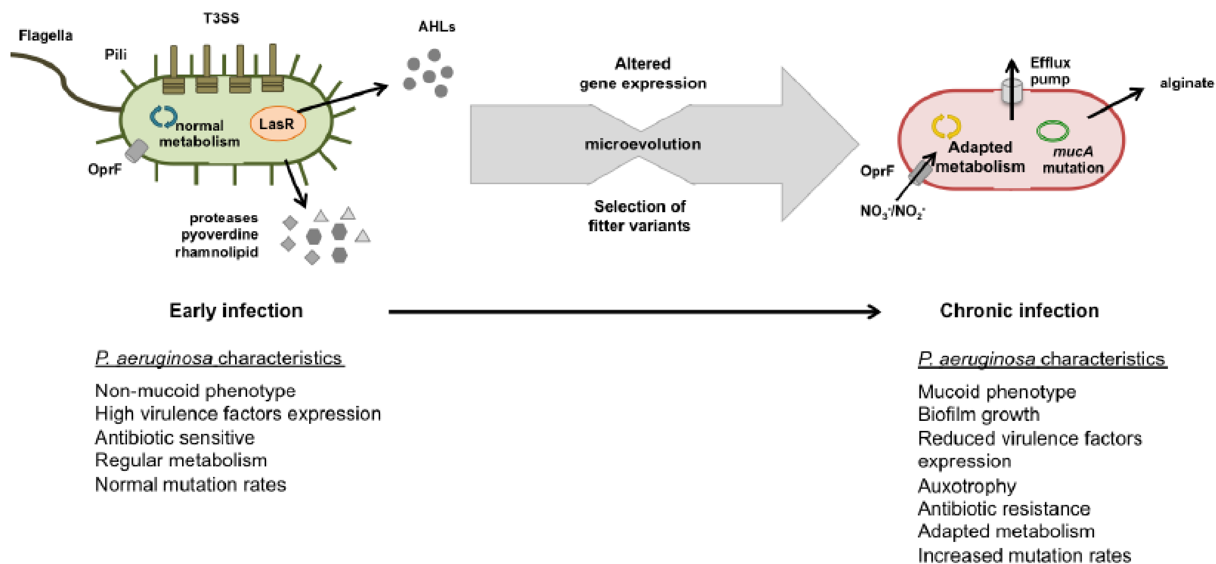

Figure 8. Schematic representation of *P. aeruginosa* microevolution during CF lung infection. Some phenotypes that appears after lung adaptation are reported. Sousa and Pereira, 2014.

One of the most striking features of *P. aeruginosa* adaptation to the airways of patients with CF is the frequent conversion to a mucoid phenotype. This is caused by mutation of the *mucA* gene, leading to overproduction of the biofilm matrix component alginate. Alginate is a polysaccaride

protecting *P. aeruginosa* from both immune system and antibiotics action (Meshulam *et al*., 1982; Meshulam *et al*., 1984; Cabral *et al*., 1987; Simpson *et al*., 1989). The mucoid phenotype correlates with the onset of significant deterioration in lung function, in fact the appearance of mucoid clones is often associated with the instauration of chronic infection (Lyczak *et al*., 2002; Folkesson *et al*., 2012).

The SCVs are often isolated as a consequence of inhaled antibiotics therapy. The name SCV (Small Colony Variants) reflects the small colonies of about 1 to 3 mm after 24-48 hours of growth on solid media (Haussler *et al*., 1999). This phenotype has been implicated in persistent and recurrent infections. In comparison with the fast growing wild type, SCVs exhibit increased antibiotic resistance. At the microscopic level SCVs are heterogeneous, indicating that this phenotype could be caused by different mutations. For instance, a subgroup of *P. aeruginosa* SCV were shown to be hyperpiliated, exhibited autoaggregative properties and showed increased biofilm formation capability (Haussler *et al*., 2003; Haussler 2004). The current state of research in clinical *P. aeruginosa* SCV is extensively discussed in a recent review (Malone 2015).

The hypermutator strains have defects in their ability to correct mistakes in DNA replication because they have a range of mutations such as in the mismatch repair gene *mutS* or *mutY* (Oliver *et al*., 2000; Macià *et al*., 2005). They may have a mutation rate as high as 1 x 10^{-5} to 10^{-4} compared with the normal rates of 1 x 10^{-8} to 10^{-7} (Foweraker 2009). The hypermutator phenotype has been found in 36% of CF patients with chronic infection with *P. aeruginosa* and were not found in non-CF clinical isolates supporting the notion that this phenotype represents an adaptation to the CF lung (Oliver *et al*., 2000). An hypermutator phenotype could be an adaptive advantage in the CF lung, an ever-changing ecological niche repeatedly exposed to various stress (Foweraker 2009).

One of the most common mutation increasing the antibiotics resistance occur in *mexZ*, coding for a negative regulator of MexXY-OprM, a multidrug resistance pump that confers resistance to aminoglycosides (Smith *et al*.,2006). Other mutations in the repressor of the MexCD-OprJ operon (*nfxB*), result in increased resistance to fluoroquinolones, while altered lipid moieties confers enhanced resistance to aminoglycoside antibiotics, cationic antimicrobials such as polymyxin E and potentially to cationic peptides produced by the host (Rau *et al*., 2010)

Other common mutations found in chronic CF isolates occur in the *lasR* gene, with consequent lack of QS response (Smith *et al*., 2006; D'Argenio *et al*., 2007; Hoffman *et al*., 2009; Feltner *et al*., 2016). The loss of social and cooperative behaviour could confer an adaptive advantage because the production of QS signal molecules and the relative factors released in response to QS activation are costly in terms of energetic demand. Avoiding these costs, *P. aeruginosa* can ensure its persistence for the long-term. Moreover, it seems that *lasR* mutants preferentially use nitrate and nitrite as the

terminal acceptor of electrons, allowing *P. aeruginosa* growth in anaerobic niches and also have growth advantage in presence of amino acids as sole carbon source, with respect to the wild type (D'Argenio *et al.*, 2007; Fothergill *et al.*, 2007; Hasset *et al.*, 2010). Interestingly the isolation of *lasR* mutants seems to be associated with enhanced host inflammatory response and a more unfavourable prognosis (Hoffman *et al.*, 2009; LaFayette *et al.*, 2015).

Also *P. aeruginosa* strains disclosing an auto-lytic phenotype on colony biofilm (Fig. 9) are frequently isolated from CF patients. This phenotype was reported for the first time in 1924 in *P. aeruginosa* confluent cultures, highlighting the resemblance of the lytic halos with phages plaques (Hadley 1924). Subsequently, this phenotype has been occasionally reported by different studies, indicating that it is common among *P. aeruginosa* isolates, including a mucoid strain (Berk 1963; Berk 1965; Holloway 1969). More recently, a study analyzing 191 CF clinical isolates showed that 59 of these (31%) disclosed the lytic phenotype. Interestingly, in a collection of transposon insertion mutants generated in the PAO1 genetic background, it was discovered that 2 out of 3 of the lytic strains were mutated in the *pqsL* gene (D'Argenio *et al.*, 2002). Since *pqsL* encodes the enzyme converting the QS signal HHQ into HQNO (Heeb *et al.*, 2011), this result suggests the existence of a link between the lytic phenotype and the *pqs* QS system.

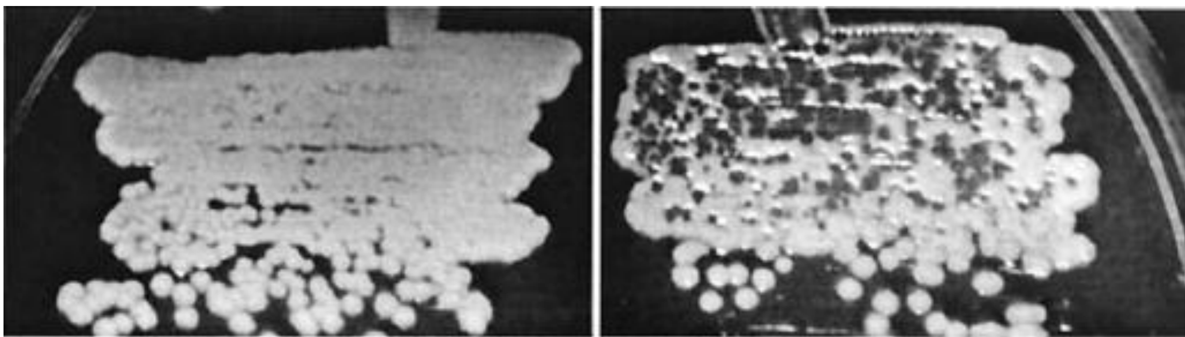

Figure 9. Image showing *P. aeruginosa* PAO1 *wild type* (left panel) and its isogenic *pqsL* mutant (right panel) grown on agar plates. The lytic phenotipe is evident in the *pqsL* mutant, as phage plaque-like halos present where the bacterial growth is confluent. D'Argenio *et al.*, 2002.

AIMS OF THE WORK

As described in the above chapter, mutations in *P. aeruginosa* CF isolates conferring resistance to antibiotics and/or increased biofilm formation are clearly correlated with the evolution and the adaptation to the CF lung environment (Folkesson *et al*., 2012; Winstanley *et al*., 2016). Conversely the emergence of mutants expressing a lytic phenotype, postulated to be related to a mutation in the *pqsL* gene (D'Argenio *et al*., 2002), and the emergence of *lasR*-defective mutants (Smith *et al*., 2006; Hoffman *et al*., 2009) in *P. aeruginosa* populations chronically infecting the CF lung are phenomenon not yet well understood. The *lasR* and *pqsL* genes belong to the *las* and *pqs* QS systems, respectively. These QS systems are highly important in *P. aeruginosa* physiology, adaptive response and virulence and well established targets for the development of anti-virulence therapies (Rasko and Sperandio, 2010; Rampioni *et al*., 2016). On these basis, the general aim of this thesis has been to increase our understanding about the *las* and *pqs* QS systems in *P. aeruginosa,* with special focus on CF infection.

The research described in chapter 3 has been specifically aimed at investigating the role played by the *pqsL* gene in the expression of the lytic phenotype of the model strain *P. aeruginosa* PAO1 and the impact of this mutation on biofilm susceptibility to antibiotics. Moreover, a collection of *P. aeruginosa* CF isolates has been screened for their ability to express the lytic phenotype and for the presence of the *pqsL* mutation, in order to assess whether a correlation exists between this mutation and the lytic phenotype, in this group of clinical *P. aeruginosa* strains.

The research described in chapter 4 has been specifically aimed at investigating the suitability of anti-virulence drugs targeting the *las* QS system in CF therapy. As previously described, anti-virulence compounds are promising as alternative or adjuvants of the conventional antibiotics, and the *las* QS system is considered a very promising target for the development of anti-virulence drugs against *P. aeruginosa* (Rasko and Sperandio, 2010; Rampioni *et al*., 2014). However, as described in the introduction, the frequent isolation of *lasR* mutations in *P. aeruginosa* CF strains has opened a debate about the suitability of drugs targeting the *las* QS system in CF therapy (Ciofu *et al*., 2015; Winstanley *et al*., 2016). Here, a collection of 100 *P. aeruginosa* isolates from CF patients infected for different years has been tested to evaluate the suitability of anti-QS therapy in CF lung infection. In particular these strains have been characterized for: *i)* antibiotic susceptibility; *ii)* QS signal molecules production; *iii)* susceptibility to niclosamide (NCL; Imperi *et al*., 2013), a strong QS inhibitor targeting the *las* QS system in the model strain PA14.

The *pqs* quorum sensing system is involved in prophage-activation and antibiotic resistance in *Pseudomonas aeruginosa*

3.1 Background

As described in chapter 1, HHQ, PQS and HQNO are the most relevant AQs and a recent study showed that, once activated by HHQ or PQS, the transcriptional regulator PqsR promotes exclusively the transcription of *pqsABCDE* operon, triggering a positive feedback loop leading to increased expression of PqsE. This protein, in turn, controls the expression of many genes involved in virulence factors production and in biofilm formation, by a mechanism still unknown. Moreover, PQS has iron-chelating activity and induces the expression of iron starvation response genes by a PqsR-independent mechanism. Finally, HQNO has no effect on *P. aeruginosa* transcriptome, hence this molecule cannot be considered a QS signal (Rampioni *et al.*, 2016). HQNO production has been mainly considered so far as a factor involved in environmental competition. Indeed HQNO inhibits of the cytochrome bc_1 complex in Gram-positives bacteria, hence it is believed to be involved in competition between *P. aeruginosa* and *S. aureus* during CF lung infections (Heeb *et al.*, 2011; Machan *et al.*, 1992; Qazi *et al.*, 2006; Voggu *et al.*, 2006). Recently, HQNO has also been shown to trigger autolysis in late stationary phase cultures of *P. aeruginosa* by self-perturbing the electron transfer reaction of the cytochrome bc_1 complex (Hazan *et al.*, 2016)

Interestingly, a previous study showed that a *P. aeruginosa* Δ*pqsL* mutant (impaired in HQNO synthesis) formed lytic plaques when grown on solid media, in areas were bacterial cells merged (Fig. 9), and produced data suggesting that the lytic phenotype might be caused by the accumulation of PQS. Moreover, this phenotype was found in 31% of a collection of *P. aeruginosa* CF isolates, suggesting that it could be selected during CF infection (D'Argenio *et al.*, 2002). Interestingly, the lytic plaques characterizing this phenotype resemble phage plaques, suggesting that prophage activation could be involved in the expression of this phenotype (Fig. 9).

The general aim of the study described in this chapter has been to better understand the processes underlying the expression of the lytic phenotype disclosed by the *P. aeruginosa* Δ*pqsL* mutant and whether this mutation could be relevant in *P. aeruginosa* chronic infection.

3.2 Results and discussion

3.2.1 HHQ accumulation induces the expression of the lytic phenotype in *Pseudomonas aeruginosa*

A *P. aeruginosa* PAO1 in frame deletion mutant in the *pqsL* gene, encoding the PqsL monooxygenase, has been generated and named PAO1 Δ*pqsL* (details in Materials and Methods). The deletion of *pqsL* causes the formation of lytic plaques when this strain is grown as colony biofilm (Fig. 10A), in agreement with a previous study (D'Argenio *et al.*, 2002). As expected, PAO1 Δ*pqsL* does not produce HQNO and accumulates PQS (Fig. 10B; D'Argenio *et al.*, 2002). Moreover, our results show that PAO1 Δ*pqsL* also accumulates HHQ (Fig. 10B). Since 2-ABA is a common precursor of HQNO, HHQ and PQS (Fig. 6; Dulcey *et al.*, 2013), it is likely that the amount of 2-ABA directed toward the synthesis of HHQ and PQS increases in the absence of the enzyme for HQNO synthesis (*i.e.* PqsL). The wild type colony phenotype (not autolytic) and normal AQs levels could be restored in PAO1 Δ*pqsL* by introduction of a pME6032-derived plasmid (named pME*pqsL*) expressing PqsL *in trans* (Fig. 10A).

It should be noticed that the formation of auto-lysis plaques in PAO1 Δ*pqsL* colony biofilms does not correspond to impaired growth rate of this mutant in liquid medium, despite the strain overproduces HHQ and PQS under this growth condition (Fig. 10B). An explanation for the apparent lack of lysis in PAO1 Δ*pqsL* planktonic cultures could be that AQs could reach very high local concentrations during growth on solid medium, while they homogeneously diffuse in liquid cultures. Moreover, the lytic plaques formed by PAO1 Δ*pqsL* seem to appear only at the centre of the colony biofilm, suggesting that high cellular density and nutritional stress could induce the production of lytic plaques (Fig. 10A; D'Argenio *et al.*, 2002).

As mentioned above, a previous study suggested that the mutation in *pqsL* induces autolysis as a consequence of PQS accumulation, while HHQ was not taken into consideration (D'Argenio *et al.*, 2002). In order to better understand the link between AQs accumulation and the lytic phenotype of PAO1 Δ*pqsL,* a set of multiple mutants impaired in different steps of AQs synthesis has been constructed and analysed.

First, the gene involved in the conversion of HHQ to PQS (named *pqsH*) was deleted in PAO1 Δ*pqsL*, originating the double mutant strain PAO1 Δ*pqsH* Δ*pqsL* (Δ*pqsHL*). Results showed that PAO1 Δ*pqsHL* disclosed a lytic phenotype more evident than PAO1 Δ*pqsL* (Fig. 11A). Notably, PAO1 Δ*pqsL* and PAO1 Δ*pqsHL* produce HHQ levels about 2.5- and 3.6-fold higher with respect to the wild type, respectively (Fig. 11B). In addition, no lytic plaques were detectable when a deletion in the *pqsA* gene, necessary for the synthesis of 2-ABA (the HHQ precursor), was introduced in

PAO1 Δ*pqsL* and PAO1 Δ*pqsHL*, generating strains PAO1 Δ*pqsAL* and PAO1 Δ*pqsAHL* (Fig. 11A e B). Finally, lytic plaques formation was restored in the *P. aeruginosa* triple mutant PAO1 Δ*pqsAHL* by the plasmid-driven expression of the genes *pqsABCD,* required for HHQ synthesis (Fig. 11C).

Overall, these results support the hypothesis that the lytic phenotype expressed by PAO1 Δ*pqsL* is correlated to HHQ rather than to PQS accumulation.

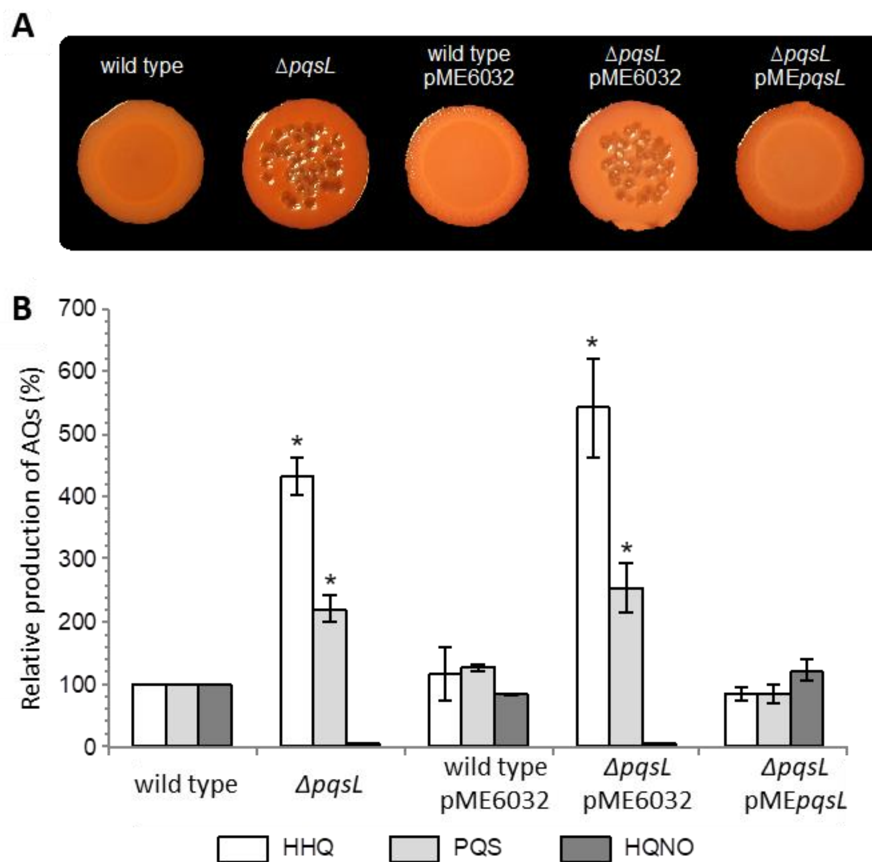

Figure 10. (A) Colony biofilm formed by the indicated *P. aeruginosa* PAO1 strains grown on Congo-Red agar plates containing IPTG (1mM) for the plasmid-driven expression of *pqsL*. (B) Levels of HHQ, PQS and HQNO measured in the indicated strains of *P. aeruginosa* PAO1grown in liquid cultures with the addition of IPTG (1mM). Mean values and standard deviations are from three independent experiment. *, $p < 0.01$.

