SCUOLE DOTTORALE IN BIOLOGIA

SEZIONE: Biologia Applicata alla Salute dell'Uomo (BASU)

CICLO DEL CORSO DI DOTTORATO

XXV

Identification of compounds affecting *Pseudomonas aeruginosa* social behaviour and virulence

Identificazione di composti inibitori della virulenza in *Pseudomonas aeruginosa*

Dottorando

Cejoice Ramachandran Pillai

Docente guida/Tutor: **Prof.ssa Livia. Leoni**

Coordinatore: **Prof. Paolo Visca**

December 2012

CONTENTS

SUMMARY I

1. INTRODUCTION 1
1.1 *Pseudomonas aeruginosa* 1
1.1.1 Quorum sensing-dependent regulation of virulence in *P. aeruginosa* 3
1.2 RND family efflux pumps in *P. aeruginosa* 7
1.2.1 RND efflux pumps and bacterial virulence 11
1.2.2 RND efflux pumps inhibitors 12

2. GENERAL RATIONALE AND AIMS 15

3. CHAPTER III
New life for an old drug: the antihelmintic drug niclosamide inhibits *Pseudomonas aeruginosa* quorum sensing 17
3.1 Background 17
3.2 Results 19
3.2.1 Identification of FDA-approved compounds inhibiting *P. aeruginosa* QS 19
3.2.2 Niclosamide inhibits the $3OC_{12}$-HSL-dependent QS system of *P. aeruginosa* 21
3.2.3 Niclosamide represses QS-activated gene 24
3.2.4 Niclosamide strongly reduces the virulence potential of *P. aeruginosa in vitro* 27
3.2.5 Niclosamide protects *G. mellonella* from *P. aeruginosa* Infection 30
3.3 Discussion 31

4. CHAPTER IV
Beyond antibiotics: efflux pumps inhibition as anti-virulence strategy against *Pseudomonas aeruginosa*. 35
4.1 Background 35
4.2 Results 36
4.2.1 *In vitro* anti-virulence activity of PAβN 36
4.2.2 *In vivo* anti-virulence activity of PAβN 43
4.3 Discussion 45

5. REFERENCES 48
6. LIST OF ABBREVIATIONS 61
7. ACKNOWLEDGMENTS 62

SUMMARY

Pseudomonas aeruginosa is the most common Gram-negative bacterium responsible for hospital-acquired infections and is a serious threat to immune compromised individuals. *P. aeruginosa* infections are difficult to eradicate because this pathogen is resistant to conventional antibiotic therapies. Resistance to antibiotics is mainly due to the ability of this microorganism to form biofilm and to express efflux pumps that reduce the intracellular concentration of the drug.

Targeting the bacterial pathogenic potential rather than bacterial growth has the advantage of reducing the bacterial adaptability to the host environment and the severity of the infection without creating the selective pressure generally caused by conventional antibiotics. The use of virulence inhibitors could ultimately provide the host immune system with a better chance of clearing the infection.

The overall rationale and aim of this project has been to pave the way for the development of innovative anti-virulence therapies specifically targeting *P. aeruginosa*.

The objective of the study described in Chapter 3 has been the identification of compounds inhibiting *P. aeruginosa* quorum sensing (QS), a cell-cell communication process playing a key role in the expression of virulence factors and biofilm development.

It is well known that searching for new side activities in drugs which use in humans has already been approved is an intelligent strategy for the development of novel drugs. This strategy is expected to reduce the time and cost associated with standard drug discovery processes.

About thousand compounds already used as drugs in humans were screened for their anti-QS activity, using a biosensor developed in our laboratory. Seven compounds with anti-QS activity were identified; the most effective drug was the anthelmintic drug niclosamide.

Microarray analysis showed that niclosamide (at 20 μM) affects the transcription of 258 genes, with a high degree of target specificity towards the QS-dependent genes. 69 genes were up-regulated and 189 genes were down-regulated, 121 of the latter group were previously included within the QS regulon. Phenotypic assays demonstrated that niclosamide suppresses surface motility, production of secreted virulence factors (elastase, pyocyanin and rhamnolipids) and reduces biofilm formation. In accordance with the strong anti-virulence activity disclosed *in vitro*, niclosamide

prevented *P. aeruginosa* pathogenicity in the *Galleria mellonella* insect model of acute infection.

To our knowledge, this is the first study in which a drug-repurposing strategy has been applied to the development of *P. aeruginosa* anti-virulence drugs. Besides the finding that niclosamide, an FDA-approved drug has a promising anti-virulence activity against one of the most antibiotic-resistant bacterial pathogens, this work provides a proof of concept that a lateral anti-QS activity can be detected among drugs already used in humans, validating a new approach to identify virulence inhibitors that could easily move into clinical applications.

Chapter four of this thesis describes a study aimed at investigating the inhibition of *P. aeruginosa* virulence *via* chemical inactivation of Resistance-Nodulation-Cell Division (RND) efflux pumps. Efflux pumps of this family are important for multiple drug-resistance in many pathogenic bacteria, including *P. aeruginosa*.

Since no close human homologues of RND transporters have been described, efflux pumps inhibitors (EPI) are considered promising scaffolds and lead compounds for the development of drugs aimed at potentiating antibiotic activity. Since in some bacterial pathogens RND efflux pumps are also involved in virulence, EPIs have been proposed as virulence inhibitors. However, no literature data are available concerning the potential anti-virulence activity of EPIs.

The main aim of this study has been to provide a first proof of concept that, apart from their role in antibiotic resistance, EPIs may act as anti-virulence drugs against *P. aeruginosa,* by using phenyl-arginine β-naphthylamide hydrochloride (PAβN) as model compound.