The 2-ABA precursor is converted into HHQ by PqsB and PqsC enzymes and can spontaneously convert in DHQ and 2-AA (Fig. 6; Dulcey *et al*., 2013). Therefore the possibility that the lytic phenotype of PAO1 Δ*pqsL* and PAO1 Δ*pqsHL* could be caused by the accumulation of 2-ABA, DHQ or 2-AA, cannot be ruled out. To clarify this issue, the *pqsB* and *pqsC* genes were independently deleted into PAO1 Δ*pqsL,* generating PAO1 Δ*pqsLB* and PAO1 Δ*pqsLC*. These

strains are capable of synthetizing 2-ABA, DHQ and 2-AA but unable of converting 2-ABA into HHQ (Fig. 12A). Interestingly, the introduction of either *pqsB* or *pqsC* mutations into the Δ*pqsL* background suppressed the expression of the lytic phenotype. Finally, the *pqsR* mutation was introduced in the lytic strains PAO1 Δ*pqsL* and PAO1 Δ*pqsHL*, generating strains PAO1 Δ*pqsLR* and PAO1 Δ*pqsHLR*. Since the AQs receptor PqsR is required for transcriptional activation of the *pqsABCDE* operon (Gallagher *et al*., 2001; Deziel *et al*., 2004), PAO1 Δ*pqsLR* and PAO1 Δ*pqsHLR*

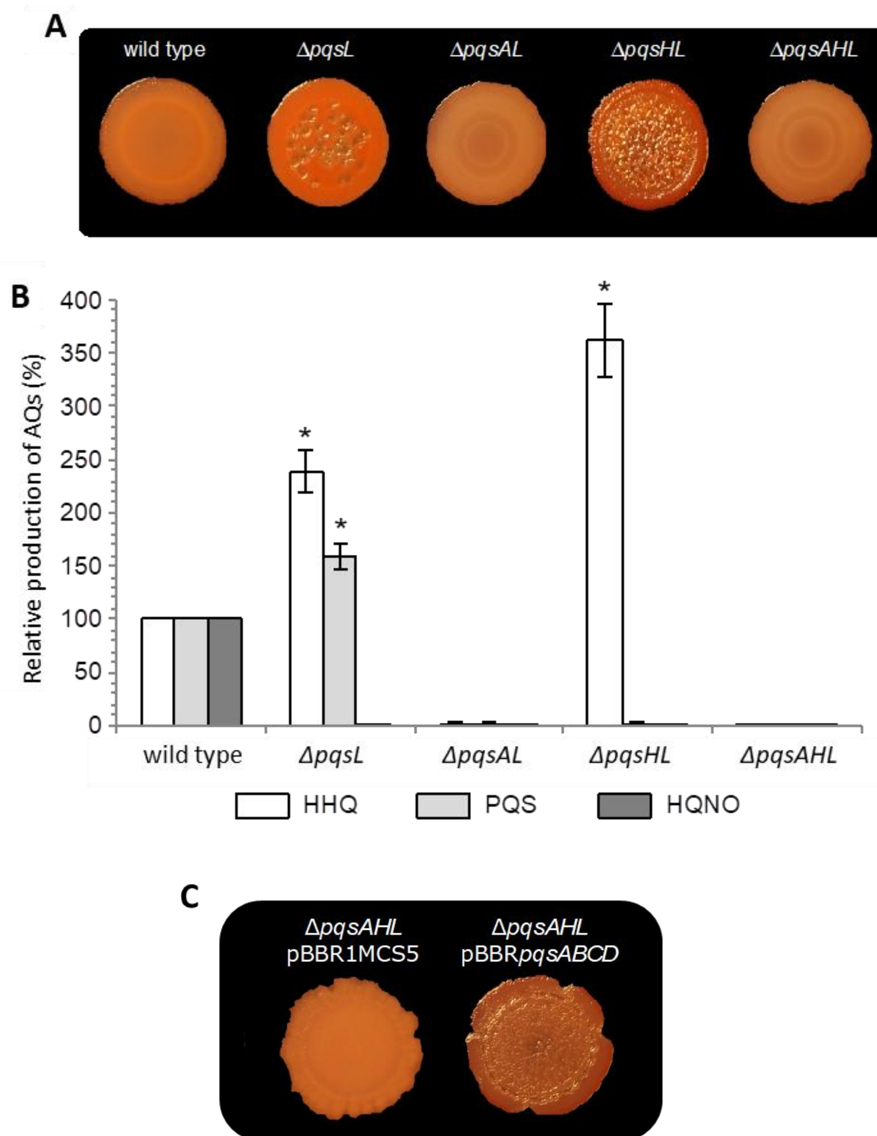

Figure 11. (A) Colony biofilm formed by the indicated *P. aeruginosa* PAO1 strains grown on Congo-Red agar plates. (B) Levels of HHQ, PQS and HQNO measured in the indicated *P. aeruginosa* PAO1 strains grown in liquid cultures. Mean values and standard deviations are from three independent experiment. *, $p <$ 0.01. (C) Colony biofilm formed by the indicated *P. aeruginosa* PAO1 strains grown on Congo-Red agar plates.

are unable to produce 2-ABA, DHQ, 2-AA, HHQ, PQS, PqsE, and HQNO. Notably, the deletion of *pqsR* mutation into a Δ*pqsL* genetic background suppressed the lytic phenotype, which could be restored by *in trans* expression of *pqsABCD* genes but not of *pqsAD* genes, required for 2-ABA synthesis (Fig. 12B). These data show that the accumulation of 2-ABA, DHQ or 2-AA is not sufficient to cause the lysis, and strongly supports the hypothesis that the lytic phenotype is induced by an accumulation of HHQ. Moreover these results also demonstrate that neither PqsR nor PqsE are required for the expression of the lytic phenotype in PAO1 Δ*pqsL*.

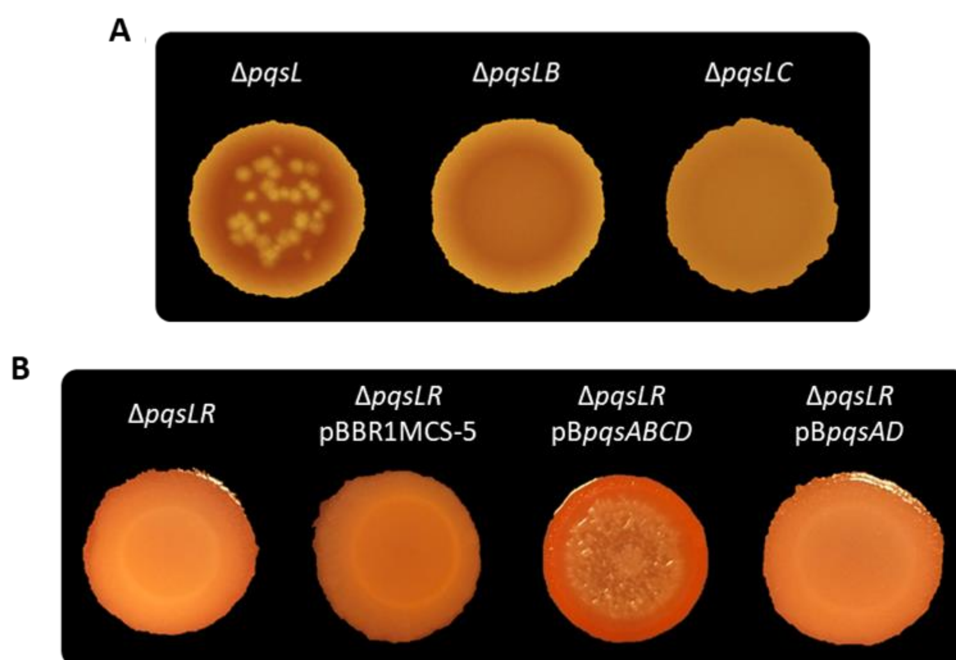

Figure 12. (A) and (B) colony biofilm formed by the indicated *P. aeruginosa* PAO1 strains grown on Congo-Red agar plates.

To further support the hypothesis that the lytic phenotype expressed by PAO1 Δ*pqsL* is caused by the higher HHQ levels accumulated by this mutant in comparison to the wild type, exogenous HHQ was provided to the PAO1 wild type strain grown in soft-agar. Results showed that exogenous HHQ promotes the appearance of lysis areas, which are not present upon the addition of DHQ or PQS (Fig. 13). Moreover, to exclude the possibility that HHQ could be converted in other AQs by the wild type strain, the exogenous addition of the molecules has been tested also in the PAO1 Δ*pqsH* and PAO1 Δ*pqsHR* strains. As mentioned above, the Δ*pqsH* mutant is unable to convert HHQ in PQS, while the introduction of *pqsR* mutation totally abolish AQs synthesis. In accordance

with our hypothesis, results showed that HHQ addition induced the production of lytic plaques in both the Δ*pqsH* and Δ*pqsHR* mutant strains, while DHQ and PQS did not (Fig. 13).

As mentioned in the background, a preliminary study showed that the exogenous addition of PQS triggered the lytic phenotype in a *P. aeruginosa pqsL pqsH* double mutant grown in soft-agar, leading the authors to conclude that PQS induced the expression of the lytic phenotype in PAO1 Δ*pqsL* (D'Argenio *et al*., 2002). However, *P. aeruginosa pqsL pqsH* double mutant strain expressed a milder lytic phenotype with respect to the corresponding *pqsL* mutant, and HHQ was not taken into consideration in this study (D'Argenio *et al*., 2002). Here we show that in our hands PAO1 Δ*pqsHL* strongly overproduces HHQ and discloses higher autolysis on colony biofilm with respect to PAO1 Δ*pqsL* (Fig. 11). Hence, since the authors of the previous study did not perform the experiment with a wild type strain and did not determined the HHQ produced by the *P. aeruginosa pqsL pqsH* mutant, they could have been mislead in the interpretation of their results.

Overall, our results strongly support the hypothesis that the lytic phenotype disclosed by PAO1 Δ*pqsL* is dependent upon HHQ accumulation, while PQS seems to be not involved in this phenomenon. The possibility that HHQ congeners could also have a role in the expression of this phenotype cannot be ruled out.

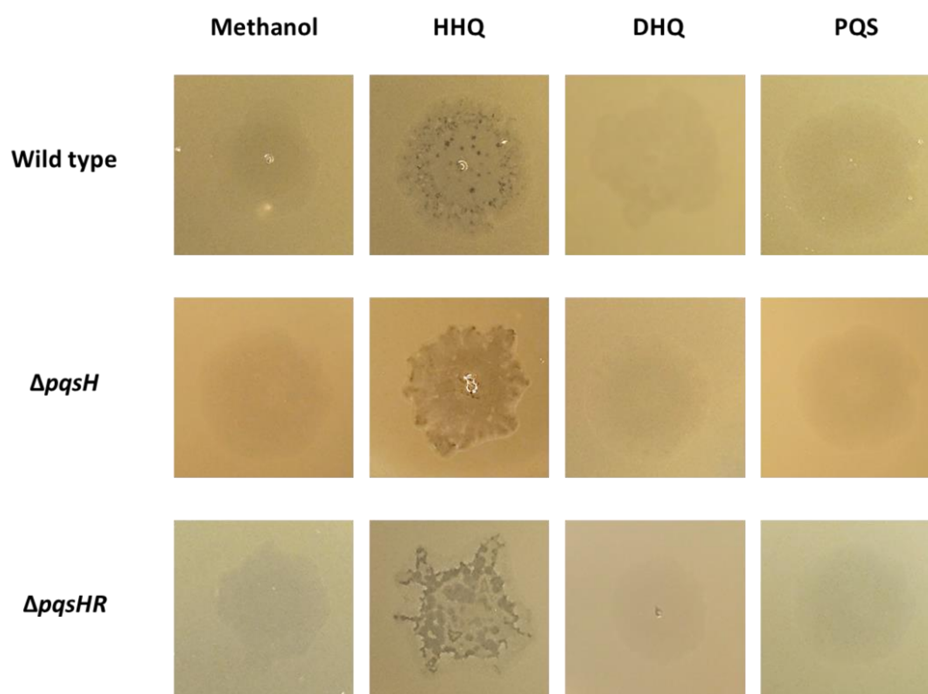

Figure 13. 5 µl of 10 mM HHQ, DHQ or PQS were spotted onto soft-agar laws formed by the indicated *P. aeruginosa* PAO1 strains.

3.2.2 Activation of Pf4 prophage is involved in the lytic phenotype expressed by PAO1 Δ*pqsL*

P. aeruginosa PAO1 has two prophages integrated in its genome, Bacto1 and Pf4 (formerly Pf1) (Stover *et al*., 2000; Finnan *et al*., 2004). Since the lytic plaques formed by PAO1 Δ*pqsL* resemble plaques formed as a consequence of phage-mediated bacterial lysis, the expression of this phenotype could involve the activation of a prophage lytic cycle. Several attempts to delete Bacto1 or Pf4 prophages in the PAO1 strain used in this study were unsuccessful. However, a *P. aeruginosa* PAO1 strain deleted in the Pf4 prophage was kindly provided by Prof. Kjelleberg (Rice *et al*., 2009). Since this mutant was not generated in our laboratory, and it is well known that PAO1 strains maintained in different laboratories disclose genotype variability (Klockgether *et al*., 2010), in this study this strain has been named PAO1-K ΔPf4 and its isogenic wild type has been named PAO1-K (Table 1, Materials and Methods).

PAO1-K ΔPf4 provides a valuable tool to evaluate Pf4 release, since the wild type strain is resistant to re-infection by the Pf4 phage, while Pf4 infection provokes lysis in the *P. aeruginosa* Pf4 knock-out mutant; therefore, the levels of Pf4 in supernatants from *P. aeruginosa* cultures can be easily detected by testing their ability to induce plaque formation when spotted onto soft-agar lawns of *P. aeruginosa* ΔPf4 (Rice *et al*., 2009). Phage particles were isolated from soft-agar lawns formed by PAO1 wild type and PAO1 Δ*pqsL* (details in Materials and Methods), and these suspensions were spotted on soft-agar lawns formed by PAO1-K wild type and PAO1-K ΔPf4. As shown in Fig. 14A, the suspension retrieved from both PAO1 wild type and PAO1 Δ*pqsL* provoked lysis in PAO1-K ΔPf4 mutant, but not in the parental strain PAO1-K, indicating Pf4 release from the tested strains.

Hence, the levels of Pf4 phage released by soft-agar lawns of the non lytic strains PAO1 wild type, PAO1 Δ*pqsAL* and PAO1 Δ*pqsAHL* and of the lytic strains PAO1 Δ*pqsL* and PAO1 Δ*pqsHL* were compared, using PAO1-K ΔPf4 for the detection of Pf4 particles (details in Materials and Methods). Results showed that the Pf4 phage was released at wild type levels by the non lytic strains unable to produce AQs (*i.e.* PAO1 Δ*pqsAL* and PAO1 Δ*pqsAHL*), indicating that AQs synthesis is not required for Pf4 release when these molecules are synthesized at physiological levels. However, the lytic strains PAO1 Δ*pqsL* and PAO1 Δ*pqsHL* released about 30- and 800-fold higher levels of Pf4 with respect to the non-lytic strains, respectively (Fig. 14B). The high level of Pf4 release in the lytic strains clearly parallels their increased HHQ levels (Fig. 11B), strongly suggesting that the lytic phenotype observed in the HHQ-overproducing strains (Fig. 11A) is caused by a transition of the Pf4 prophage from a lysogenic to a lytic cycle.

Finally, the Δ*pqsL* mutation has been introduced in the PAO1-K wild type and PAO1-K ΔPf4, generating strains PAO1-K Δ*pqsL* and PAO1-K ΔPf4 Δ*pqsL*. Deletion of the *pqsL* gene in strains

PAO1-K and PAO1-K ΔPf4 induces the expression of a lytic phenotype only in PAO1-K, hence the Pf4 prophage is required for the expression of this phenotype (Fig. 14C).

Overall, the above results show that the formation of lytic plaques in a *pqsL* defective background is due to the induction of prophage Pf4 lytic cycle and strongly suggest that this phenomenon is a consequence of HHQ overproduction.

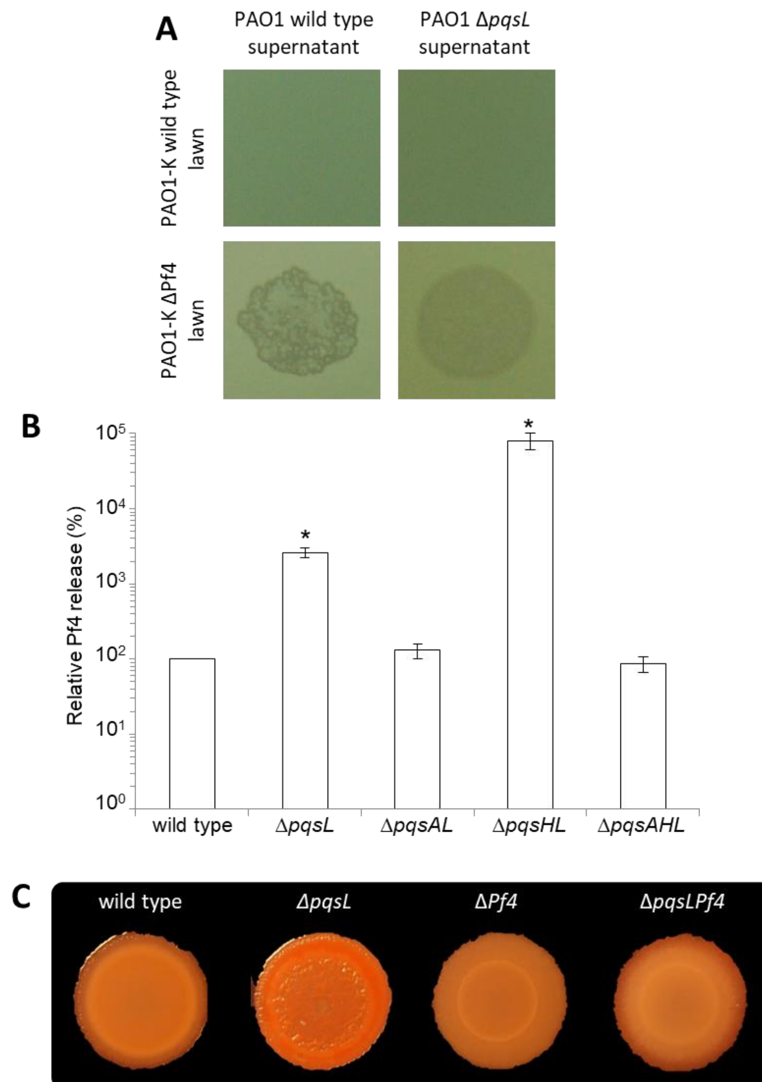

Figure 14. (A) Soft-agar LB lawns of *P. aeruginosa* PAO1-K wild type (top panels) and PAO1-K ΔPf4 (lower panels) on which 5 µl of filtered supernatants from overnight LB cultures of *P. aeruginosa* PAO1 wild type (left panels) or PAO1 Δ*pqsL* (right panels) were spotted before 24 hours incubation at 37°C. (B) Relative Pf4 release quantification from soft-agar LB lawns of the indicated *P. aeruginosa* PAO1 strains with respect to PAO1 wild type. Mean values and standard deviation are from three independent cultures. *, p value < 0.01. (C) Colony biofilms formed by the indicated PAO1-K strains grown on Congo-Red agar plates.