Here, we demonstrate that PAβN 6.5 μM abrogates swarming motility, an *in vitro* phenotype strictly related to virulence *in vivo*. Moreover, microarray analysis showed that PAβN affects the transcription of about 109 genes. *P. aeruginosa* transcriptome is affected by PAβN in a specific way, since it negatively affects the expression of particular groups of genes, mainly related to iron and phosphate acquisition, while it increases the expression of genes involved in nitrogen metabolism.

Overall, our results demonstrate that in *P. aeruginosa* PAβN has pleiotropic effects that go far beyond the increased susceptibility to antibiotics. In particular, PAβN decreases the transcription of virulence related genes, resulting in a reduced pathogenic potential in the *G. mellonella* infection model system.

In conclusion, the use of EPIs in therapy is a promising challenge since these compounds, besides their effect as antibiotic adjuvants, may display anti-virulence properties.

1. INTRODUCTION

1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the most common Gram-negative bacterium responsible for hospital-acquired infections, and is a serious threat to immune compromised individuals such as neutropenic, cancer, bone marrow transplant and AIDS patients. This bacterium is known to cause pneumonia, urinary tract, surgical wound, bloodstream infections, bacteremia in severe burn victims and keratitis in contact lenses users. In addition, *P. aeruginosa* chronic lung infection is the major cause of death in cystic fibrosis (CF) patients, a genetic disease affecting about 1/3000 newborns in the Caucasian population (Driscoll *et al.,* 2007; Rosenthal *et al.,* 2012).

Antibiotics are often poorly effective against *P. aeruginosa*, especially in the treatment of chronic infections where this microorganism forms biofilm. Moreover, the strong selective pressure exerted by antibiotics prompts to the emergence of resistant strains. For these reasons, *P. aeruginosa* infections are characterized by high morbidity and mortality rates (Breidenstein *et al.,* 2011; Rosenthal *et al.,* 2012).

Overall, *P. aeruginosa* causes 140,000 infections in the EU region per year, with more than 10,000 deaths and around 800,000 extra hospital days costing billions of Euros. The ability of *P. aeruginosa* to acquire genetic determinants of antibiotic resistance results in the generation of pan-resistant strains for which currently available antibiotic therapies are no longer effective (Latifi *et al.,* 1995), calling for the development of new anti-*P. aeruginosa* drugs.

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

Some of the virulence factors that confer to the pathogen the ability to colonize the host are cell surface virulence factors. Actually the cell surface itself is a virulence factor because it contains very immunogenic compounds, like the lipopolysaccharide (LPS). Flagella and pili involved in different kind of motility and in chemotaxis, display a critical role in pathogenesis, by adhering to epithelial cells and stimulating an inflammatory response (Adamo *et al*., 2004; DiMango *et al*., 1995). When *P. aeruginosa* chronically infects patients, it adapts to the biofilm mode of

growth. The generally accepted definition of a biofilm is a community of cells attached to a surface or to each other, embedded in a self-made, protective matrix of extracellular polymeric substances (Kirisits and Parsek, 2006). The clinical implications of bacterial biofilms are particularly pronounced. Biofilms may form on any foreign object inserted into the human body, and also in the lungs of CF patients *P. aeruginosa* forms biofilm during chronic infection. In the biofilm mode the bacteria are highly tolerant to the action of several antimicrobial agents including antibiotics, disinfectants, and to the action of the immune system (Davies, 2002; Donlan and Costerton, 2002; Drenkard, 2003).

P. aeruginosa also produces several extracellular products that, after the initial step of colonization, cause extensive tissue damage, bloodstream invasion and dissemination. Some of these extracellular products, besides having the role of favouring pathogen dissemination, also provide nutrients to *P. aeruginosa* by causing host tissues damage. These secreted factors are elastases, alkaline proteases, exotoxins and hemolysins that also contribute to the infection in lung disease by destroying the protective glicocalix of the respiratory epithelium (Kipnis *et al*., 2006). Other secreted factors are toxic compounds such as hydrogen cyanide and pyocyanin: hydrogen cyanide is a potent poison that blocks cytochrome oxidase, leading to the inhibition of mitochondrial respiration (Gallagher and Manoil, 2001). Pyocyanin is a blue pigment metabolite of *P. aeruginosa* that has been shown to have numerous pathogenic effects such as increasing IL-8, depressing host-response, and inducing apoptosis in neutrophils (Allen *et al*., 2005; Denning *et al*., 1998). In animal models of acute and chronic lung infection, pyocyanin was shown to be essential for *P. aeruginosa* virulence (Lau *et al*., 2004). Additionally, due to its known oxidoreductive properties, pyocyanin oxidizes glutathione and inactivates catalase in respiratory epithelial cells thus participating in oxidative-stress related damage (O'Malley *et al*., 2004). The need for iron in *P. aeruginosa* is supported by the production of the two siderophores, pyoverdine and pyochelin, that are small molecules chelating iron from the iron-poor environment encountered in the host, allowing its utilization in *P. aeruginosa* metabolism (Buckling *et al*., 2007).

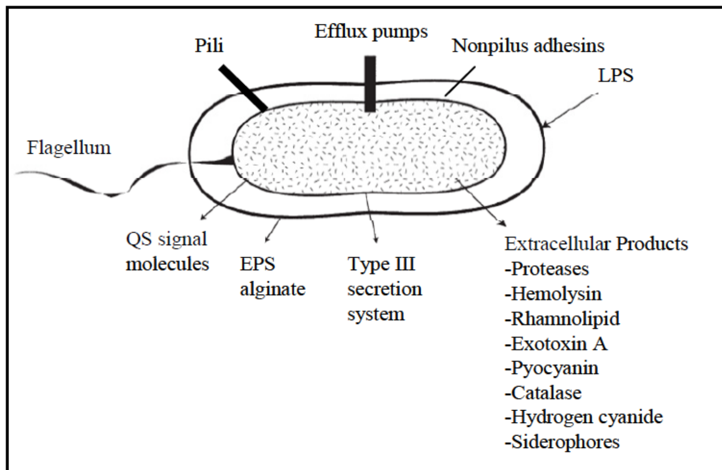

Figure 1. Virulence and antibacterial resistance factors in *P. aeruginosa* (modified from Van Delden and Iglewski, 1998).

1.1.1. Quorum sensing-dependent regulation of virulence in *P. aeruginosa.*

Many bacteria coordinate their behaviour via the secretion of specific signalling molecules in a population density-dependent manner. During growth the bacteria secrete the signal molecule that accumulates in the surrounding environment as the population density increases until a critical threshold concentration is reached, which then triggers expression of certain sets of genes (Fig. 2). This type of cell-to-cell communication is termed "quorum sensing" (QS), in order to emphasize the fact that a sufficient number of bacteria, the bacterial "quorum", is needed to induce or repress expression of target genes (Whitehead *et al.,* 2001).

QS is involved in the regulation of a wide variety of different bacterial processes including bioluminescence, horizontal gene transfer, antibiotic biosynthesis, motility, biofilm formation, and virulence in plant, animal and human pathogens (Càmara *et al*., 2002; Lazdunski *et al*., 2004).

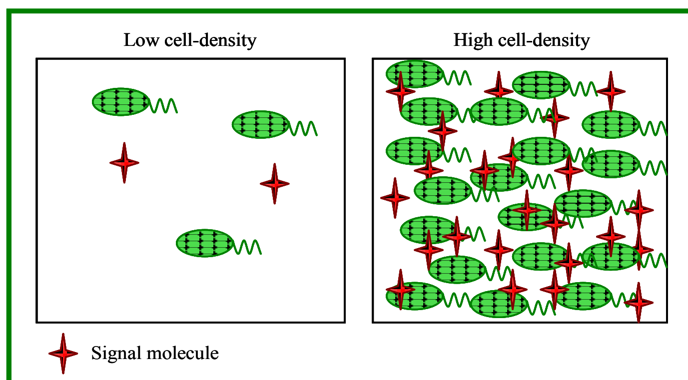

Figure 2. A simple representation of QS. The signal molecule is constitutively produced at a basal level by the bacterial cells. At low-cell densities very little signal molecule is present. As cell density increases, the signal molecule accumulates until a threshold level is reached. This signal molecule concentration is responsible for a coordinate transcriptome reprogramming in the whole bacterial population.

In *P. aeruginosa* there are three interconnected QS systems, each one relying on different signal molecule. The first two systems, named *las* and *rhl*, belong to the Lux-family of QS systems and employ 3-oxo-docecanoyl homoserine lactone ($3OC_{12}$-HSL) and butanoyl homoserine lactone (C_4-HSL) as signal molecules, respectively (Fig. 3). The second chemically distinct class of auto inducers are the 4-quinolones which consists of more than 50 compounds (Lepine *et al.*, 2004) and includes the most active signal molecule 2-heptyl-3-hydroxy-4-quinolone (PQS), which is commonly referred to as the *Pseudomonas* quinolone signal. It is important to notice that the $3OC_{12}$-HSL-dependent QS system (*i.e.* the *las* system) positively controls the other two QS systems (Williams and Càmara, 2009).

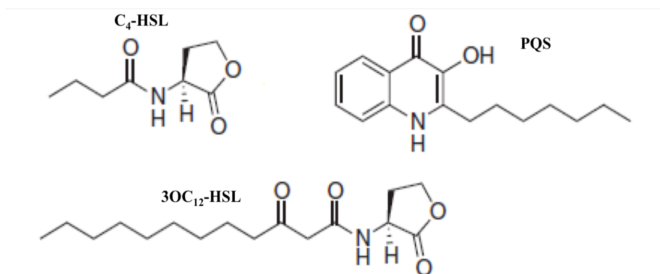

Figure 3. Structure of QS molecules exploited by *P. aeruginosa* for cell-to-cell communication, (modified from Piddock, 2006.).

When the bacterial population has reached a certain density, the signal molecules accumulate, bind to their respective regulator proteins, and initiate the transcription of a distinct set of genes, including own biosynthetic genes (Fig. 4). Many of the quorum sensing-regulated genes are virulence factors, and the extra cellular accumulation of acyl homoserine lactones (acyl-HSL)-signalling molecules has also been shown to impact on the structural development and stabilization of *P. aeruginosa* biofilms (Davies *et al*., 1998; Kirisits and Parsek, 2006).

The multiple extracellular pathogenic traits positively regulated by QS in *P. aeruginosa* include elastases, alkaline protease, exoenzyme S, neuraminidase, haemolysin, lectins, pyocyanin, rhamnolipids, hydrogen cyanide and oxidative stress-responsive enzymes catalase and superoxide dismutase (Williams and Camara, 2009). All these extracellular virulence factors are crucial for the competence of *P. aeruginosa* to establish and maintain the infection. Mutants defective in QS are typically compromised in their ability to establish a successful infection. QS mutants show reduced virulence in a number of mammalian and non-mammalian infection models (Cosson *et al*., 2002; Rahme *et al*., 1995; Rumbaugh *et al*., 1999; Tan *et al*., 1999; Tang *et al*., 1996; Wu *et al*., 2000). Additionally, the specific signal molecules of all three QS systems of *P. aeruginosa* were detected in the sputum of CF patients, thus suggesting that QS is functionally active in the CF chronic lung infections (Collier *et al.,* 2002; Erickson *et al.,* 2002; Middleton *et al*., 2002). The QS systems of *P. aeruginosa* contribute to its pathogenesis not only by regulating expression of virulence factors, but also by inducing host cellular responses like for instance inflammation and apoptosis (Rumbaugh, 2007; Smith *et al*., 2002). The rapid increase of multidrug-resistant strains is demanding novel therapeutic approaches.