3.2.3 The expression of the lytic phenotype correlates with increased biofilm resistance to antibiotics

Different authors reported that prophages have a role in biofilm formation and in the resistance of biofilm to antibiotics. In particular, activation of the lytic cycle of a prophage seems to be involved in killing and lysis of a subpopulation of *P. aeruginosa* cells during biofilm formation, a pivotal step to promote the release of extracellular DNA (eDNA) (Webb *et al*., 2003; Allesen-Holm *et al*., 2006; Rice *et al*., 2009). eDNA is a main component of the biofilm matrix, and *P. aeruginosa* biofilms rich in eDNA are more resistant to antibiotics as gentamicin and tobramycin (Murakawa 1973a and b; Whitchurch *et al*., 2002; Nemoto *et al*., 2003; Chiang *et al*., 2013). Interestingly a previous study showed that a *P. aeruginosa* Δ*pqsL* mutant strain released more eDNA with respect to the wild type strain (Allesen-Holm *et al*., 2006). On these basis it could be postulated that the expression of the lytic phenotype by PAO1 Δ*pqsL* could correlate with increased biofilm resistance to antibiotics, hence experiments aimed at verifying this hypothesis have been performed.

Biofilms of PAO1 wild type and PAO1 Δ*pqsL* were grown for 16 hours and then incubated for 6 hours with different concentrations of tobramycin and gentamicin. The effect of the antibiotics on the mass and on the viability of the biofilm was determined by crystal violet staining and by Fluorescein Diacetate (FDA) assay, respectively (as detailed in Materials and Methods). Results showed that biofilms formed by both PAO1 wild type and PAO1 Δ*pqsL* strains are very similar in the absence of antibiotics, indicating that deletion of *pqsL* does not affect biofilm formation or viability (Fig. 15). Moreover biofilm mass is not significantly affected in the presence of tobramycin or gentamicin (Fig. 15A and C). Conversely, the viability of biofilms formed by PAO1 wild type was strongly reduced by both antibiotics, in comparison to the biofilms formed by PAO1 Δ*pqsL* (Fig. 15B and D). Notably, the susceptibility to antibiotics of biofilms formed by PAO1 Δ*pqsL* is restored to the wild type levels by expressing *in trans pqsL* (Fig. 16).

In addition, the Minimum Inhibitory Concentration (MIC) of tobramycin and gentamicin were identical (0.78 μg/ml and 0.27 μg/ml, respectively) for both PAO1 and PAO1 Δ*pqsL* planktonic cultures grown in the same medium used for biofilm growth (M9 minimal medium).

Overall, our results show that the expression of the lytic phenotype by PAO1 Δ*pqsL* confers increased resistance to antibiotics only when this strain is grown as a biofilm. The increased resistance to antibiotics of PAO1 Δ*pqsL* with respect to PAO1 wild type biofilms is likely due to the activation of the lytic cycle of Pf4 prophage and consequent increased release of eDNA by PAO1 Δ*pqsL* with respect to the wild type strain. This conclusion is supported by a number of studies on *P. aeruginosa* biofilms, showing a strong correlation among phage-mediated cells lysis, eDNA release and increased resistance to antibiotics (Webb *et al*., 2003; Allesen-Holm *et al*., 2006; Rice *et*

al., 2009; Murakawa 1973 a and b; Whitchurch *et al*., 2002; Nemoto *et al*., 2003; Chiang *et al*., 2013).

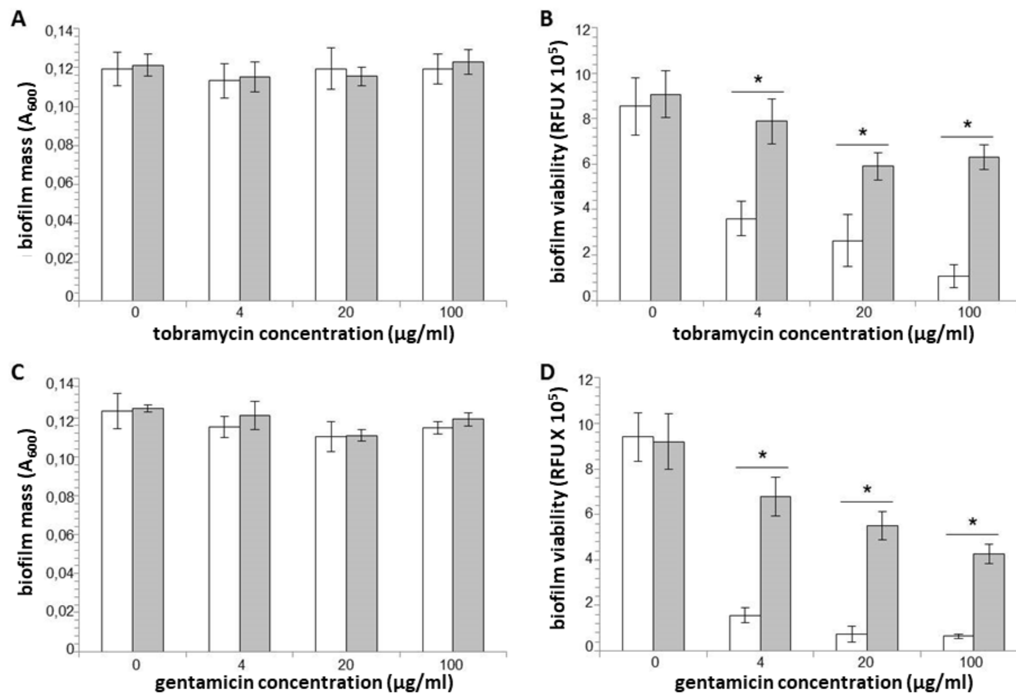

Figure 15. Graphs representing biofilm mass (A and C) and viability (B and D) of *P. aeruginosa* PAO1 wild type (white bar) and *P. aeruginosa* PAO1 Δ*pqsL* (grey bar) after 6 hours of treatment with tobramycin (A and B) or gentamicin (C and D). Biofilm mass was measured by a standard CV assay, while viability was measured by an FDA assay (as described in Materials and Methods). Mean values and standard deviations are from three independent experiment. In each experiment the biofilm has been grown in 8 wells. *, p value < 0.01.

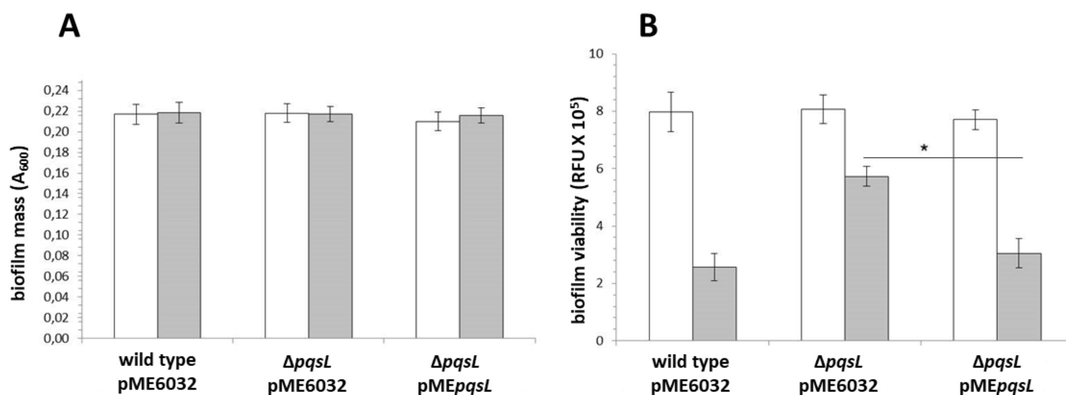

Figure 16. Graphs representing biofilm mass (A) and viability (B) of the indicated *P. aeruginosa* PAO1 strains. White bar, without tobramycin; grey bar, after 6 hours of treatment with 100 µg/ml tobramycin. Biofilm mass was measured by a standard CV assay, while viability was measured by an FDA assay (as described in Materials and Methods). Mean values and standard deviations are from three independent experiment. In each experiment the biofilm has been grown in 8 wells. *, p value < 0.01.

3.2.4 The expression of the lytic phenotype is correlated to *pqsL* inactivation in *P. aeruginosa* cystic fibrosis clinical isolates

As previously described, *P. aeruginosa* CF strains often express a lytic phenotype similar to PAO1 Δ*pqsL* (Berk 1963; D'Argenio *et al*., 2002). In order to investigate if the *pqsL* mutation correlates with the expression of the lytic phenotype in *P. aeruginosa* CF clinical isolates, a collection of 101 *P. aeruginosa* strains isolated from CF patients has been analysed. 27 out of 101 strains belong to our laboratory collection and derive from CF patients with no information about how many years they have been colonized (Supplementary information, Table S2). The remaining 74 strains belong to the collection described in chapter 4 (Supplementary information, Table S1); of these 29 correspond to the first isolation of *P. aeruginosa* from a CF patient, 21 are strains isolated from CF patients with chronic infection up to 3 years of chronic infection, 15 are strains isolated from CF patients with chronic infection from 4 to 7 years, and 9 are strains isolated from CF patients with chronic infection for more than 15 years (Supplementary information, Table S1).

Results showed that 34 out of the 101 strains tested express a lytic phenotype when grown as colony biofilms, a value in accordance with a previous study (31%; D'Argenio *et al*., 2002; Fig. 17). In the 74 strains in which data about the years of infection are available, there are not statistically significant differences between the expression of lytic phenotype and the years of infection (data not shown).

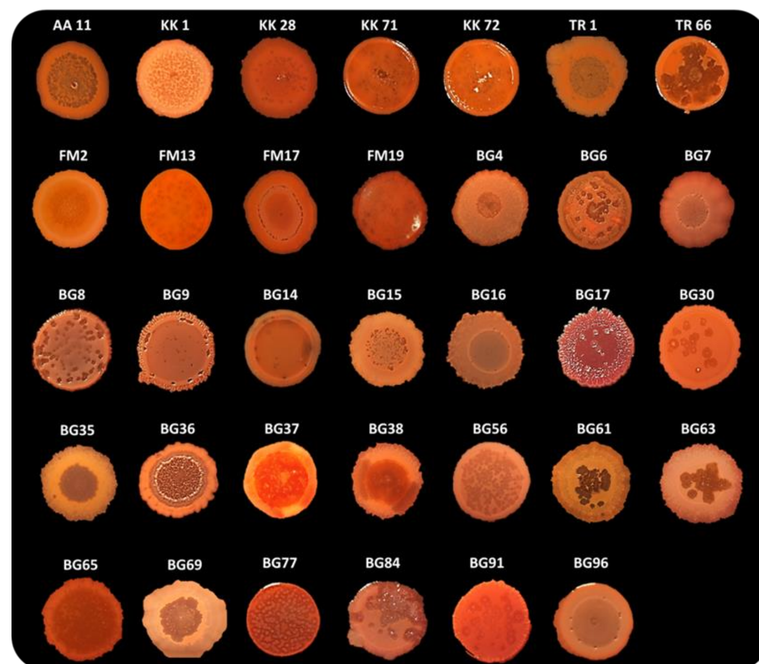

Fig. 17. Colony biofilm formed by the indicated *P. aeruginosa* CF clinical strains, grown on Congo-Red agar plate.

The *pqsL* gene was amplified by PCR from the genomes of the 34 lytic strains present in our collection. As shown in figure 18, the PCR generated a DNA fragment of expected molecular weight (~ 1200 bp) in the PAO1 wild type (positive control) and in 25 out of 34 lytic strains. On the contrary, in 9 out of 34 strains there was no PCR product (Fig. 18). These 9 strains have been further analysed by PCR using different pairs of primers and testing different PCR conditions. However, any attempt to amplify the *pqsL* gene in these strains was unsuccessful. This strongly suggest that in 9 CF clinical isolates (26% of the lytic strains in our collection) the *pqsL* sequence could be deleted or severely rearranged.

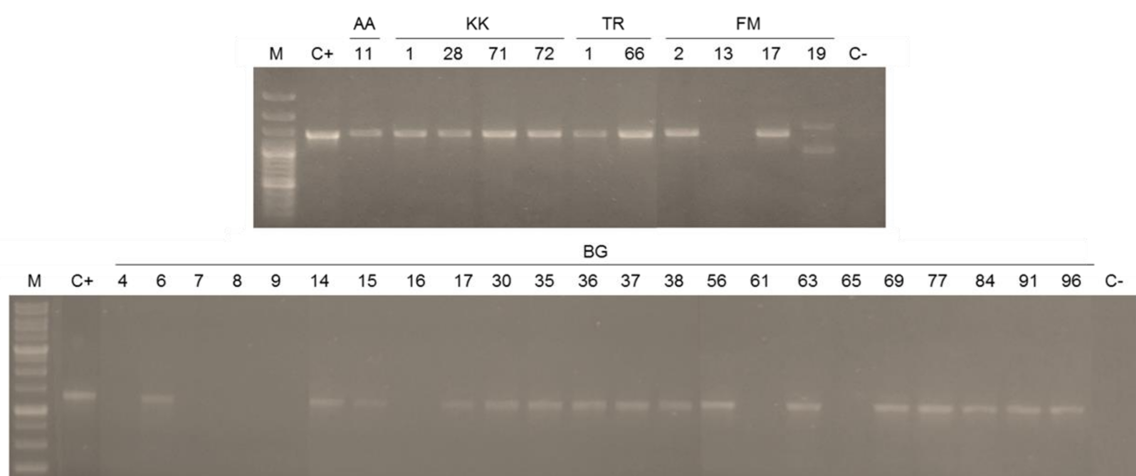

Figure. 18. Electrophoresis analyses of the *pqsL* gene amplified by PCR from the genome of the indicated *P. aeruginosa* CF strains by using primers FW374*pqsL*-RV375*pqsL* and treated by electrophoresis gel analyses. M, ladder ”GeneRuler 100bp Plus DNA Ladder” (Fermentas); C+, positive control generated from *P. aeruginosa* PAO1 genome; C-, negative control in which DNA has not been added.

The DNA fragment amplified in the 25 CF strains with lytic phenotype was sequenced and compared with the *pqsL* sequence of our PAO1 laboratory strain, which is identical to the *pqsL* sequence deposited for *P. aeruginosa* PAO1 in the *Pseudomonas* database (Stover *et al*., 2000; Winsor *et al*., 2011). All 25 lytic strains carry nucleotide substitutions in the *pqsL* ORF, and in 17 of these the substitution is nonsynonymous figure 19A. The conservative nucleotide substitutions found in the *pqsL* ORF of the remaining 8 lytic strains do not introduce rare codons, suggesting that the lytic phenotype in these strains do not depend upon loss of PqsL functionality. However, the possibility that in these strains *pqsL* could not be expressed due to mutations in its promoter region cannot be ruled out.

Notably, the substitution of glutamic acid in position 330 to glycine (E330G) is very common (figure 19A). For this reason the importance of this mutation has been preliminarily investigated by generating an *in silico* model of the 3D structure of PqsL (in collaboration with Prof. Fabio Polticelli, University Roma Tre) (Fig. 19B). According to this model, the Glu 330 residue is located in an alpha helix which could be important for the structural stability of the PqsL protein, suggesting that mutations affecting Glu 330 could destabilize PqsL structure, hence hampering its enzymatic activity (Fig. 19B).

A

Lytic CF clinical isolates	PqsL mutations							
AA11		D117E	D175E	R179H		A232S		
KK1							E330G	
KK28							E330G	
KK71							E330G	
KK72							E330G	
TR1							E330G	
TR66							E330G	
BG6							E330G	
BG14							E330G	
BG15							E330G	
BG30			D175E	R179H	S206A	A232S		
BG37	A60D							
BG38	A60D							
BG69			D175E				E330G	
BG84								T378A
BG91			D175E				E330G	
BG96							E330G	

B

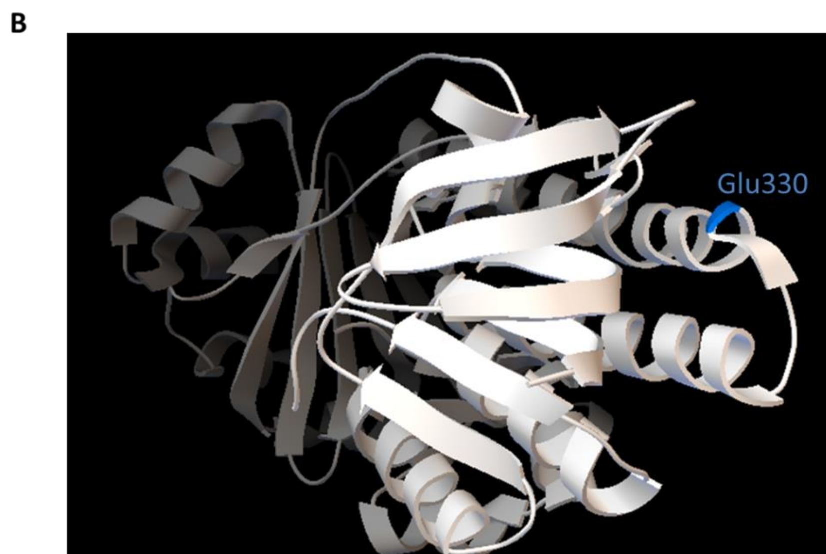

Figure. 19. (A) PqsL mutations in the indicated *P. aeruginosa* CF strains (B) *In silico* 3D structure of PqsL protein. E330 residue is highlighted in blue.

Overall, the *pqsL* gene is likely deleted or rearranged in 9 lytic strains, while it carries a nonsynonymous nucleotide substitution in 17 lytic isolates. Hence, it is likely that in these 26 strains, accounting for 76 % of the lytic CF strains analysed, the lytic phenotype could depend upon loss of PqsL expression. In the lytic strains for which data about the years of infection are available (n =23), there are not statistically significant differences between the inactivation of *pqsL* and the years of infection (data not shown).