Given that the QS circuitry in *P. aeruginosa* plays an important role in controlling pathogenicity as well as biofilm formation, it represents a highly attractive target for the development of novel antimicrobial agents (Reviewed in Warren *et al.,* 2012). It has been suggested that the use of QS inhibitors (QSI) that specifically inhibit expression of pathogenic traits without affecting growth of the bacterium has the advantage of minimizing the possibility of selecting resistant mutants. Although such mutants may eventually arise, they would not have a selective growth advantage and thus would not out-compete the parental strain. Therefore, the emergence of resistance to QSI is an unlikely event (Darch *et al.,* 2012; Hentzer and Givskov, 2003).

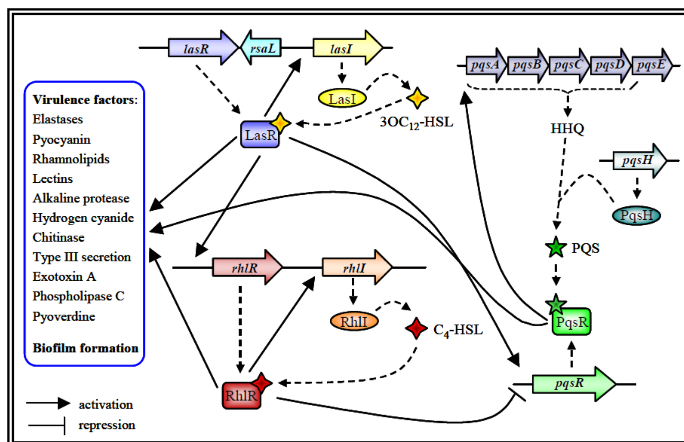

Figure 4. Schematic representation of the central core of the QS network in *P. aeruginosa*, with *las*, *rhl*, and *pqs* systems. Relevant QS-regulated virulence factors are listed (modified from Camara *et al*., 2002 and Diggle *et al*., 2003).

1.2. RND family efflux pumps in *P. aeruginosa*

Today, the treatment of bacterial infections is severely compromised by the emergence of bacteria, including those belonging to the *P. aeruginosa* species, that are resistant to multiple antibiotics. The dissemination of multidrug-resistant (MDR) Gram-negative bacteria drastically impairs the efficacy of antibiotic families and limits their clinical use (Falagas and Bliziotis, 2007; Gandhi *et al*., 2010). The active efflux of antibiotics via efflux pumps is a major cause of the bacterial MDR phenotype. Efflux pumps have been reported to play a key role in mediating multidrug resistance in clinical isolates from varied geographic locations and populations (Martinez *et al*., 2009; Nikaido, 2009; Piddock, 2006; Poole, 2007).

Efflux pumps have been categorized into five families, based primarily on amino acid sequence identity, on the energy source required to drive export, and on substrate specificity (Lister *et al.,* 2009). The families include (i) the ATP-binding cassette (ABC) family, (ii) the small multidrug resistance family (SMR), (iii) the major facilitator superfamily (MFS), (iv) the resistance-nodulation-division (RND) family, and (v) the multidrug and toxic compound extrusion family (MATE).

Although sequence analysis of the *P. aeruginosa* genome revealed the presence of efflux systems from all five families, the largest number of predicted pumps belong to the RND family, with a total of 12 RND systems (including two divalent metal cation transporters) (Stover *et al.,* 2000). Unlike the primary active transporters of the ABC family, which utilize ATP hydrolysis for energy, the RND family (as well as the remaining super families) are secondary active transporters (symporters, antiporters, and uniporters) that derive the energy required for compound extrusion by proton motive force (Nikaido, 1996; Poole, 2007).

RND pumps typically exist as a tripartite system consisting of a periplasmic membrane fusion protein (MFP), an outer membrane factor (OMF), and a cytoplasmic membrane (RND) transporter (Fig. 5). This complex forms a channel spanning the entire membrane, allowing for the transportation of lipophilic and amphiphilic drugs from the periplasmic space and cytoplasm to the extracellular environment. The genes encoding these pumps are organized into operons on the *P. aeruginosa* chromosome (Fig. 6). Each operon is composed of at least two genes, coding for the MFP and the RND transporter. Six of the 12 operons possess an OMF gene, completing the tripartite system, while the remaining operons are devoid of an OMF gene. Several operons have an adjacent regulatory gene transcribed in the same orientation or divergently from the operon, whose product

functions as a repressor or activator of pump expression. Operons may contain additional genes besides those coding for the efflux pump. For example, *mexG* in the *mexGHI-opmD* operon encodes an integral membrane protein, and PA2528-PA2527-PA2526-*opmB* possesses a second RND transporter gene, PA2526 (Lister *et al.,* 2009).

The 10 RND pumps of *P. aeruginosa* known to be involved in antibiotic efflux are named MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK, MexGHI-OpmD, MexVW, MexPQ-OpmE, MexMN, and TriABC (Fig. 6). Mex is an acronym for *m*ultiple *e*fflu*x*, and “Tri” refers to triclosan efflux. While several of these pumps share common substrates, they are also responsible for unique resistance phenotypes inherent to their expression. Substrates of these pumps include antibiotics, biocides, dyes, detergents, organic solvents, aromatic hydrocarbons (Scweizer, 2003).