To provide a genetic evidence of the correlation between *pqsL* inactivation and the expression of the lytic phenotype, we aimed at expressing in the 26 lytic strains carrying mutations in *pqsL* a functional *pqsL* gene, carried by the pME*pqsL* plasmid. Despite multiple attempts, this plasmid could be successfully introduced only in 8 out of the 26 strains. This is not surprising, since the difficulty to transfer plasmidic DNA in *P. aeruginosa* CF isolated is well known (Diver *et al*., 1990; Hentzer *et al*., 2001). However, the lytic phenotype was abrogated or strongly reduced in 6 out of 8 CF lytic strains carrying the pME*pqsL* plasmid (figure 20).

Overall, it is reasonable to conclude that the lytic phenotype depend upon loss of PqsL expression or functionality in the majority of the lytic CF strains analysed.

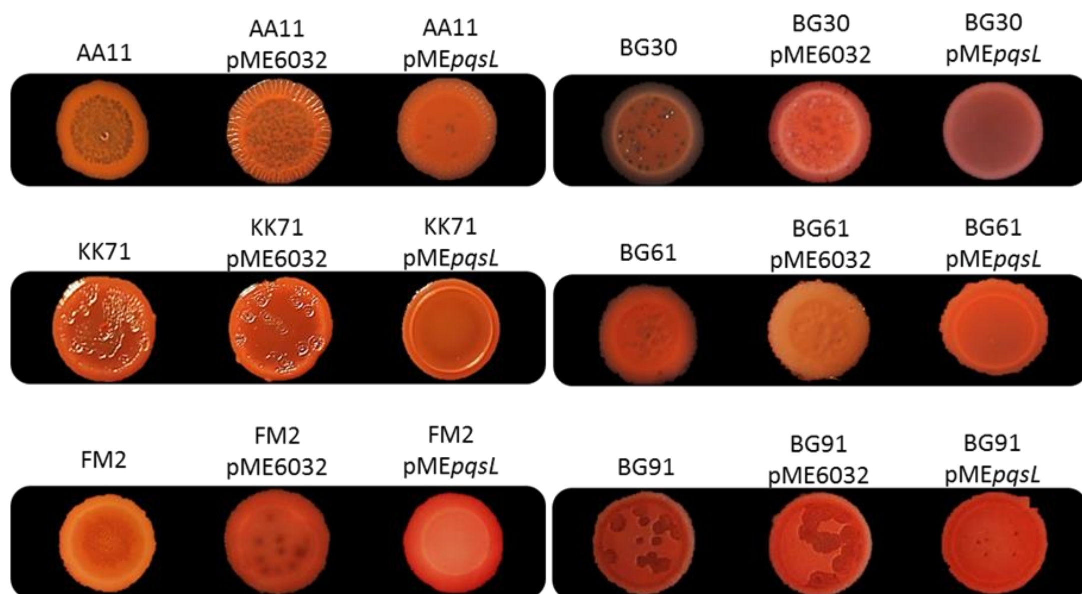

Figure 20. Colony biofilm formed by the indicated *P. aeruginosa* CF clinical isolates grown on Congo-Red agar plates, containing IPTG (1mM) for the plasmid driven expression of *pqsL*.

3.3 Conclusions

The isolation of *P. aeruginosa* clinical strains expressing a lytic phenotype has intrigued researchers since 1924 (Hadley 1924; Berk 1963; Berk 1965; Holloway 1969). About this, Holloway wrote "*For the present, the genetic basis of autoplaque formation remains obscure*" (Holloway, 1969). Even if a preliminary study published more than 15 years ago suggested a correlation between the lytic phenotype and the inactivation of the *pqsL* gene (D'Argenio *et al*. 2002), the mechanisms underlying this phenomenon and the possible adaptive advantage causing the emergence of the lytic phenotype in *P. aeruginosa* clinical strains have not been further investigated so far.

Other recently published puzzling observations concern HQNO, the product of the PqsL enzyme. In fact, this molecule does not affect *P. aeruginosa* transcriptome and cannot be considered a QS signal (Rampioni *et al*., 2016), even if this AQ is produced at levels similar to the HHQ and PQS QS signals (Lépine *et al*., 2003; Déziel *et al.*, 2004). HQNO is toxic for Gram-negative bacteria and the production of this molecule could confer a competitive advantage to *P. aeruginosa* in polymicrobial communities, partly explaining the predominance of *P. aeruginosa* over *S. aureus* in early CF infections (Filkins *et al*., 2015; Nguyen *et al*., 2016).

This study has been aimed at shedding new light on the role played by PqsL and HQNO synthesis in *P. aeruginosa* physiology. It has been shown that the abolition of HQNO synthesis in *P. aeruginosa* PAO1 *pqsL* causes accumulation of HHQ. This induces the activation of the lytic cycle in the Pf4 prophage, with consequent formation of lytic plaques in colony biofilms.

Finally, 26 out of 34 (76%) CF strains expressing the lytic phenotype carry a deleted or mutated *pqsL* gene, and the wild type (non-lytic) phenotype is restored in 6 out of the 8 CF strains in which a functional *pqsL* could be expressed *in trans*. To the best of our knowledge, this is the first evidence of a clear genetic correlation between the expression of the lytic phenotype and the emergence of the *pqsL* mutation in clinical *P. aeruginosa* strains. It is also important to highlight that Pf4 prophage is very common among *P. aeruginosa* CF clinical isolates (Webb *et al*., 2003), suggesting that the molecular mechanism observed in PAO1 could be conserved. Further studies will be needed in the future to assess whether HHQ accumulation and prophage activation occurs even in the CF strains carrying the *pqsL* mutation, and which are the genes and mechanisms involved in the expression of the lytic phenotype by CF strains carrying a functional *pqsL* gene.

Overall, our results suggest that the production of HQNO confers an adaptive advantage to *P. aeruginosa* wild type not only by inhibiting the growth of competitors in polymicrobial communities (Heeb *et al*., 2011), but also by limiting HHQ accumulation and consequent cellular lysis due to prophage activation. However, it is tempting to speculate that when *P. aeruginosa* prevails upon *S. aureus* in the CF lung and establishes a single-species infection, the selective

pressure for the maintenance of HQNO synthesis could decrease and the emergence of a sub-population of *pqsL* mutants could became advantageous in this challenging environmental niche. In this view, the increased antibiotic resistance of the *P. aeruginosa* PAO1 *pqsL* biofilm with respect to the wild type shown in this and other studies suggests the existence of a link between prophage activation, bacterial autolysis and biofilm resistance to antibiotics (Webb *et al*., 2004; Allesen-Holm *et al*., 2006; Rice *et al*., 2009; Chiang *et al*., 2013), and it could at least in part explain the emergence of *pqsL* mutants during the infection in antibiotic-treated CF patients.

Assessing the suitability of quorum sensing inhibition in cystic fibrosis therapy

4.1 Background

As described in the introduction, *P. aeruginosa* strains that chronically infect the lung of CF patients undergo a patient-related evolutionary process. CF strains isolated from late chronic infections develop peculiar phenotypical characteristics, including the inability to produce virulence factors or the loss of QS functionality (Bjarnsholt *et al.*, 2010; Folkesson *et al.*, 2012; Jiricny *et al.*, 2014; Winstanley *et al.*, 2016; Feltner *et al.*, 2016). Since *P. aeruginosa* QS-defective mutants should be considered resistant to anti-QS drugs, the suitability of QS-inhibition for cystic fibrosis therapy is under debate. Nevertheless, the research aimed at identifying anti-QS drugs is very active world-wide and the effectiveness of antivirulence compound targeting QS is seldom tested in CF isolates (Ciofu *et al.*, 2015; Rampioni *et al.*, 2014, Winstanley *et al.*, 2016).

Several molecules inhibiting the $3OC_{12}$-HSL-dependent QS system (the *las* system) have been identified so far (Lasarre and Federle, 2013; Rampioni *et al.,* 2014). A relevant example is NCL, discovered in 2013 by a drug repurposing approach (Imperi *et al.*, 2013). Drug repurposing is based on the screening of library of compounds already approved for use in humans by the US Food and Drug Administration (FDA) with the aim to identify side activities that could be exploited for new therapeutic applications, in molecules with low toxic activity. By this way, the time necessary to translate new drugs to therapy should be reduced (Rangel-Vega *et al.*, 2015).

The screening leading to NCL discovery was performed by co-cultivating the PA14-R3 biosensor strain of *P. aeruginosa* and its isogenic wild type strain (PA14). PA14-R3 is a mutant unable to produce $3OC_{12}$-HSL and containing a transcriptional fusion between a promoter LasR/$3OC_{12}$-HSL dependent and the *luxCDABE* operon, encoding the enzymes required for light emission. As PA14-R3 is deleted in *lasI*, the gene coding for the synthase responsible for $3OC_{12}$-HSL production, it is able to emit bioluminescence only when grown in presence of a strain producing $3OC_{12}$-HSL or when this molecule is exogenously added (Massai *et al.* 2011; Rampioni *et al.*, 2018; see also Supplementary information). In the co-culture based screening PA14 provides the $3OC_{12}$-HSL activating PA14-R3 light emission, hence the addition of an anti-QS compound to the co-culture provokes an emission of bioluminescence lower with respect to the untreated co-culture (Imperi *et al.*, 2013; Rampioni *et al.*, 2018; Supplementary information). Criteria used for hits selection were the ability to inhibit light emission $\geq 50\%$ and growth $\leq 20\%$, with respect to the untreated control. The screening led to the identification of 7 compounds that were further tested at different concentrations, showing an IC_{50} from 10 to 150 μM. As the anthelmintic drug NCL disclosed the highest anti-QS activity (lowest IC_{50}), it was selected for further studies (Imperi *et al.*, 2013).

Dose-response experiments carried out by measuring $3OC_{12}$-HSL levels in supernatants of PA14 grown in the presence of increasing NCL concentrations showed that the maximum inhibition of $3OC_{12}$-HSL production reached a plateau at the concentration of 5 μM (Imperi *et al*., 2013). Notably NCL was able to reduce the production of C_4-HSL both in PA14 and in a Δ*lasI* mutant strain, indicating that this drug has an inhibitory effect also on the *rhl* QS system, independent on its effect on the *las* system. On the contrary, NCL had no effect on the *pqs* QS system. Moreover, NCL was able to inhibit light emission in PA14-R3 when $3OC_{12}$-HSL was exogenously added, indicating that NCL targets *lasR* expression or activity rather then the synthesis of $3OC_{12}$-HSL (Imperi *et al*., 2013). In addition, microarray analysis showed that 20 μM NCL affects the transcription of 258 genes in PA14, 73.2 % of which are repressed, including genes responsible for the production of QS-dependent virulence factors, such as pyocyanin, LasA protease, LasB elastase, rhamnolipids and others. Among the genes repressed by NCL, 96 of these are activated by $3OC_{12}$-HSL and/or C_4-HSL. In accordance with microarray analysis, 5-10 μM NCL strongly reduces the production of QS-dependent virulence factors (e.g. pyocyanin, elastase and rhamnolipids) and protects *Galleria mellonella* larvae from *P. aeruginosa* infection (Imperi *et al*., 2013).

Overall the study summarized above supports the anti-QS and anti-virulence activity of NCL and suggests that NCL could be a good candidate to combat *P. aeruginosa* infections. However, as an anthelmintic, NCL is administered orally and is not adsorbed by the gut. With the aim of making this drug suitable for aerosol administration, the rough power of NCL was re-formulated as a mannitol-based dry powder named T80_10 DP, containing NCL nanoparticles that can be reconstituted in saline. Analysis of the T80_10 DP pharmacological properties showed that this formulation has an optimal *in vitro* aerosol performance, and low toxicity in CF bronchial epithelial cells and in acute murine model of toxicity. Moreover, formulation of T80_10 DP and NCL (rough powder) have comparable ability to inhibit $3OC_{12}$-HSL production in *P. aeruginosa* PA14 (Costabile *et al*., 2015).

The study described in this chapter has been carried out in order to understand if a drug able to affect the *las* QS system in a laboratory strain could be suitable for the treatment of CF patients, highlighting possible limitations of this kind of therapy. To this aim, a collection of *P. aeruginosa* CF strains expressly enriched in CF strains isolated from patients with intermittent or early chronic infection has been assembled and NCL has been used as model inhibitor of the *las* QS system.

4.2 Results and discussion

4.2.1 Collection of *Pseudomonas aeruginosa* cystic fibrosis clinical isolates

A previous study considering 238 CF clinical strains suggested that strains isolated 12 years after the onset of the chronic lung infection have high probability to express deficiencies in $3OC_{12}$-HSL production (Bjarnsholt *et al*., 2010). Since QS-defective strains should be considered resistant to anti-QS drugs, a main outcome of this study is that anti-QS drugs would not be effective in the majority of CF patients with chronic infection established from more than a decade. On the other hand, this also implies that the probability of isolating strains susceptible to drugs targeting the $3OC_{12}$-HSL-based QS system should be higher in patients with intermittent or early chronic infection. On these basis, we assembled a collection of 100 *P. aeruginosa* CF clinical strains deliberately enriched with strains isolated from CF patients for the first time or with chronic infection up to 7 years (Supplementary information, Table S1; in collaboration with Dr. E. Fiscarelli, Hospital Bambino Gesù, Rome, Italy).

The clinical isolates of the collection have been grouped in four categories with respect to years of lung infection. The "first isolate" group contains 40 strains that correspond to the first isolation of *P. aeruginosa* from a patient. The "early" and "middle" groups contain 25 strains from chronic infection up to 3 years and from 4, up to 7 years, respectively; the "late" group contains 10 strains isolated from patients with more than 15 years of chronic infection (Figure 21).

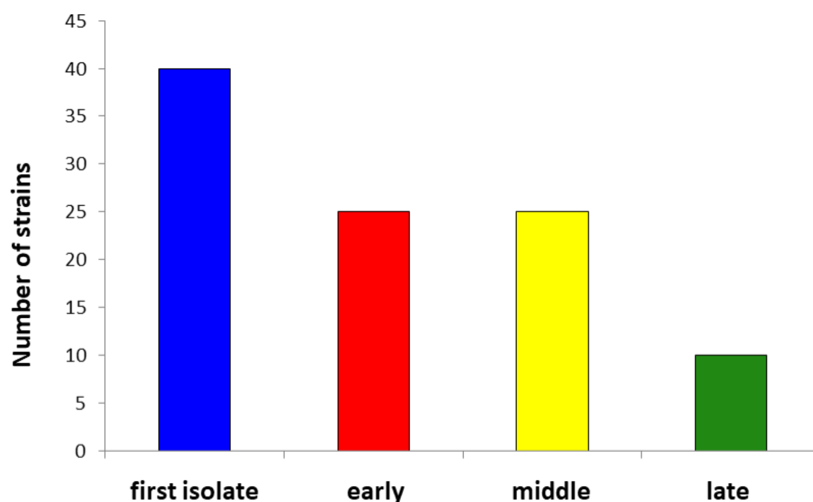

Figure 21. Collection of *P. aeruginosa* CF strains used in this study. "fist isolate", strains isolated for the first time from CF patients; "early", strains isolated from CF patients with chronic infection up to 3 years; "middle", strains isolated from CF patients with chronic infection from 4 up to 7 years; "late", CF strains isolated from CF patients with chronic infection for more than 15 years.

The strains belonging to the collection have been characterized for susceptibility to different antibiotic classes (Supplementary information, Table S3) and the overall pattern of resistance to antibiotics has been analysed for each group of strains of the collection, according to the following criteria: susceptible, strains susceptible to all antibiotic classes; resistant, strains that are not susceptible to antibiotics belonging to one or two different classes; multi-drug resistant (MDR), strains not susceptible to one or more antibiotics belonging to at least 3 different classes; extensively-drug resistant (XDR), strains not susceptible to one or more antibiotics belonging to all classes, except two. MDR and XDR strains have been classified following ECDC (European Centre for Disease Control, http://ecdc.europa.eu/en/Pages/home.aspx) criteria. As shown in figure 22 the percentage of susceptible strains decreases in parallel with years of chronic infection, while the percentage of resistant strains increases up to 3 years of chronic infection and then decreases in parallel with the rise of MDR and XDR strains percentage. These observations are in accordance with literature data and are likely caused by intensive antibiotics treatment to which CF patients are exposed along years (Oliver and Mena, 2010; Breideinstein *et al*., 2011; Winstanley *et al*., 2016).

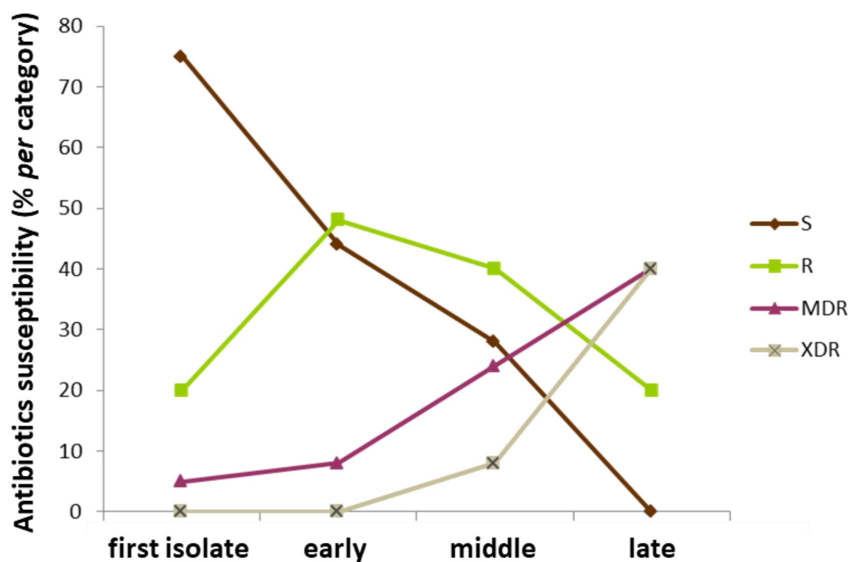

Figure 22. Pattern of antibiotic resistance in the CF strain collection. S, strains susceptible to all antibiotic classes; R, strains not susceptible to antibiotics belonging to one or two different classes; MDR, strains not susceptible to one or more antibiotics belonging to at least 3 different classes; XDR, strains not susceptible to one or more antibiotics belonging to all classes save two. Are statistically significant (p value ≤ 0,0232) differences between: fist isolate and early category, first isolate and middle, first isolate and late for the S group; first isolate and early for the R group; first isolate and middle, first isolate and late, early and late for the MDR group; first isolate and late, early and late, middle and late, for the XDR group. Statistical analyses ANOVA.

4.2.2 Production of quorum sensing signal molecules in *P. aeruginosa* cystic fibrosis isolates

The strains of the collection have been characterized for their ability to produce QS signal molecules by co-cultivation with two specific whole cell biosensors. In particular, biosensor PA14-R3 has been used for $3OC_{12}$-HSL detection, while PAO1-Δ*rhlI* (pKD-*rhlA*) has been used for C4-HSL detection (Massai *et al.*, 2011; Imperi *et al.*, 2013; Rampioni *et al.*, 2018; more details in Materials and Methods).

Overall, 63 strains of the collection produce both $3OC_{12}$-HSL and C_4-HSL, 6 strains produce exclusively $3OC_{12}$-HSL, 22 strains produce exclusively C_4-HSL and 9 strains cannot produce both QS molecules (Fig. 23 and Supplementary information, Table S4).

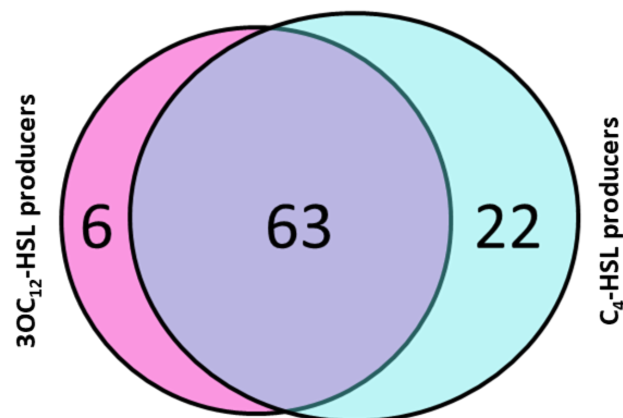

Figure 23. Number of strains producing $3OC_{12}$-HSL, C_4-HSL, or both signal molecules in the CF strain collection.

As shown in figure 23, the overall number of strains producing exclusively C_4-HSL (n = 22) is 3.7 folds higher than the number of strains producing exclusively $3OC_{12}$-HSL (n = 6). This value is similar to what reported by Bjarnsholt *et al* (3.9) and supports the notion that loss of $3OC_{12}$-HSL production is more frequent with respect to the loss of C_4-HSL production (Bjarnsholt *et al.*, 2010).

The percentage of producers of both $3OC_{12}$-HSL and C_4-HSL decreases in parallel with the years of infection, even if there are not statistically significant differences between the four categories of strains (Fig.24A; Supplementary information, Table S5). However, it is worth to highlight that the "first isolate" and "late" categories are close to be significantly different (p = 0.0538; Fig. 24A; Supplementary information, Table S5). Also when considering the total $3OC_{12}$-HSL producers (irrespective if a strain also produce C_4-HSL), the percentage of producers decreases in parallel with the increase of years of infection and the differences between "first isolate" and "late" category is

close to be statistically significant (p = 0.0546) (Fig. 24B; Supplementary information, Table S5). Since the "late" category contains much less strains than each one of the other categories (figure 21), it is likely that statistical significance could be increased by analysing and higher number of strains in the "late" category, in order to reach a number comparable to the other categories.

Overall, our results are in agreement with the study by Bjarnsholt *et al*, supporting the notion that the loss of $3OC_{12}$-HSL production parallels the years of chronic infection and begin to be significant only after more than one decade of infection (Bjarnsholt *et al*., 2010). A similar conclusion was reached by Jiricny *et al*, by considering a collection of 40 strains (Jiricny *et al*., 2014).

The percentage of total C_4-HSL producers (irrespective if they also produce $3OC_{12}$-HSL), is similar among the four categories (Fig. 24B). This result is in accordance with the studies by Jiricny *et al* (2014) and by Bjarnsholt *et al* (2010). In particular, the latter showed that the loss of C_4-HSL signal production parallels the years of chronic infection and starts to be significant after 17 years of chronic infection (Bjarnsholt *et al*., 2010). Overall, our results are in agreement with previous studies and indicate that the loss of $3OC_{12}$-HSL and C_4-HSL producers is not significant up to 7 years of chronic infection.

To the best of our knowledge, the actual levels of $3OC_{12}$-HSL produced by CF clinical isolates has been previously quantified in few studies (Erickson *et al*., 2002), considering limited numbers of strains likely due to the lack of methods allowing a rapid and reliable quantification of $3OC_{12}$-HSL (Massai *et al.,* 2011; Rampioni *et al.,* 2018). Here $3OC_{12}$-HSL levels have been determined in the supernatants of the 69 producing strains present in our collection (more details in Materials and Methods and in Supplementary information). The levels of $3OC_{12}$-HSL produced is highly variable (from ≈ 0.8 μM to ≈ 15 μM; Fig. 25A), even if the majority of the strains produces $3OC_{12}$-HSL levels comparable to the model strain PA14 (≈ 3 μM). Statistical analyses showed that levels of $3OC_{12}$-HSL produced are not correlated with years of infection or with antibiotic susceptibility (Fig. 25A and B). Also in this case, it should be noted that the total number of MDR and XDR is little (n= 5 and 2, respectively) in comparison to S and R strains (n= 38 and 24, respectively). In the future statistical significance of this analysis could be increased by analysing and higher number of strains belonging to these categories.

Overall the majority of strains in our collection produces $3OC_{12}$-HSL at levels comparable or higher to the reference strain PA14. This suggest that the majority of *P. aeruginosa* strains isolated from CF patients for the first time or with chronic infection established up to 7 years should be considered susceptible to drugs targeting the *las* QS system.

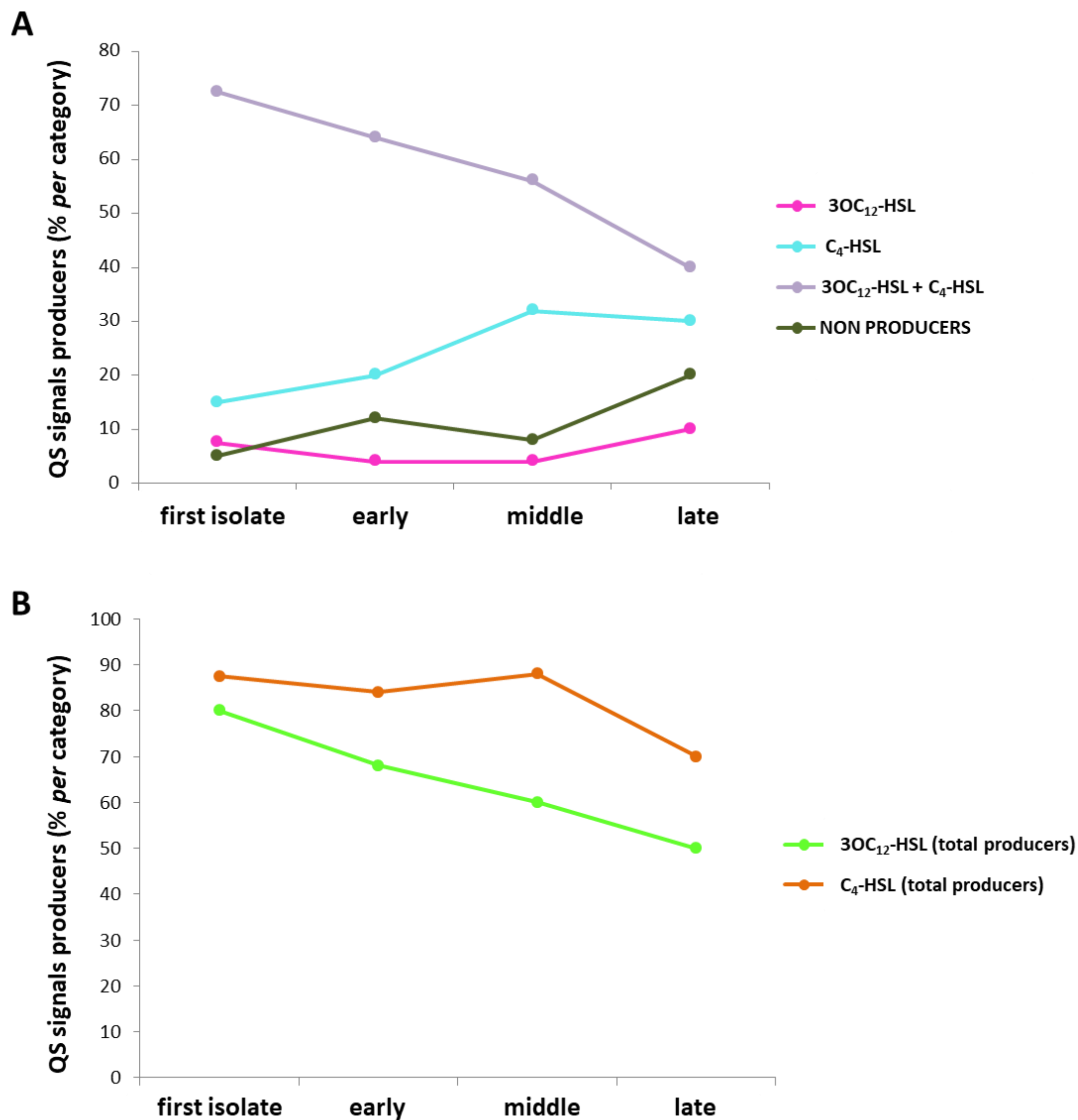

Figure 24. Percentages of strains producing $3OC_{12}$-HSL and C_4-HSL QS signal molecules in the CF strain collection, for the indicated group of strain. (A) strains have been grouped in: producers of both signals (violet line); producers of $3OC_{12}$-HSL (pink line); producers of C_4-HSL (sky blue line); non producers (dark green line). (B) strains have been grouped in: total producers of $3OC_{12}$-HSL (strains that produces both signal molecules plus strains that produces only $3OC_{12}$-HSL; light green line); in total producers of C_4-HSL (strains that produces both signal molecules plus strains that produces only C_4-HSL; orange line). Differences among categories are not statistically significant (ANOVA; see also Supplementary information, Table S5).

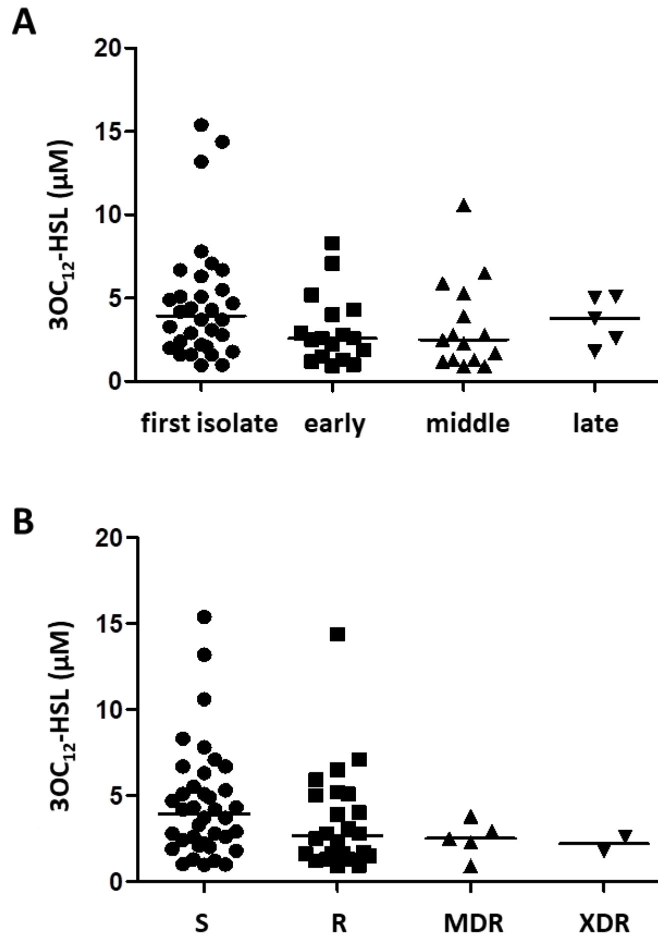

Figure 25. Levels of $3OC_{12}$-HSL produced by the strains of the CF isolate collection, in relation with years of infection (A) and with antibiotic susceptibility (B). There are not statistical differences among levels of $3OC_{12}$-HSL produced and antibiotic susceptibility (ANOVA). Black lines represent the median values.

4.2.3 Susceptibility of *P. aeruginosa* cystic fibrosis isolates to niclosamide

As described in the introduction of this chapter, the anthelmintic drug NCL inhibits $3OC_{12}$-HSL production and virulence in the reference strain *P. aeruginosa* PA14 (Imperi *et al.*, 2013). Therefore it could be a good candidate to treat *P. aeruginosa* lung infections, also considering that this drug can be successfully be reformulated for aerosol administration (Costabile *et al.*, 2015). In addition, the results shown above suggest that the majority of strains isolated from CF patients infected for the first time or with early chronic infections are $3OC_{12}$-HSL producers and should be susceptible to anti-QS drugs targeting the *las* QS system. However it is true that the CF lung environment could also select strains with increased resistance to antibiotics and other toxic factors produced by the immune system. Indeed strains isolated from CF patients frequently disclose overexpression of efflux pumps, increased biofilm matrix production, altered cell surface properties

(Hancock *et al*., 1983; Walsh *et al*., 2000; Lee *et al*., 2005; Bragonzi *et al*., 2006; Poole, 2005; Cigana *et al*., 2009; Kamath *et al*., 2016; Winstanley *et al*., 2016). This raises the doubt that a QS inhibitor active against a non-CF strains could be not effective against a number of CF strains. Here this issue has been studied by using NCL as model QS inhibitor. Hence, the efficacy of this molecule has been tested in the $3OC_{12}$-HSL producer strains of the collection (n = 69), in comparison to the non-CF strain PA14.

The $3OC_{12}$-HSL producer CF strains of the collection and PA14 have been grown in the absence and in the presence of 20 μM NCL, and $3OC_{12}$-HSL levels were measured in culture supernatants (details in Materials and Methods and Supplementary information). NCL 20 μM has not effect in inhibiting the growth of the tested strains, as determined by preliminary experiments (data not shown).

Results showed that NCL susceptibility is highly variable and overall low among the CF strains analysed (Fig. 26). In fact, in 31 out of 69 strains NCL affected $3OC_{12}$-HSL production (residual activity ≤ 80%) and only in 16 strains among these, the inhibition was higher or comparable to what measured in PA14 (residual activity ~ 55%).

In order to establish a possible correlation between inhibition of $3OC_{12}$-HSL and inhibition of virulence factors controlled by this QS signal, the levels of pyocyanin, elastase and proteases were determined in the supernatants of NCL-treated and untreated strains (details in Materials and Methods and Supplementary information). Results showed that 50 out of 69 strains produce pyocyanin, protease and elastase; 13 strains produce only proteases and elastase; only 6 strains do not express these three virulence traits. In accordance with the scarce effect shown on $3OC_{12}$-HSL production, NCL had a low effect also in reducing virulence factors production in the strains of the collection. As shown in figure 27, 50 % of strains are not susceptible to NCL and in only about 25% of the strains the production of virulence factors is reduced ≥ 20%. Overall, the effect of NCL on the production of pyocyanin, elastase and proteases is very low in comparison to the effect observed in PA14.

Finally, statistical analyses demonstrated that NCL susceptibility does not correlate with years of infection (Fig. 28A) or with the levels of $3OC_{12}$-HSL produced in the untreated sample (data not shown). Moreover there is no correlation between NCL susceptibility and antibiotic resistance. However, the low number of MDR and XDR strains in the collection does not rule out the possibility that a significant correlation could be found by considering an higher number of strains with MDR and XDR phenotype (Fig. 28B).

Overall, the above results suggest that NCL could be pumped out by efflux pumps or could not reach its target due to changes in the cell permeability to this compound, in CF strains. Further

studies aimed at understanding the mechanisms of action and of resistance to NCL could drive medicinal chemistry studies aimed at potentiating NCL activity and at expanding its range of activity in CF strains.

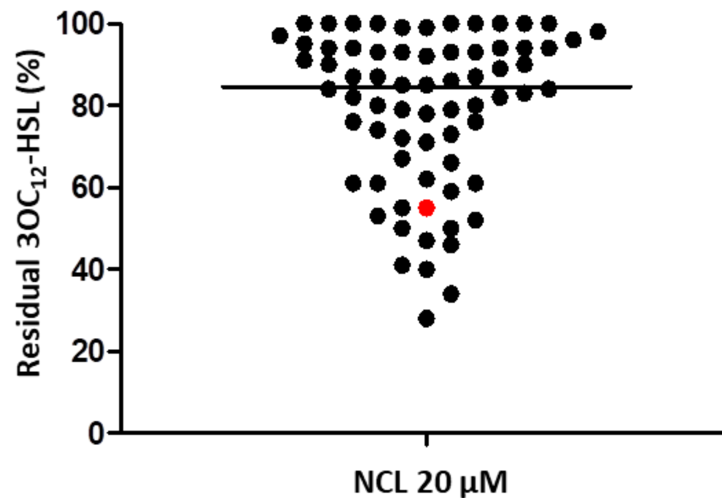

Figure 26. Levels of $3OC_{12}$-HSL in supernatants of CF strains grown in presence of 20 µM NCL shown as percentage with respect to the untreated control. Red dot represent the PA14 wild type. Black line represent the median values.

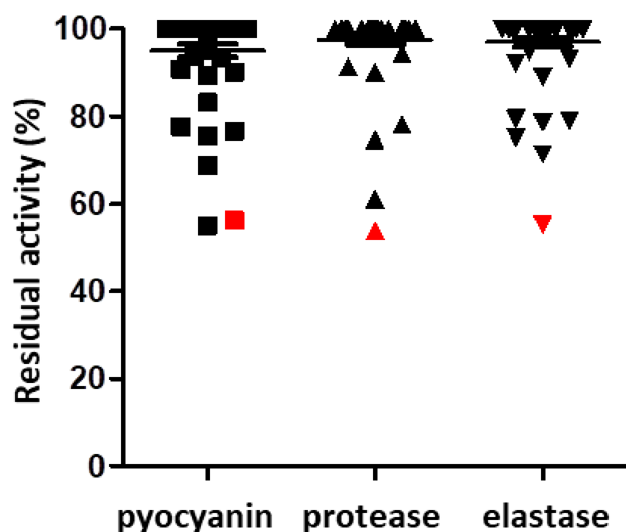

Figure 27. Levels of pyocyanin, protease and elastase in supernatants of each CF strains grown in presence of 20 µM NCL shown as percentage with respect to the untreated control. Red dots represent the PA14 wild type. Black line represent the median values.

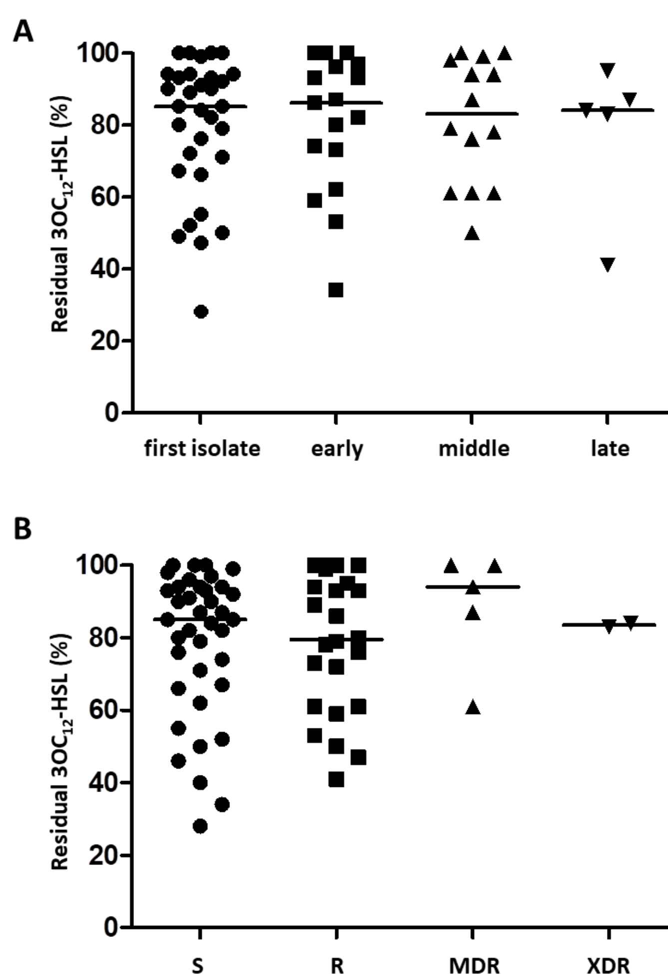

Figure 28. Levels of $3OC_{12}$-HSL produced by strains in presence of 20 µM NCL, in percentage with respect to untreated control. (A), in relation with years of infection; (B), in relation with antibiotic susceptibility. Black lines represent median values. Differences among A and B are not statistically significant (ANOVA).