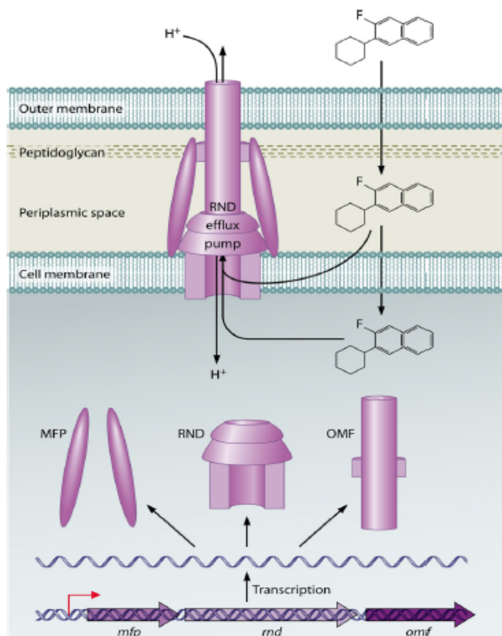

Figure 5. Structure of an RND efflux pump. RND pumps typically exist in a tripartite system consisting of an RND cytoplasmic membrane transporter (RND), a membrane fusion protein (MFP) and an outer membrane factor (OMF). The tripartite complex forms a channel spanning the entire membrane, allowing for the proton-driven transport of lipophilic and amphiphilic drugs from the cytoplasm of the cell across the cytoplasmic membrane, through the periplasmic space, across the peptidoglycan, and across the outer membrane. The RND efflux pumps can also extrude drugs from the periplasmic space before they cross the cytoplasmic membrane (Lister *et al.,* 2009).

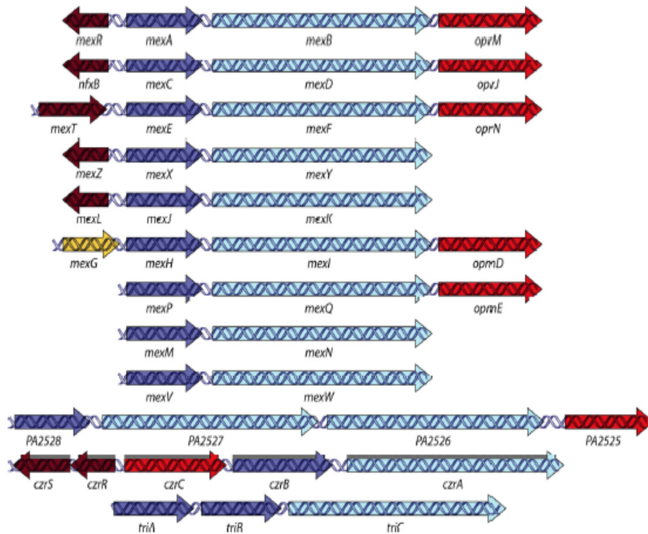

Figure 6. Operons encoding RND efflux pumps in *P. aeruginosa*. Genes which encode protein components or characterized pumps are denoted by their gene names, and genes encoding protein components of uncharacterized pumps are designated with the *P. aeruginosa* (PA) numbers assigned in the annotated *P. aeruginosa* genome sequence (www.pseudomonas.com). Genes are depicted with the following colour scheme: dark red arrow, transcriptional regulator-encoding gene; dark blue arrow, membrane fusion protein-encoding gene; light blue arrow, RND transporter-encoding gene; red arrow, outer membrane protein-encoding gene; and gold arrow, gene encoding a protein with unknown function (Schweizer, 2003).

1.2.1. RND efflux pumps and bacterial virulence

Some bacterial efflux pumps, besides antibiotics, dyes and detergents, export antimicrobial agents and metabolites produced by the bacterium itself, including adhesins, toxins and other factors that are important for the colonization and infection of human and animal cells (Piddock, 2006).

So far, evidence of a role for the RND family pumps in pathogenicity has been obtained for some animal and plant pathogens (Piddock, 2006). For example, the *Campylobacter jejuni* RND family efflux pump CmeABC is essential for the colonization of 1-day-old chickens (Lin *et al.,* 2003) and *Neisseria gonorrhoeae* RND-family efflux pump MtrCDE is important for infection of the genito-urinary tract of female mice (Jerse *et al.*, 2003). The AcrAB–TolC RND efflux pump is important for the ability of *Salmonella typhimurium* to infect BALB/c mice (Lacroix *et al.,* 1996; Stone and Miller, 1995), to colonize chickens, to adhere to, invade and survive in mouse macrophages and to invade and survive in human embryonic intestinal cells (Baucheron *et al.,* 2005; Buckley, 2006).

Burse and colleagues (2004[a]) have shown that the genome of *Erwinia amylovora* (which causes the disease fire-blight in apple and pear trees) contains an RND efflux pump homologous to AcrAB–TolC of *S. typhimurium*. Disruption of *E. amylovora acrB* gene was found to cause a significant impairment in the survival of *E. amylovora* in apple plantlets and a marked reduction in its ability to cause fire blight symptoms. Furthermore, the expression of *acrAB* was shown to increase two fold during growth of the bacteria in apple tissue contained antimicrobial secondary metabolites, supporting the hypothesis that AcrAB has a role in the colonization of the host plant (Burse *et al.,* 2004 [b]).