4.3 Conclusions

Anti-virulence compounds targeting the QS system are generally considered promising candidates for the development of innovative therapies aimed at eradicating the antibiotic-resistant infections caused by *P. aeruginosa*, including lung infection in patients affected by CF (Rasko and Sperandio, 2010; Jacobsen *et al*., 2013; Rampioni *et al*., 2014). Accordingly, researchers aiming at identifying *P. aeruginosa* QS inhibitors are very active worldwide and the number of papers describing the discovery of molecules with anti-QS activity is exponentially increasing (Rampioni *et al*., 2014). As an example, a search in "Web of Science" (apps.webofknowledge.com) using the query "quorum AND sensing AND inhibitor AND *aeruginosa*" retrieved more than 700 papers, with about 100 papers published per year in the last three years (at the date of October 27th, 2017).

The effectiveness of newly discovered anti-QS compounds is commonly validated in *P. aeruginosa* laboratory strains, while strains isolated from CF patients are seldom taken into consideration. This is surprising given the frequent isolation of CF strains carrying mutations in genes coding for QS systems, in particular in the *lasR* $3OC_{12}$-HSL receptor, and the overall high phenotypic and genetic strain diversity within patients, which is dynamic over time (Berk 1963; Oliver *et al*., 2000; Lyczak *et al*., 2002; Smith *et al*.,2006; Foweraker 2009; Hoffman *et al*., 2009; Folkesson *et al*. 2012; Malone 2015; Feltner *et al.,* 2016;Winstanley *et al*., 2016).

Phenotypes frequently studied among *P. aeruginosa* CF isolates are antibiotic resistance, lack of specific virulence factors, overproduction of alginate and of other factors associated to the biofilm matrix (Winstanley *et al*., 2016). However, to the best of our knowledge, the ability of *P. aeruginosa* strains isolated from a significant number of CF patients to actually synthetize $3OC_{12}$-HSL has been determined only in two studies, both showing that the loss of $3OC_{12}$-HSL production parallels the years of chronic infection and starts to be significant only after more than one decade of infection (Bjarnsholt *et al*., 2010; Jiricny *et al*., 2014).

Our analysis is in agreement with previous studies, showing that about 69% of the *P. aeruginosa* strains isolated from CF patients with the first infection or with chronic infection up to 7 years are able to synthetize $3OC_{12}$-HSL and should be in principle susceptible to an anti-virulence drug targeting the *las* QS system. However, our results show that the production of $3OC_{12}$-HSL and of $3OC_{12}$-HSL-dependent virulence factors is overall scarcely affected in CF isolates treated with the model QS inhibitor NCL. This could be explained by the peculiarity of CF strains, often disclosing intrinsic resistance or tolerance to disinfectants and antibiotics, due to overexpression of efflux pumps and/or to inaccessibility of the drug into the bacterial cell (Kamath *et al*., 2016; Winstanley *et al*., 2016).

Overall, this study shows that CF strains isolated from patients that have never been treated with a QS inhibitor could be resistant to NCL. These results are in agreement with the only other study published so far analysing the effect of an anti-QS drug (furanone C-30) on CF isolates (Garcìa-Contreras *et al*., 2015). Also in accordance with this previous study, the highly variable effect of NCL and the lack of correlation with the MDR phenotype suggest that strain-specific changes in surface permeability rather than mutations leading to overexpression of efflux pumps could be involved in resistance to NCL. However, further studies will be needed to clarify this issue.

The observation that the anthelmintic drug NCL strongly inhibits QS and virulence only in *P. aeruginosa* PA14 and in a little number of CF strains, should not discourage but rather stimulate further research aimed at: i) understanding the NCL mechanism of action and resistance mechanisms; ii) developing more effective analogs able to penetrate a larger number of CF strains,

by traditional medicinal chemistry as well as parallel synthesis. This would be in line with the SOSA (Selective Optimization of Side Activity) strategy which inspired the study firstly describing the QS inhibitory activity in NCL (Wermuth, 2006; Imperi *et al*, 2013).

Overall, this study suggests that future anti-virulence drugs targeting the *las* QS system could be successfully developed and used in CF therapy only if shown to be active against a large proportion of CF isolates. This validation should be carried out before starting more advanced pre-clinical studies. In any case, the high variability of CF strains will require to preliminarily test the susceptibility of the specific *P. aeruginosa* strain(s) colonizing a patient toward the QS inhibitor, a practice routinely used before administering antibiotics. This is achievable, since convenient methods for the fast detection of QS signal molecules have already been developed and could be further improved and translated to the diagnostic laboratory. As an example, the biosensor-based method used in this study and described in the book chapter attached to this thesis (Rampioni *et al*., 2018; Supplementary information).

CONCLUDING REMARKS

Mutations conferring antibiotic resistance, lack of specific virulence factors, overproduction of alginate and of other factors associated to the biofilm matrix are frequently studied among *P. aeruginosa* CF isolates and could be correlated with the evolution and the adaptation of the *P. aeruginosa* population to the CF lung environment (Folkesson *et al*., 2012; Winstanley *et al*., 2016). The emergence of mutants expressing a lytic phenotype, postulated to be related to a mutation in the *pqsL* gene (D'Argenio *et al*., 2002), and of *lasR*-defective mutants (Smith *et al*., 2006; Hoffman *et al*., 2009) has been also being described.

Chapter 3 of this thesis has been focused on the investigation of the role played by the *pqsL* gene in *P. aeruginosa* physiology and CF infection. The product of the enzyme coded by this gene is HQNO, which is produced at high (μM) concentration as well as the QS signals HHQ and PQS. HQNO is not a QS signal (Rampioni *et al*., 2016), but rather an antibacterial toxic for *S. aureus,* which is an earlier colonizer of the CF lung (Filkins *et al*., 2015; Nguyen *et al*., 2016). A major novel finding of this study is that HQNO could also act as a molecular sink to avoid the cellular lysis caused by HHQ accumulation and prophage activation, in *P. aeruginosa* wild type. Notably, a first evidence of a clear genetic correlation between the expression of the lytic phenotype and the emergence of the *pqsL* mutation in clinical *P. aeruginosa* strains has also been provided.

The observation that *P. aeruginosa* PAO1 *pqsL* biofilms are more resistant to antibiotics gives a cue to explain why this mutation could be advantageous in the CF lung environment. It is reasonable that once *P. aeruginosa* has overpowered *S. aureus* and established a single-species biofilm in the CF lung, the selective pressure for the conservation of PqsL function could decrease while the emergence of an autolytic subpopulation with increasing biofilm resistance to antibiotics (likely via massive eDNA release) could be positively selected.

Chapter 4 of this thesis has been focused on *lasR*, a *P. aeruginosa* QS gene frequently found mutated in strains isolated from CF patients (Bjarnsholt *et al*., 2010; Jiricny *et al*., 2014). Despite the debated role of the *las* QS system in the CF infection (Ciofu *et al*., 2015; Winstanley *et al*., 2016; Feltner *et al.*, 2016), only one study tested the efficacy of an anti-QS drug (furanone C-30) in CF strains, founding that the susceptibility of these strains to furanone-C30 is higher variable. However, in this study the susceptibility to furanone C-30 was not correlated to the length of the chronic infection (Garcìa-Contreras *et al*., 2015).

Our analysis showed that the majority of *P. aeruginosa* strains isolated from CF patients with first infection or with chronic infection established up to 7 years are able to synthetize $3OC_{12}$-HSL and should be susceptible to an anti-virulence drug targeting the *las* QS system, at least in principle.

However, the production of $3OC_{12}$-HSL and of $3OC_{12}$-HSL-dependent virulence factors is overall scarcely affected in CF isolates treated with the model QS inhibitor NCL. This result is in agreement with the results obtained with furanone C-30 (Garcìa-Contreras *et al.*, 2015) and indicates that future anti-virulence drugs targeting the *las* QS system could be successfully developed and used in CF therapy only if shown to be active against a large proportion of CF isolates. This validation should be carried out before starting more advanced pre-clinical studies. Moreover, understanding the mechanisms underlying the lack of susceptibility of CF strains to an anti-QS compound could drive further medicinal chemistry studies aimed at potentiating these drugs and expanding their range of activity. It should also be highlighted that even if a QS inhibitor would reach the clinical use, the high variability of CF strains will require to preliminarily test the susceptibility of the specific *P. aeruginosa* strain(s) colonizing a patient toward the drug, a practice routinely used before administering antibiotics. This is achievable, since convenient methods for the precise detection of QS signal molecules have already been developed (Rampioni *et al.*, 2018, Supplementary information) and could be further improved and translated to the diagnostic laboratory.

Overall the research carried out in this thesis has clarified some aspects of the *pqs* QS system which could be relevant to understand and combat the lethal *P. aeruginosa* infections in CF patients, and also highlighted the limitations that should be overcome for translating the use of anti-QS compounds to the therapy of CF patients.

MATERIALS AND METHODS

6.1 Bacterial strains, media and chemicals

The bacterial strains used in this study are listed in Table 1. The list of *P. aeruginosa* CF clinical isolates used in this study is provided in Table S1 and S2. Bacterial strains were routinely grown at 37 °C in Luria-Bertani broth (LB; Sambrook *et al.*, 1989) with aeration and when necessary antibiotics were added at the concentrations of 25 µg/ml Kanamycin (Km), 30 µg/ml Chloramphenicol (Cm), 10 µg/ml Tetracyclin (Tc) and 20 µg/ml Gentamicin (Gm) for *Escherichia coli*; 200 µg/ml Kanamycin (Km), 375 µg/ml Chloramphenicol (Cm), 200 µg/ml Tetracyclin (Tc), 150 µg/ml Carbenicillin (Cb) and 100 µg/ml Gentamicin (Gm) for *P. aeruginosa*. When required were added: Tobramycin (Tb) or Gm at indicated concentrations; 10 % (w/v) sucrose; 1mM isopropyl-β-D-1-thiogalactopyranoside (IPTG); 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS, pH 7.0). M9 minimal medium supplemented with 20 mM Glucose was used in the MIC assay and in the biofilm assay. Synthetic $3OC_{12}$-HSL stock solutions (10 mM) was prepared in MeOH. Niclosamide (NCL) was purchased from Sigma-Aldrich and suspended in dimethyl sulfoxide (DMSO) at 10 mM final concentration.

Table 1 – Bacterial strains used in this study.

Strains	Description	References
E. coli DH5α	Cloning strain	Bethesda Research Laboratories
E. coli S17.1λ*pir*	Cloning strain, used for conjugation in *P. aeruginosa*; *tra*$^{+}$.	Simon *et al.*, 1983
P. aeruginosa PAO1	*P. aeruginosa* PAO1 wild type strain from Nottingham collection, kindly provided by Prof. P. Williams (University of Nottingham, UK).	P. Williams collection (University of Nottingham, UK)
P. aeruginosa PAO1 Δ*pqsL*	*P. aeruginosa* PAO1 mutant strain deleted in *pqsL* gene by using plasmid pDM4Δ*pqsL*; does not produce HQNO.	Rampioni *et al.*, 2016
P. aeruginosa PAO1 Δ*pqsHL*	*P. aeruginosa* PAO1 mutant strain deleted in *pqsL* and *pqsH* genes gene; does not produce HQNO and does not convert HHQ in PQS.	Rampioni *et al.*, 2016
P. aeruginosa PAO1 Δ*pqsAL*	*P. aeruginosa* PAO1 mutant strain deleted in *pqsL* and *pqsA* genes; does not produce AQs.	Rampioni *et al.*, 2016
P. aeruginosa PAO1 Δ*pqsAHL*	*P. aeruginosa* PAO1 mutant strain deleted in *pqsL*, *pqsA and pqsH* genes; does not produce AQs and does not convert HHQ in PQS.	Rampioni *et al.*, 2016
P. aeruginosa PAO1-K	PAO1-K wild type strain, kindly provided by Prof. S. Kjelleberg (University of New South Wales, Australia).	Rice *et al.*, 2009
P. aeruginosa PAO1-K Δ*Pf4*	*P. aeruginosa* PAO1-K mutant strain deleted in Pf4 prophage genetic *locus*, kindly provided by Prof. S. Kjelleberg (University of New South Wales, Australia).	Rice *et al.*, 2009
P. aeruginosa PAO1 Δ*pqsLR*	*P. aeruginosa* PAO1 mutant strain deleted in *pqsL* and *pqsR* genes, generated by mutating *pqsR* gene in *P. aeruginosa* PAO1 Δ*pqsL* mutant strain by using pDM4Δ*pqsR* plasmid (Table 2).	This study

P. aeruginosa PAO1 Δ*pqsLB*	*P. aeruginosa* PAO1 mutant strain deleted in *pqsL* e *pqsB* genes, generated by mutating *pqsB* gene in *P. aeruginosa* PAO1 Δ*pqsL* strain by using pDM4Δ*pqsB* plasmid (Table 2).	This study
P. aeruginosa PAO1 Δ*pqsLC*	*P. aeruginosa* PAO1 mutant strain deleted in *pqsL* and *pqsC* genes, generated by mutating *pqsC* gene in *P. aeruginosa* PAO1 Δ*pqsL* strain by using pDM4Δ*pqsC* plasmid (Table 2).	This study
P. aeruginosa PAO1-K Δ*pqsL*	*P. aeruginosa* PAO1-K mutant strain generated mutating *pqsL* gene in *P. aeruginosa* PAO1-K strain by using pMEΔ*pqsL* plasmid (Table 2).	This study
P. aeruginosa PAO1-K Δ*pqsLPf4*	*P. aeruginosa* PAO1-K mutant strain deleted in *pqsL* gene and in the genetic *locus* of Pf4 prophage, generated mutating *pqsL* gene in *P. aeruginosa* PAO1-K Δ*Pf4* strain by using pMEΔ*pqsL* plasmid (Table 2).	This study
P. aeruginosa PA14	PA14 wild type strain	Rahme *et al.*, 1995
P. aeruginosa PA14 *ΔlasI ΔrhlI*	*P. aeruginosa* PA14 mutant strain deleted in *lasI* and *rhlI* genes; does not produce $3OC_{12}$-HSL or C_4-HSL; Gm^R	Laboratory collection Generated by G. Rampioni
P. aeruginosa PA14-R3,	*P. aeruginosa* PA14 biosensor strain able to emit bioluminescence as function of $3OC_{12}$-HSL concentration.	Massai *et al.*, 2011
P. aeruginosa PAO1-*ΔrhlI* (pKD-*rhlA*)	*P. aeruginosa* PAO1 biosensor strain able to emit bioluminescence as function of C_4-HSL concentration. Km^R.	Duan and Surette, 2007

6.2 Recombinant DNA techniques

Plasmid used or generated in this study and details on their construction are reported in Table 2. Oligonucleotides used in this study are reported in Table 3.

Preparation of plasmid DNA, purification of DNA fragments, restrictions, ligations and transformation in *E. coli* competent cells and in *P. aeruginosa* were performed with standard procedures (Sambrook *et al.,* 1989). The DNA amplification was performed by Polymerase Chain Reaction (PCR) using the GoTaq® Polymerase (Promega). All plasmids generated in this study were checked by restriction analysis and by sequencing *via* the Genechron Sequence Service. The same service was used for sequencing of *pqsL* gene amplified with oligonucleotides FW374*pqsL* e RV375*pqsL* (Table 3) from the chromosome of *P. aeruginosa* strains. Sequences alignment was performed by the programme "ClustalW2" (www.ebi.ac.uk/Tools/msa/clustalw2). The conversion of nucleotidic sequence in amino acidic sequence was performed by the programme "ExPASy-translate tool" (web.expasy.org/translate).

Plasmids were introduced into *P. aeruginosa* by transformation or by bi-parental conjugation from *E. coli* S17.1 λpir strains (Sambrook et al. 1989).

Table 2 – Plasmids used in this study.

Plasmids	Description and construction	References
pME6032	Vector for protein expression, IPTG-inducible; *lacI*^{Q}, Tc^{R}.	Heeb *et al*., 2000
pBBR1MCS-5	Vector for constitutive protein expression; Gm^{R}.	Kovach *et al*., 1995
pME3087	Suicide plasmid for site-directed mutagenesis; Tc^{R}.	Voisard *et al*., 1994
pDM4Δ*pqsR*	Suicide plasmid for mutagenesis of *pqsR* gene in *P. aeruginosa*; *sacB*, Cm^{R}.	Ilangovan *et al*., 2013
pBBR2-AD	Plasmid for constitutive expression of PqsA e PqsD proteins in *P. aeruginosa*; Km^{R}. Kindly provided by Prof. S. Fetzner (University of Munster, Germany).	Prof. S. Fetzner (University of Munster, Germany)
pB*pqsABCD*	pBBR1MCS-5 derived plasmid for constitutive expression of PqsA, PqsB, PqsC e PqsD proteins in *P. aeruginosa*; Gm^{R}.	Rampioni *et al*., 2016
pDM4Δ*pqsB*	pDM4 derivative plasmid for mutagenesis of *pqsB* gene in *P. aeruginosa*; *sacB*, Cm^{R}.	Laboratory collection Generated by G. Rampioni
pDM4Δ*pqsC*	pDM4 derivative plasmid for mutagenesis of *pqsC* gene in *P. aeruginosa*; *sacB*, Cm^{R}.	Laboratory collection Generated by G. Rampioni
pME*pqsL*	pME6032 derivative plasmid for IPTG-inducible expression of PqsL in *P. aeruginosa*. Generated by EcoRI-SalI cloning of a DNA fragment, corresponding to *pqsL,* amplified from *P. aeruginosa* PAO1 genome with oligonucletotides FW31*pqsL* e RV67*pqsL* (Table 3)	This study
pB*pqsAD*	pBBR1MCS-5 derivative plasmid for the constitutive expression of PqsA and PqsD proteins in *P. aeruginosa*. Generated by SacI-SalI cloning of a DNA restriction fragment extracted from pBBR2-AD plasmid.	This study
pMEΔ*pqsL*	pME3087 derived plasmid for mutagenesis of *pqsL* gene in *P. aeruginosa*. Generated by BamHI-HindIII, the DNA fragment containing up and down regions of *pqsL* gene amplified from *P. aeruginosa* PAO1 Δ*pqsL* genome with oligonucleotides FW623Δ*pqsL* and RV626Δ*pqsL* (Table 3).	This study

Table 3 - Oligonucleotides used in this study.

Name	Sequences (5'-3') [a]	Restriction site
FW31*pqsL*	CCGGAATTCATGACGGACAACCATATCG	EcoRI
RV67*pqsL*	TACGAGCTCGCTGGCGGGTTCAGCCG	SalI
FW623Δ*pqsL*	TATGGATCCACCGTGGTCGATGGCGT	BamHI
RV626Δ*pqsL*	TATAAGCTTCCTGTTCAATTACCCGAGCC	HindIII
FW374*pqsL*	CAAGTGCAGACAACACCCATT	-
RV375*pqsL*	GTGGCCAAGGTATTCCCGC	-

[a] **Restriction sites are underlined.**

6.3 Lytic phenotype, phage quantification and biofilm assays

To analyse the expression of the lytic phenotype in colony biofilms, 5 µl of overnight cultures grown at 37°C in LB were dropped off on Congo Red plates [10g/L Triptone (Acumedia), 15 g/L

Bacto Agar (Difco), 40 mg/L Congo Red (VWR International), 10 mg/L Comassie Brilliant Blue R-250 (Fluka)] and incubated for 24 h at 37°C. When required, IPTG (1mM) was added to the plates.
To perform experiments with soft-agar lawn, 100 µl of *P. aeruginosa* overnight cultures grown at 37°C in LB were diluted in 5 ml of LB Top Agar [LB plus 7,5 g/L Bacto Agar (Difco)]. Cultures in LB top agar were spread onto LB agar plates and 5 µl of 10mM HHQ, DHQ, PQS (or methanol as control) were spotted before incubating for 24 h at 37°C. Synthetic molecules were kindly provided by Prof. P. Williams (University of Nottingham, UK).
Phage quantification was performed as described in Frangipani, 2014. Briefly the soft-agar lawn prepared as described above, were scraped from the plate and suspended in 2 ml of TNM buffer [1.21g/L Tris, 8.77 g/L NaCl, 2.46 g/L $MgSO_4X7H_2O$, pH 7.4]. After vortexing, the filtrated supernatants were recovered and sequentially diluted in TNM buffer. 5 µl of each dilution were spotted onto soft-agar formed by the PAO1-K ΔPf4 strain (kindly provided by Prof. Kjelleberg, University of New South Wales, Australia) and PFU (Plaques Forming Unit) were counted (Frangipani, 2014).
The strains used in assays of biofilm formation were grown in M9 minimal medium plus 20 mM glucose for 8 h at 37°C in shaking condition. The cultures were refreshed in M9 minimal medium plus 20 mM glucose to optical density A_{600} 0.014, 100 µl were dispensed into 96-wells polystyrene U-bottom microtiter plates (Sarstedt), and then incubated at 30°C in static condition for 16 h. After 16 h of incubation the planktonic phase was removed and the biofilm was refreshed with 100 µl of M9 minimal medium with no antibiotics or with different concentrations of tobramycin or gentamicin (4 µg/ml, 20 µg/ml, 100 µg/ml). Microtiter plates were incubated at 30°C for 6 h. Overall, 16 identical biofilms were grown in parallel for each strain and each condition, then in 8 samples was determined biofilm mass by using crystal violet (CV) assay (O'Toole and Kolter, 1998) and in the remaining samples biofilm viability was determined by fluorescein diacetate assay (FDA) with minor modifications (Peeters *et al.*, 2008). Briefly, the M9 minimal medium was removed and 200 µl of FDA (Sigma-Aldrich) working solution in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer freshly prepared before each assay were dispensed in all wells. The working solution was prepared diluting 1:100 in 100mM MOPS pH 7.0, the 10 mg/ml FDA stock solution prepared in acetone. The microtiter was incubated in the dark at 37°C (static condition). Fluorescence was measured after 1 h through the automated VICTOR^{3V} luminometer-spectrophotometer (Perkin Elmer).
All the experiments have been performed at least in duplicate.

6.4 Determination of quorum sensing molecules production and other phenotypic assays

For the measurement of AQs levels produced by *P. aeruginosa* strains, 1 ml of filtered supernatant coming from overnight cultures grown at 37°C in LB was extracted with 3 ml of ethyl acetate. Evaporation of ethyl acetate was accelerate by centrifugation at 37°C in SpeedVac RC10.22 (Jouan), and samples were analysed by LC-MS/MS analyses (Ortori *et al*., 2011) in the laboratory of Prof. P. Williams laboratory (University of Nottingham, UK).

Protease production was measured basically as described in (Kessler *et al*., 1982).

A detailed description of the methods used for the measurement of acyl-HSL levels, elastase and pyocyanin produced by *P. aeruginosa* strains (in the presence and in the absence of 20 μM NCL) has been reported in a book chapter co-authored by Giulia Giallonardi (provided in the Supplementary information; Rampioni *et al*., 2018)

6.5 Statistical analysis

Statistical analysis was performed with the software GraphPad, Instat, using an unpaired t-test (ANOVA). Differences having a p *value* of < 0.05 were considered statistically significant.

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SUPPLEMENTARY INFORMATION

Table S1

P. aeruginosa CF isolates belonging to the collection kindly provided by Dr. E. Fiscarelli (Hospital Bambino Gesù, Rome). All the strains reported in this table were used in the experiments described in chapter 4; some of these strains (in bold) were used also in the experiments described in chapter 3. Lytic strains are marked with an asterisk. Lytic strains inactivated in *pqsL* gene are marked with two asterisks.

Isolates	Colonization[a]	Years of chronic colonization	Patient ID	Year of isolation	Antibiotics resistance[b]
BG 1	first isolate	first isolation	MaDa	2006	R
BG 2	early	3		2009	R
BG 3	middle	6		2012	MDR
BG 4**	first isolate	first isolation	PaLu	2006	R
BG 5	early	2		2009	R
BG 6**	middle	6		2013	R
BG 7**	first isolate	first isolation	GaCr	2008	S
BG 8**	early	2		2010	R
BG 9*	middle	5		2013	S
BG 10	first isolate	first isolation	DeZuFr	2008	S
BG 11	early	3		2011	R
BG 12	middle	5		2013	S
BG 13	first isolate	first isolation	TaEn	2008	MDR
BG 14**	early	3		2011	MDR
BG 15**	middle	5		2013	MDR
BG 16**	first isolate	first isolation	FrCh	2006	S
BG 17*	early	2		2009	R
BG 18	middle	6		2013	R
BG 19	first isolate	first isolation	AnVe	2009	R
BG 20	early	3		2012	R
BG 21	middle	5		2013	R
BG 22	first isolate	first isolation	RuFr	2007	S
BG 23	early	2		2009	S
BG 24	first isolate	first isolation	PoCh	2009	MDR
BG 25	early	2		2011	MDR
BG 26	middle	5		2013	XDR
BG 27	first isolate	first isolation	HaRe	2008	S
BG 28	early	2		2010	S
BG 29	middle	5		2013	R
BG 30**	first isolate	first isolation	ToDo	2007	S
BG 31	early	3		2010	S
BG 32	middle	5		2012	S
BG 33	first isolate	first isolation	BaDa	2006	R
BG 34	early	2		2012	R
BG 35*	first isolate	first isolation	GrOl	2010	S
BG 36*	early	3		2013	S
BG 37**	first isolate	first isolation	CoCl	2010	R

BG 38**	early	3		2013	R
BG 39	first isolate	first isolation	SaAr	2010	S
BG 40	early	3		2013	R
BG 41	first isolate	first isolation	ViSi	2010	S
BG 42	early	3		2013	R
BG 43	first isolate	first isolation	TrAn	2009	S
BG 44	early	3		2012	R
BG 45	first isolate	first isolation	BuNi	2006	S
BG 46	early	2		2008	S
BG 47	first isolate	first isolation	AdGi	2009	R
BG 48	first isolate	first isolation	MaCr	2010	S
BG 49	early	2		2012	S
BG 50	first isolate	first isolation	ArCo	2010	S
BG 51	early	2		2013	R
BG 52	first isolate	first isolation	SaGa	2010	S
BG 53	early	2		2013	S
BG 54	first isolate	first isolation	MoAn	2008	S
BG 55	early	3		2012	S
BG 56*	first isolate		SeSo	2006	R
BG 57	first isolate	first isolation	FiSo	2007	R
BG 58	early	2		2010	S
BG 59	first isolate		TaDa	2006	S
BG 60	first isolate	first isolation	SeCr	2007	S
BG 61*	early	2		2013	S
BG 62	first isolate	first isolation	DeMoDi	2008	S
BG 63*	early	2		2011	S
BG 64	first isolate	first isolation	CrCr	2007	S
BG 65**	first isolate	first isolation	DiGiJa	2008	S
BG 66	first isolate	first isolation	PaLuJu	2006	S
BG 67	first isolate	first isolation	AcSt	2007	S
BG 68	first isolate	first isolation	ViMa	2008	S
BG 69**	first isolate	first isolation	MaEm	2009	S
BG 70	first isolate	first isolation	CaGi	2009	S
BG 71**	first isolate	first isolation	CoDa	2007	S
BG 72	first isolate	first isolation	CaGin	2008	S
BG 73	first isolate	first isolation	TrRoMi	2008	S
BG 74	first isolate	first isolation	FeGi	2008	S
BG 75	first isolate	first isolation	SaNi	2009	S
BG 76	middle	7	RiEl	2012	R
BG 77*	middle	6	MoSa	2010	XDR
BG 78	middle	5	PiAl	2009	MDR
BG 79	middle	7	BaAn	2010	R
BG 80	middle	5	BaMo	2013	R
BG 81	middle	5	BaFi	2010	S
BG 82	middle	6	BaDe	2010	MDR
BG 83	middle	5	CoVi	2013	R
BG 84**	middle	5	CaVe	2012	R
BG 85	middle	6	CeRo	2013	R
BG 86	middle	6	ReRa	2013	S
BG 87	middle	6	StDo	2013	S
BG 88	middle	5	FaLo	2010	MDR
BG 89	middle	6	ToFr	2012	MDR
BG 90	middle	7	GiMo	2011	S

BG 91**	late	≥ 15	RaDa	2013	XDR
BG 92	late	≥ 15	VoCl	2013	XDR
BG 93	late	≥ 15	RoAl	2013	MDR
BG 94	late	≥ 15	BaBa	2009	XDR
BG 95	late	≥ 15	PaMi	2011	MDR
BG 96**	late	≥ 15	CoCl	2013	R
BG 97	late	≥ 15	CoEl	2013	XDR
BG 98	late	≥ 15	AnAn	2013	MDR
BG 99	late	≥ 15	MiAl	2013	MDR
BG 100	late	≥ 15	BuDa	2013	R

[a] Strains named "first isolate" represent *P. aeruginosa* first isolation from the patient (i.e. from intermittent infections); "early" and "middle" categories represent strains up to 3 and from 4 up to 7 years of chronic infection respectively; to "late" category belong strains from more than 15 years of chronic infections.
[b] Criteria to define multi-drug resistant (MDR) and extensively-drug resistant (XDR) bacteria come from ECDC site (http://ecdc.europa.eu/en/Pages/home.aspx) and respectively indicate: non susceptible to one or more antibiotics belonging to at least 3 different classes, and non susceptible to one or more antibiotics belonging to all the classis except 2 or less. Susceptible (S) indicate strains susceptible to all antibiotic classes and resistant (R) correspond to strains non susceptible to one or more antibiotics belonging to less than 3 different classes.

Table S2

P. aeruginosa CF isolates belonging to our laboratory collection. All the strains reported in this table were used in the experiments described in chapter 3. Lytic strains are marked with an asterisk. Lytic strains inactivated in *pqsL* gene are marked with two asterisks.

Isolates	References
AA2	Bragonzi *et al.*, 2009
AA11**	Bragonzi *et al.*, 2009
AA12	Bragonzi *et al.*, 2009
AA43	Bragonzi *et al.*, 2009
AA44	Bragonzi *et al.*, 2009
KK1**	Bragonzi *et al.*, 2009
KK2	Bragonzi *et al.*, 2009
KK27	Bragonzi *et al.*, 2009
KK28**	Bragonzi *et al.*, 2009
KK71**	Bragonzi *et al.*, 2009
KK72**	Bragonzi *et al.*, 2009
BT2	Bragonzi *et al.*, 2009
BT73	Bragonzi *et al.*, 2009
TR1**	Bragonzi *et al.*, 2009
TR66**	Bragonzi *et al.*, 2009
FM1	Massai *et al.*, 2011
FM2*	Massai *et al.*, 2011
FM4	Massai *et al.*, 2011
FM11	Massai *et al.*, 2011
FM12	Massai *et al.*, 2011
FM13**	Massai *et al.*, 2011
FM14	Massai *et al.*, 2011
FM15	Massai *et al.*, 2011

FM17*	Massai *et al.*, 2011
FM19**	Massai *et al.*, 2011
FM20	Massai *et al.*, 2011
FM21	Massai *et al.*, 2011

Table S3

Antibiotics resistance panel of strains listed in Table S1 provided by Dr. E. Fiscarelli following "*European Committee on Antimicrobial Susceptibility Testing*" (EUCAST) criteria.

Isolates	Antibiotics resistance[a]									
	PIP/TAZ	ATM	CAZ	MEM	IMP	AK	TM	CIP	LV	COL
BG 1							X			
BG 2						X	X			
BG 3	X	X	X				X			
BG 4										X
BG 5							X			
BG 6							X			
BG 7										
BG 8						X				
BG 9										
BG 10										
BG 11		X								
BG 12										
BG 13	X	X	X	X	X		X			
BG 14			X	X	X		X			
BG 15			X	X	X	X	X			
BG 16										
BG 17						X				
BG 18						X				
BG 19					X	X	X			
BG 20				X	X	X	X			
BG 21				X	X	X	X			
BG 22										
BG 23										
BG 24			X	X	X	X	X	X	X	
BG 25			X	X	X	X	X	X	X	
BG 26	X	X	X	X	X	X	X	X	X	
BG 27										
BG 28										
BG 29								X	X	
BG 30										
BG 31										
BG 32										
BG 33						X				
BG 34						X	X			
BG 35										
BG 36										
BG 37							X			
BG 38						X	X			
BG 39										
BG 40					X					
BG 41										

BG 42				X	X					
BG 43										
BG 44			X			X	X			
BG 45										
BG 46										
BG 47	X						X			
BG 48										
BG 49										
BG 50										
BG 51								X		
BG 52										
BG 53										
BG 54										
BG 55										
BG 56						X	X			
BG 57					X					
BG 58										
BG 59										
BG 60										
BG 61										
BG 62										
BG 63										
BG 64										
BG 65										
BG 66										
BG 67										
BG 68										
BG 69										
BG 70										
BG 71										
BG 72										
BG 73										
BG 74										
BG 75										
BG 76			X		X					
BG 77	X	X	X	X	X	X	X			
BG 78			X	X	X		X	X	X	
BG 79							X			
BG 80						X			X	
BG 81										
BG 82				X			X	X	X	
BG 83		X					X			
BG 84						X				
BG 85				X		X	X			
BG 86										
BG 87										
BG 88			X	X	X	X				
BG 89				X	X	X		X	X	
BG 90										
BG 91	X	X	X	X	X	X	X	X	X	
BG 92	X	X	X	X	X	X	X	X	X	
BG 93				X	X		X	X	X	
BG 94	X	X	X	X	X	X	X	X	X	
BG 95			X	X	X	X		X	X	
BG 96						X	X			

BG 97	X	X	X	X	X	X	X	X	X	
BG 98						X	X	X	X	X
BG 99		X	X	X	X	X				
BG 100						X	X	X	X	

[a] **Tobramycin (TM), amikacin (AK), meropenem (MEM), imipenem (IMP), ceftazidime (CAZ), levofloxacin (LV), ciprofloxacin (CIP), piperacillin/tazobactam (PIP/TAZ), aztreonam (ATM) e colistin (COL). The X indicates the resistance to antibiotic according to EUCAST criteria.**

Table S4

P. aeruginosa CF clinical isolates (Table S1) grouped for their ability to produce both QS signal molecules $3OC_{12}$-HSL and C_4-HSL, one of them, or none of them.

Producers of	Total strains	Strains				
$3OC_{12}$-HSL and C_4-HSL	63	BG 4	BG 23	BG 48	BG 69	BG 93
		BG 5	BG 27	BG 49	BG 70	BG 97
		BG 6	BG 28	BG 50	BG 72	BG 100
		BG 7	BG 29	BG 51	BG 73	
		BG 8	BG 31	BG 52	BG 74	
		BG 9	BG 33	BG 53	BG 75	
		BG 10	BG 34	BG 54	BG 76	
		BG 11	BG 35	BG 55	BG 78	
		BG 12	BG 36	BG 56	BG 80	
		BG 13	BG 42	BG 57	BG 82	
		BG 15	BG 43	BG 60	BG 83	
		BG 16	BG 44	BG 62	BG 84	
		BG 17	BG 45	BG 66	BG 85	
		BG 18	BG 46	BG 67	BG 86	
		BG 22	BG 47	BG 68	BG 92	
$3OC_{12}$-HSL	6	BG 39				
		BG 40				
		BG 59				
		BG 64				
		BG 81				
		BG 96				
C_4-HSL	22	BG 14	BG 38	BG 90		
		BG 19	BG 41	BG 91		
		BG 20	BG 61	BG 95		
		BG 21	BG 65	BG 98		
		BG 24	BG 71			
		BG 25	BG 77			
		BG 26	BG 87			
		BG 32	BG 88			
		BG 37	BG 89			
Non producers	9	BG 1	BG 79			
		BG 2	BG 94			
		BG 3	BG 99			
		BG 30				
		BG 58				
		BG 63				

Table S5

p *value* calculated among categories, by statistical analysis performed with GraphPad t-test (ANOVA). **(A-D)** refer to Figure 24A; **(E,F)** refer to Figure 24B.

A

$3OC_{12}$-HSL + C_4-HSL producers				
category	first isolate	early	middle	late
first isolate	-	0.4778	0.1767	0.0538
early	0.4778	-	0.5730	0.2060
middle	0.1767	0.5730	-	0.4072
late	0.0538	0.2060	0.4072	-

B

$3OC_{12}$-HSL producers				
category	first isolate	early	middle	late
first isolate	-	0.5749	0.5749	0.7994
early	0.5749	-	1	0.5041
middle	0.5749	1	-	0.5041
late	0.7994	0.5041	0.5041	-

C

C_4-HSL producers				
category	first isolate	early	middle	late
first isolate	-	0.6076	0.1080	0.2788
early	0.6076	-	0.3435	0.5385
middle	0.1080	0.3435	-	0.9116
late	0.2788	0.5385	0.9116	-

D

non producers				
category	first isolate	early	middle	late
first isolate	-	0.3103	0.6308	0.1227
early	0.3103	-	0.6455	0.5549
middle	0.6308	0.6455	-	0.3278
late	0.1227	0.5549	0.3278	-

E

$3OC_{12}$-HSL total producers				
category	first isolate	early	middle	late
first isolate	-	0.2817	0.0819	0.0546
early	0.2817	-	0.5651	0.3339
middle	0.0819	0.5651	-	0.6020
late	0.0546	0.3339	0.6020	-

F

C_4-HSL total producers				
category	first isolate	early	middle	late
first isolate	-	0.6965	0.9533	0.1841
early	0.6965	-	0.6909	0.3643
middle	0.9533	0.6909	-	0.2132
late	0.1841	0.3643	0.2132	-

Chapter 22

A Coculture-Based Approach for Screening Campaigns Aimed at Identifying Novel *Pseudomonas aeruginosa* Quorum Sensing Inhibitors

Giordano Rampioni, Giulia Giallonardi, Francesca D'Angelo, and Livia Leoni

Abstract

Quorum sensing (QS) is recognized as a promising target for the identification of anti-virulence drugs hampering *Pseudomonas aeruginosa* adaptability to the host environment and pathogenicity. Consequently, a number of studies in the last decade focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *las* QS system, which is based on *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL) as signal molecule. Different experimental approaches have been successfully used to identify QS blockers interfering with the activity/stability of the $3OC_{12}$-HSL receptor LasR, with the functionality of the $3OC_{12}$-HSL synthase LasI, or with the stability/bioavailability of the $3OC_{12}$-HSL signal molecule itself.