Literature data are also available suggesting a role for *P. aeruginosa* RND-family efflux pumps in virulence. *P. aeruginosa* lacking the MexAB–OprM efflux pump could not invade epithelial cells [Madin–Darby canine kidney (MDCK) cells] and invasion could be restored by complementation with MexAB–OprM or by supplementation with culture supernatant obtained from MDCK cells infected with wild-type *P. aeruginosa*. These findings suggested that MexAB–OprM exports virulence determinants that allow *P. aeruginosa* to be invasive and cause infection (Hirakata *et al.,* 2002). Further support for the hypothesis that RND efflux pumps may be involved in *P. aeruginosa* virulence comes from an experimental model of acute pneumonia in rats, in which, even in the absence of antibiotic treatment, isolates of *P. aeruginosa* that over express MexCD-OprJ and MexEF-OprN efflux pumps were identified (Join-Lambert *et al.*, 2001). Moreover, it was also reported that MexAB-OprM participates in the efflux

of $3OC_{12}$-HSL (Evans *et al.*, 1998; Minagawa *et al.*, 2012; Pearson *et al.,* 1999) and that MexEF-OprN and MexGHI-OprM might be involved in transport of PQS and other 2-alkylquinolones (Lamark and Deziel, 2011).

1.2.2. RND efflux pumps inhibitors

The continuous increase in the development of multidrug resistance by many pathogens has resulted in difficult fighting of many infectious diseases. Since the majority of multidrug resistant pathogens expresses RND efflux pumps that are responsible for the extrusion of the antibiotics from inside the cells, a new direction for chemotherapeutics is the use of efflux pump inhibitors (EPIs) (Pagès and Amaral, 2009). The inhibition of the efflux pumps is promising in order to increase the intracellular drug concentration, to restore the drug activity against the resistant strains, and to minimize further development of resistant strains (Askoura *et al.,* 2011; Zhang and Mah, 2008).

The inhibition of efflux pumps can be achieved by different mechanisms as shown in figure 7: interference with the regulatory steps needed for the expression of the efflux pump (Lister *et al.,* 2011); chemical changes in the antibiotic structure, hence hindering its recognition as the specific substrate (Alyssa *et al.,* 2009); disruption of the assembly of the efflux pump-components (McGowan, 2006); inhibition of the substrate (antibiotic) binding by either competitive or non-competitive binding (Hancock and Speert, 2000); blocking the outer pores responsible for the efflux of antibiotic compound (Falagas and Bliziotis, 2007); interference with the energy required for the pump activity (Pagès and Amaral, 2009).

Peptidomimetic compound molecules with phenyl-arginine β-naphthylamide hydrochloride (PAβN; also named MC-207,110) as a leading compound (Fig. 8) were discovered by MPEX pharma- ceuticals in a screen for adjuvants of levofloxacin activity against a *P. aeruginosa* strain that over-expressed the levofloxacin-specific efflux pump MexAB-OprM (Askoura *et al.,* 2011). It has been demonstrated that PAβN is very potent as an efflux inhibitor against the major RND pump of *P. aeruginosa* (MexAB-OprM) when compared to quinoline derivatives (another class of EPIs; Davin-Regli *et al.,* 2006). This compound also shows activity against MexCD-OprJ and MexEF-OprN (Lomovskaya *et al.,* 2001). It was also shown that PAβN, besides quinolones, can restore the activity of other unrelated antibiotics such as chloramphenicol and macrolides; hence, it can be considered a broad spectrum efflux pump inhibitor (Lee *et al.,* 2001; Lomovskaya and Bostian, 2006).

The mechanism of action of PAβN and related compounds is believed to occur through competitive inhibition mechanism (competitively binding to the substrate site of RND efflux pump), where the efflux pumps recognize them as a substrate instead of the target antibiotics. It has been shown that PAβN can compete with certain antibiotics but not with others depending on the nature of the efflux pump, in particular of the substrate-binding site (Lee *et al.,* 2001; Sangalang *et al.,* 2002). In addition to the competitive binding to the RND efflux pumps, Iino and co-workers (2012) recently suggested a membrane-permeabilising effect of PAβN.

The main drawbacks associated with the PAβN-derived EPI compounds are their toxic properties hindering their clinical applications. At present, they are used in order to evaluate the different efflux mechanisms expressed by different pathogenic bacteria (Lomovskaya and Bostian, 2006; Lomovskaya *et al.,* 2001).

Overall, PAβN-derived compounds are the most studied and developed EPIs against *P. aeruginosa*, though more studies concerning their structure-activity relationship, pharmacokinetics, and stability in biological fluids are required.

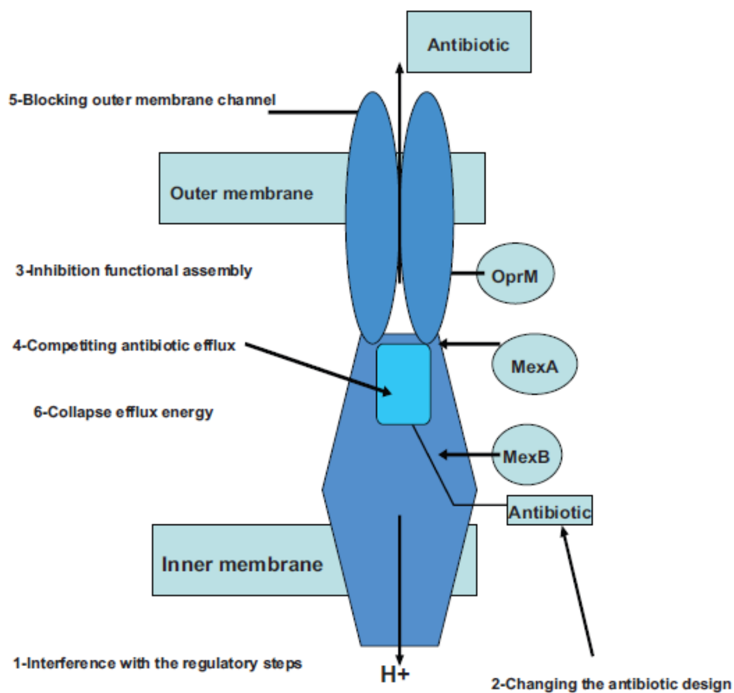

Figure 7. Schematic illustration showing the general mechanisms of efflux pump inhibition (and the targets that can be affected) using MexAB-OprM efflux pump as an example (Askoura *et al.,* 2011).

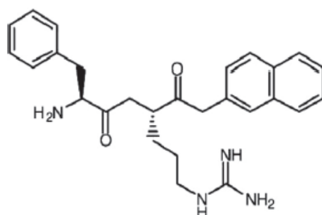

Figure 8. PAβN (MC-207,110)

2. GENERAL RATIONALE AND AIMS

P. aeruginosa was selected as model organism for this project because it is one of the most dreaded pathogens in developed countries. Indeed, current treatments, based on traditional antibiotics that kill or inhibit the growth, are often thwarted by *P. aeruginosa* ability to develop resistance to all known classes of antibacterial agents (Gilbert, 2010).

The anti-virulence drugs are able to specifically inhibit the bacterial capability to establish the infection rather than bacterial growth, and represent an innovative therapeutic approach against difficult-to treat bacteria, either when used alone or in combination with antibiotics. The use of anti-virulence drugs has the advantage of reducing the bacterial adaptability to the host environment, facilitating the host immune system to clear the infection, without creating, in principle, the strong selective pressure generally caused by antibiotics (Rasko and Sperandio, 2010).

In *P. aeruginosa,* the QS global regulatory system is necessary for full pathogenicity and represents an ideal target to develop anti-virulence drugs (Brjansholt *et al.*, 2010). In addition, recent studies supported the ground-breaking principle that the selection of mutants resistant to QS inhibitors (QSI) is an unlikely event. Indeed, despite the emergence of mutants resistant to QSI is possible, such mutants behave as social cheaters and are not able to enrich in a population of QSI-sensitive bacteria (Maeda *et al.*, 2012; Mellbye and Schuster, 2011).

Overall, the state of the art knowledge strongly supports the hypothesis that targeting QS *via* anti-virulence drugs represents a promising strategy to prevent and/or treat *P. aeruginosa* infections (Bjarnsholt *et al.*, 2010).

In addition, the notion is emerging that RND efflux pumps are involved in the secretion of endogenous bacterial products, including QS signal molecules and virulence factors (Piddock, 2006). In *P. aeruginosa,* efflux pump inhibitors (EPIs) have been mainly studied for their effect on antibiotic resistance, while, to our knowledge, nothing is known about their impact on QS and virulence-related phenotypes.

The general aim of this PhD work has been to gain new insights in the anti-virulence drug therapy against *P. aeruginosa.*

The specific aim of the work described in chapter three has been to apply a drug-repurposing approach to the identification of anti-virulence drugs targeting *P. aeruginosa* QS. The work described in this chapter has been recently published (Imperi *et al.*, 2013).

Chapter four describes a study investigating the potential of EPIs as anti-virulence drugs, apart from their role in antibiotic resistance, using PAβN as model compound. A manuscript concerning this study is in preparation.

3. CHAPTER III

New life for an old drug: the antihelmintic drug niclosamide inhibits *Pseudomonas aeruginosa* quorum sensing

3.1. Background

The introduction of antibiotics in the clinical practise at the middle of 20th century is a milestone in the history of medicine. However, the original expectation that all bacterial infections could be one day defeated by antibiotics was soon disappointed by the emergence of antibiotic resistant strains, prompting the still ongoing race for the discovery of new antibacterial agents. While the treatment of infections sustained by antibiotic resistant bacteria has high socio-economic costs and represents a major health problem worldwide, pharmaceutical industries have dramatically reduced the investments in antibiotics research. As traditional antibiotic research appears to be helpless to cope with the emergence of antibiotic resistant strain, novel scientifically sound and cost effective approaches should be undertaken in order to identify new drugs (Gilbert, 2010).

Selective Optimization of Side Activities of drug molecules (the SOSA approach) is a smart strategy for the identification of new potential drugs (Wermuth, 2006). A limited number of highly diverse drugs, whose use in humans has already been approved, are screened for side activities against unrelated diseases. Once a hit compound has been found, this could be either tested directly in clinical studies or used as the lead for drug optimization programs. This strategy has a high probability of yielding safe and bioavailable drug-like compounds, and it is thus expected to reduce the time and cost generally associated with standard drug discovery processes (Antoniani *et al.,* 2010; Ejim *et al.,* 2011; Wermuth, 2000).

An innovative strategy to combat bacterial infections relies on specific inhibition of bacterial virulence, hence the ability to cause disease, rather than bacterial growth (Cegelski *et al.,* 2008). The use of "anti-virulence drugs" could have the advantage of reducing the bacterial adaptability to the host environment, facilitating the host immune system to combat the infection and reducing the strong selective pressure exerted by conventional antibiotics (Rasko and Sperandio, 2010), although this is not yet supported by direct clinical evidence.

In many bacteria, pathogenicity is controlled and coordinated by an inter-cellular communication process named quorum sensing (QS). QS is

based on the synthesis and secretion of a signal molecule that binds to a cognate receptor. The signal-activated receptor controls the expression of target genes. Since the production of the signal molecule is proportional to the bacterial growth, QS coordinates gene expression in response to the bacterial population density (Atkinson and Williams, 2009). So far, QS is considered one of the most promising targets for anti-virulence therapies (Amara *et al.,* 2011; Njoroge and Sperandio, 2009; Rasko and Sperandio, 2009).