Here we describe the use of a high-throughput screening system for the identification of novel *las* QS inhibitors based on the cocultivation of *P. aeruginosa* wild type and the *P. aeruginosa*-derived biosensor strain PA14-R3, in which light emission relies on the ability of the wild type strain to synthesize $3OC_{12}$-HSL and of the biosensor strain to perceive this signal molecule. With respect to other screening systems, this method has the advantage of being cost-effective and allowing the identification of compounds targeting, besides $3OC_{12}$-HSL reception, any cellular process critical for the functionality of the *las* QS system, including $3OC_{12}$-HSL synthesis and secretion.

Key words Quorum sensing inhibitors, Screening, Whole-cell biosensors, Anti-virulence drugs, Niclosamide, *Pseudomonas aeruginosa*, *lasR*, *lasI*

1 Introduction

The introduction of antibiotics into clinical practice at the middle of the twentieth century is a milestone in the history of medicine. However, the original expectation that all bacterial infections could be defeated by antibiotics has been soon disregarded by the emergence of antibiotic-resistant strains. As traditional antibiotic research appears to be helpless in coping with the emergence of

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antibiotic-resistant strains, novel approaches should be undertaken in order to identify new drugs [1, 2].

An innovative strategy to combat bacterial infections relies on specific inhibition of bacterial virulence, hence the ability to cause disease rather than bacterial growth. The use of anti-virulence drugs is expected to reduce bacterial adaptability to the host environment, facilitating the host immune system to resolve the infection, and to diminish the strong selective pressure exerted by conventional antibiotics, although this is not yet supported by direct clinical evidence [3–5].

Since in many bacteria pathogenicity is controlled and coordinated by quorum sensing (QS), this communication system is considered one of the most promising targets for anti-virulence therapies [5, 6].

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most dreaded opportunistic pathogens, and represents a prototype of multidrug resistant bug for which effective therapeutic options are limited. The ability of *P. aeruginosa* to cause a wide range of both community- and hospital-acquired infections in humans is linked to its capacity to produce a large repertoire of virulence factors, form antibiotic-tolerant biofilms and, ultimately, respond and adapt to environmental fluctuations, including host immune responses and antibiotic treatments. For these reasons, *P. aeruginosa* infections are generally characterized by high morbidity and mortality rates [7, 8].

The pathogenic potential of *P. aeruginosa* relies on the coordinated expression of a large array of virulence factors, the majority of which are positively controlled by QS [9, 10]. The *P. aeruginosa* QS network consists of at least three different QS systems, *las*, *rhl*, and *pqs*, based on the production and perception of the signal molecules *N*-3-oxododecanoyl-homoserine lactone (3OC$_{12}$-HSL), *N*-butanoyl-homoserine lactone (C$_4$-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively. *P. aeruginosa* QS is hierarchically organized, since the *las* QS system is required for optimal activation of the *rhl* and *pqs* QS systems. Overall, QS controls the expression of nearly 10% of the *P. aeruginosa* genome, including genes for secreted virulence factors, biofilm formation, and immune-modulatory and pro-inflammatory agents [10, 11].

QS signal molecules can be detected in clinical samples, proving that QS is active during *P. aeruginosa* infections. Moreover, QS-defective mutants show strongly impaired virulence in several animal models of infection, corroborating the importance of QS for *P. aeruginosa* pathogenicity and its suitability as a target for the development of anti-*Pseudomonas* drugs [12, 13].

On these bases, a number of studies focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *P. aeruginosa las* QS system. Different experimental approaches have been successfully used to identify small molecules

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interfering with the activity/stability of the $3OC_{12}$-HSL receptor LasR or with the functionality of the $3OC_{12}$-HSL synthase LasI. Also the identification of enzymes that inactivate $3OC_{12}$-HSL and the development of antibodies that limit the bioavailability of this signal molecule have been reported [14, 15].

Here we describe a convenient strategy for the identification of compounds affecting the *P. aeruginosa las* QS system at multiple levels: (1) expression/activity of the signal receptor LasR; (2) activity/availability of the signal molecule $3OC_{12}$-HSL; (3) expression/activity of the signal synthase LasI [16]. This strategy is defined by a primary assay, suitable for high-throughput screening of chemical compounds, and by secondary assays, used to confirm the specific activity of the hit compounds selected in the primary assay. This approach has been successfully employed for the identification of a novel *las* QS inhibitor, the FDA-approved anthelmintic drug niclosamide [17].

As a general remark, it should be pointed out that, while we chose to illustrate a screening strategy linked to the activity of the *las* QS system in *P. aeruginosa*, some of the techniques presented here can be applied to a variety of different biological systems. For instance, reporter-based assays modelled on the one described here can be devised for the *rhl* and *pqs* QS systems of *P. aeruginosa*, as well as for different QS systems in other Gram-negatives or in Gram-positive bacteria.

2 Materials

1. Bacterial strains: *P. aeruginosa* PA14 [18] and PA14-R3 biosensor [16].
2. Growth media: Luria-Bertani broth (LB: 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract); Luria-Bertani agar (LA, as LB plus 15 g/l agar).
3. 1 M MOPS Buffer: 83.7 g/l 3-(*N*-morpholino) propanesulfonic acid (MOPS), 13.6 g/l sodium acetate trihydrate, 3.7 g/l ethylenediaminetetraacetic acid (EDTA) disodium salt, pH 7.0.
4. Protease Buffer: 100 mM Tris, 1 mM $CaCl_2$, pH 7.5.
5. Synthetic *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL).
6. Elastin-Congo Red.
7. Chloroform.
8. 0.2 N HCl.
9. Black clear-bottom 96-wells microtiter plates.

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10. Automated luminometer-spectrometer plate reader.
11. UV/Vis spectrophotometer.
12. Plastic microcuvettes for UV/Vis spectrophotometer.

3 Methods

3.1 Rationale of the Primary Screening

The screening system for inhibitors of *P. aeruginosa* QS is based on a biosensor strain (PA14-R3) able to detect the QS signal molecule $3OC_{12}$-HSL [16]. The PA14-R3 biosensor is available from the authors upon request.

The PA14-R3 strain carries a nonfunctional allele of the *lasI* gene, and is thus unable to synthesize $3OC_{12}$-HSL; however, it can respond to exogenous $3OC_{12}$-HSL provided either through supply of the purified molecule or by cocultivation with a wild type *P. aeruginosa* proficient in $3OC_{12}$-HSL production, such as the PA14 strain. The screening system is detailed in Fig. 1. The $3OC_{12}$-HSL signal synthesized by the wild type PA14 diffuses into the PA14-R3 biosensor and induces bioluminescence emission. The addition of a molecule with inhibitory activity towards any process related to the $3OC_{12}$-HSL-dependent QS system, namely, $3OC_{12}$-HSL synthesis, transport, and perception, will reduce light emission by the biosensor with respect to a control coculture grown in the absence of any chemical compound.

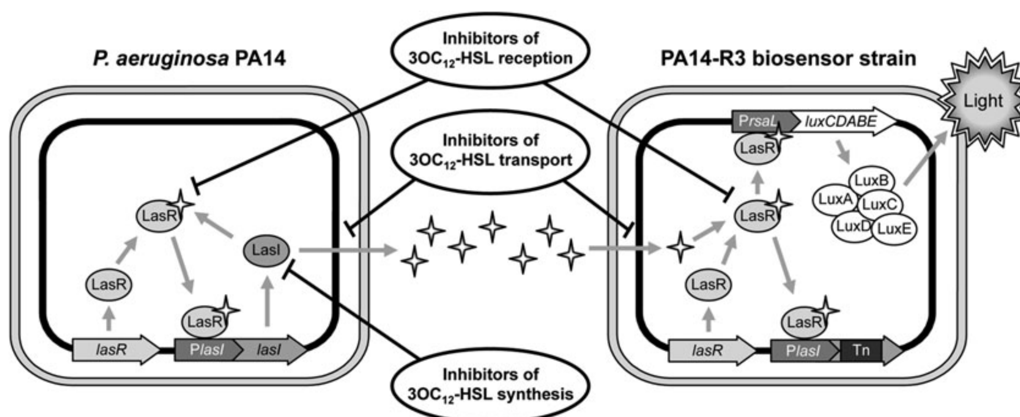

Fig. 1 Schematic representation of the PA14/PA14-R3 cocultivation screening system. The wild type PA14 produces $3OC_{12}$-HSL signal molecules that induce bioluminescence emission in the biosensor strain PA14-R3. PA14-R3 is a PA14 derivative in which a transcriptional fusion between the LasR-dependent *rsaL* promoter (P*rsaL*) and the *luxCDABE* operon was integrated at the *attB* neutral site of the chromosome. In addition, in order to avoid self-activation of the reporter strain, the *lasI* gene, encoding the $3OC_{12}$-HSL synthase LasI, was inactivated by transposon insertion (Tn). Molecules interfering with different steps of the *las* QS system are expected to cause a reduction in bioluminescence in comparison to the untreated control. Modified from [16]

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3.2 Primary Screening Procedure

1. Grow *P. aeruginosa* PA14 wild type and the $3OC_{12}$-HSL reporter strain PA14-R3 (*see* **Note 1**) overnight at 37 °C on LA plates.
2. Scrape bacteria from LA plate surfaces and dilute in 1 ml of LB supplemented with 100 mM MOPS Buffer to an absorbance at 600 nm wavelength (A_{600}) of 0.09 and 0.03 for PA14-R3 and PA14, respectively (3:1 reporter:wild type ratio). Mix isovolumes of the PA14-R3 and PA14 diluted cultures, to obtain a coculture in which the A_{600} of PA14-R3 and PA14 is 0.045 and 0.015, respectively.
3. Aliquot 100 μl per well of the coculture in a 96-well microtiter black plates with clear bottom.
4. As untreated control, add 100 μl of LB in six wells containing the coculture.
5. Set up serial dilutions of the compounds to 2× the final concentrations to be tested. For example: chemical compounds to be used in the screening assays are dissolved in 10 mM DMSO and then diluted to 400 μM, 40 μM, and 4 μM in LB medium (to obtain 200 μM, 20 μM, and 2 μM final concentrations in the assay, respectively). Aliquot 100 μl of each compound in the microtiter wells containing the coculture.
6. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.
7. Measure A_{600} and light counts per second (LCPS), simultaneously with an automated luminometer-spectrometer plate reader (*see* **Note 2**).
8. Average the A_{600} and LCPS measurements of the untreated control samples. For all samples, normalize the LCPS to the A_{600} to obtain PA14-R3 reporter activity. Compare PA14-R3 reporter activity of the treated samples to one of the untreated controls (*see* **Note 3**).

3.3 Rational of the Secondary Assays: $3OC_{12}$-HSL, Elastase, and Pyocyanin Production

As previously described, compounds reducing light emission in the primary screening could hamper the *las* QS system at different levels, including $3OC_{12}$-HSL synthesis, transport, and perception. Inhibition of any of these steps would limit $3OC_{12}$-HSL synthesis in PA14 wild type, as well as the production of $3OC_{12}$-HSL-dependent virulence factors such as elastase and pyocyanin.

Elastase is a *P. aeruginosa* secreted protease that mainly targets mammalian elastin and plays a key role in virulence [19]. Transcription of the elastase gene, *lasB*, is strongly activated by the *las* QS system [20].

Pyocyanin is a redox-active phenazine responsible for the blue-green color characteristic of *P. aeruginosa* cultures. Besides its role in virulence, pyocyanin has been also recognized as a signalling

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molecule, as an electron shuttle for bacterial respiration, and as an antibacterial and antifungal agent [21, 22].

Therefore, a convenient method to validate the *las* inhibitory activity of a hit compound is to measure its effect on $3OC_{12}$-HSL, elastase, and pyocyanin production, in comparison with an untreated control.

The assay for $3OC_{12}$-HSL production by PA14 wild type is based on the use of the same PA14-R3 biosensor strain used in the primary screening [16].

The elastase assay is based on the Elastin-Congo Red reagent, a water-insoluble powder in which elastin is bound to the Congo Red dye. Hydrolysis of elastin causes the release of the dye in the aqueous phase. The amount of released (soluble) dye is proportional to the level of hydrolyzed elastin and, ultimately, to the level of elastase present in a *P. aeruginosa* culture supernatant. The method here described is modified from ref. 23.

Pyocyanin can be easily quantified in chloroform extracts of *P. aeruginosa* culture supernatants by spectrophotometric analysis. The method here described is modified from ref. 24.

3.4 Quantification of $3OC_{12}$-HSL

1. Inoculate *P. aeruginosa* PA14 in 5 ml of LB and grow the culture overnight at 37 °C with 200 rpm shaking.
2. Refresh the overnight culture to an A_{600} of 0.015 in 30 ml of LB supplemented with 50 mM MOPS Buffer, in the presence of increasing concentrations of the test compound. Incubate at 37 °C with 200 rpm shaking.
3. Withdraw 7 ml of bacterial cultures every 3 h, up to 9 h. Measure A_{600} of the samples, harvest the cells by centrifugation, and recover the culture supernatants (*see* **Notes 4** and **5**).
4. Scrape bacteria from the surfaces of an LA plate of the biosensor strain PA14-R3 and dilute in 2 ml of LB. Measure the A_{600} of this bacterial suspension and use it to prepare an inoculum of the biosensor strain in LB supplemented with 50 mM MOPS Buffer to an A_{600} of 0.045.
5. Aliquot 195 μl per well of the biosensor culture in a 96-well microtiter black plates with clear bottom.
6. Add 5 μl of each culture supernatant from **step 3** in three wells containing the reporter culture. As untreated control, add 5 μl of LB in six wells containing the reporter culture.
7. For the calibration curve, set up 1:3 serial dilutions of synthetic $3OC_{12}$-HSL in LB, from a maximal concentration of 120 μM to a minimum concentration of ~18 nM. Add 5 μl of each diluted $3OC_{12}$-HSL sample in three wells containing the reporter culture.
8. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.

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9. Measure A_{600} and LCPS, simultaneously (*see* **Note 2**). Average the A_{600} and LCPS measurements of the replicates. Normalize the averaged LCPS to the averaged A_{600} to obtain PA14-R3 reporter activity. Extrapolate $3OC_{12}$-HSL concentration in the treated and untreated supernatants based on the values obtained for the calibration curve (*see* **Note 6**).

3.5 Elastase Assay Procedure

1. Set up 1.5 ml tubes each one containing 20 mg of Elastin-Congo Red and 1 ml of Protease Buffer (*see* **Note 7**).
2. Add 100 μl of culture supernatant collected in Subheading 3.4, **step 3** (*see* **Notes 5** and **8**) to the tube containing the Elastin-Congo Red suspension. Prepare a control sample (blank) by adding 100 μl of sterile LB instead of the culture supernatant.
3. Incubate 2 h with gentle shacking at 37 °C.
4. Centrifuge for 5 min at 11,000 × *g* at room temperature.
5. Measure absorbance at 495 nm wavelength (A_{495}) of the clear supernatants in plastic microcuvettes, using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, **step 3**.

3.6 Pyocyanin Assay Procedure

1. Add 3 ml of chloroform to 15 ml conical tubes containing 5 ml of the supernatants collected in Subheading 3.4, **step 3** (*see* **Notes 5** and **8**). Mix vigorously by vortexing for 10 s. As control sample (blank), use 5 ml of sterile LB in place of the bacterial supernatant.
2. Centrifuge the tubes at 3,000 × *g* for 5 min.
3. Transfer 2 ml of the lower organic phase in clean 15 ml conical tubes (the lower organic phase is blue if pyocyanin is present) and add 1 ml of 0.2 N HCl. Mix vigorously by vortexing for 10 s.
4. Centrifuge the tubes at 3,000 × *g* for 5 min.
5. Transfer 800 μl of the upper aqueous phase in plastic microcuvettes (the upper aqueous phase is pink if pyocyanin is present).
6. Measure the absorbance at 520 nm wavelength (A_{520}), using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, **step 3**.

4 Notes

1. By using bacterial biosensors in which light emission is induced by exogenous C_4-HSL, PQS, or other QS molecules different from $3OC_{12}$-HSL, similar coculture-based approaches can be designed to identify inhibitors of other QS systems. Please

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consider that growth of biosensor strains different from PA14-R3 may require LB supplementation with antibiotics for plasmid selection. Moreover, the use of other biosensor strains may require preliminary optimization of experimental parameters, including wild type/biosensor ratio, A_{600} of the coculture at t_0, and incubation time of the coculture at 37 °C.

2. We routinely use a Wallac 1420 Victor^{3V} multiplate reader (Perkin-Elmer) as automated luminometer-spectrometer plate reader. For the Wallac 1420 Victor^{3V} multiplate reader relevant parameters for bioluminescence measurement are: emission aperture, large; counting time, 1 s. Relevant parameters for absorbance measurement are: filter 595/60; excitation aperture, normal; reading time, 0.1 s.
3. The criteria used for the selection of hit compounds in [17] were: (a) ≥50% inhibition of PA14-R3 reporter activity; (b) ≤10% reduction of growth with respect to the untreated controls. The latter criterion was aimed at avoiding any unspecific effect of impaired growth on the QS response.
4. This step is just for harvesting cells, so speed and times for centrifugation can vary. Our standard conditions are 6,300 × *g* for 5 min at room temperature.
5. For the $3OC_{12}$-HSL quantification assay, the supernatants can be stored at −20 °C. Conversely, for elastase and pyocyanin assays it is recommended to process supernatants as soon as possible.
6. The same method can be used to quantify the amount of $3OC_{12}$-HSL produced by different strains of *P. aeruginosa* (*e.g.*, to compare a wild type and a mutant) or of *P. aeruginosa* grown in different media. Also quantification of $3OC_{12}$-HSL in *P. aeruginosa* clinical isolates has been described [16].
7. Avoid the preparation of a stock suspension of Elastin-Congo Red. This powder is highly insoluble and aliquots of a suspension could contain different amounts of the reagent. We have observed that aliquoting the powder in each sample tube enhances the assay reliability.
8. Under these conditions, *P. aeruginosa* usually starts to produce detectable levels of elastase and pyocyanin at around $A_{600} \approx 3.0$, therefore consider using only supernatants collected after 6 and 9 h incubation in Subheading 3.4, **step 3**.

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