In this study the SOSA approach has been applied to the identification of anti-virulence drugs targeting bacterial QS, using *Pseudomonas aeruginosa* as a model organism. *P. aeruginosa* is one of the most dreaded Gram-negative pathogens in developed countries, being responsible for both community- and hospital-acquired infections. In addition, *P. aeruginosa* chronic lung infection is the major cause of death in cystic fibrosis (CF) patients, a genetic disease affecting about 1/3,000 newborns in the Caucasian population (Driscoll *et al.,* 2007; Orsi *et al.,* 2005; Rosenthal *et al.,* 2012; Talbot *et al.,*2000). Besides being intrinsically resistant to several antibiotics, *P. aeruginosa* can easily acquire new resistance determinants, and indeed the emergence of pan-resistant strains has already been documented (Page and Heim, 2009). For these reasons, *P. aeruginosa* infections are generally characterized by high morbidity and mortality rates (Breidenstein *et al.,* 2011Rosenthal *et al.,* 2012).

The pathogenic potential of *P. aeruginosa* relies on the coordinated expression of a large array of virulence factors (Lee *et al.,* 2006), the majority of which are positively controlled by QS (Williams and Càmara, 2009). The *P. aeruginosa* QS network consists of three different QS systems, based on the production of specific 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). *P. aeruginosa* QS is hierarchically organized, since $3OC_{12}$-HSL is required for optimal production of the other QS signals (Williams and Càmara, 2009).

QS controls the expression of nearly 10% of the *P. aeruginosa* genome, including genes for biofilm formation, secreted virulence factors, immune-modulatory and pro-inflammatory agents (Williams and Càmara, 2009). 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 (Bjarnsholt *et al.,* 2010; Winstanley and Fothergill, 2009).

We have recently developed a convenient system for the identification of compounds affecting the *P. aeruginosa* $3OC_{12}$-HSL-based QS system at multiple levels: (i) expression/activity of the signal receptor, (ii) expression/activity of the signal synthase, and (iii) activity/availability of the signal molecule (Massai *et al.,* 2011). Here, screening a library of about one thousand compounds with known pharmacological activities has validated this system.

Seven hit compounds have been identified. Among these, we have focused our investigation on the anthelmintic drug niclosamide, which showed high inhibitory activity against *P. aeruginosa* QS and virulence both *in vitro* and *in vivo*. To the best of our knowledge, this is the first demonstration that the SOSA approach can be successfully applied to the search for anti-QS drugs.

3.2. Results

3.2.1. Identification of FDA-approved compounds inhibiting *P. aeruginosa* QS.

We recently developed a novel screening system for the identification of *P. aeruginosa* QSI. This system is based on the co-cultivation of a biosensor strain for $3OC_{12}$-HSL detection, PA14-R3, and a wild-type *P. aeruginosa* PA14 strain. The $3OC_{12}$-HSL signal synthesized by the wild-type PA14 induces bioluminescence emission by the biosensor (Massai *et al.,* 2011). The addition of a molecule with inhibitory activity towards any process related to the $3OC_{12}$-HSL-dependent QS system, including $3OC_{12}$-HSL synthesis, transport and perception, would reduce the luminescence emitted by the biosensor with respect to a control co-culture without any compound added (Massai *et al.,* 2011).

The PA14/PA14-R3 co-cultivation system was used to screen a commercial library of marketed drugs from Prestwick Chemicals (www.prestwickchemical.fr). This library contained 1,120 chemical compounds with known biological activities, selected for their high chemical and pharmacological diversity, as well as for known bioavailability and safety in humans. Each drug was tested at three different concentrations (100, 10 and 1 µg/ml) in duplicate. Criteria used for the selection of hit compounds were: (i) = 50% inhibition of bioluminescence emission and (ii) = 20% 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.

The screening assay allowed the identification of seven putative QSIs

which reproducibly inhibited the QS response of the PA14/PA14-R3 co-cultivation system, without affecting bacterial growth at the highest concentration tested. The seven hits were further tested in triplicate at 100, 80, 60, 40, 20, 10, 5 and 2.5 μg/ml final concentration, showing half maximal inhibitory concentration (IC_{50}) in the range 3-77 μg/ml (corresponding to 10-150 μM; Table 1). Four of the identified compounds are antibiotics, in agreement with the well-known negative effect of sub-inhibitory concentrations of antibiotics on the *P. aeruginosa* QS response (Jander *et al.,* 2000; Babic *et al.,* 2010). The remaining three compounds corresponded to a quaternary ammonium salt, an anti-cancer drug and a teniacide for the treatment of tapeworm infections (Table 1). Among non-antibiotic drugs, the teniacide niclosamide showed the highest anti-QS activity (lowest IC_{50}) (Table 1), and was therefore selected for further investigations.

Table 1. Hit compounds identified by screening the Prestwick Chemical Library with the PA14/PA14-R3 QSI screening system.

Prestwick code	**Compound name**	**IC_{50} (μM)[a]**	**Properties**
01D11	Niclosamide	10	Anthelmintic
02H11	Gentamycin	20	Aminoglycoside antibiotic
05G06	Mitoxantrone dihydrochloride	150	Antineoplastic agent
07E06	Rifampicin	50	Antibiotic of the rifamycin group
07H08	Dirithromycin	50	Macrolide glycopeptide antibiotic
13C08	Sanguinarine	60	Quaternary ammonium salt of the benzylisoquinoline alkaloids group
14G10	Rifabutin	10	Antibiotic of the rifamycin group

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

3.2.2. Niclosamide inhibits the $3OC_{12}$-HSL-dependent QS system of *P. aeruginosa.*

To verify the result of the screening assay, niclosamide was purchased from an alternative supplier (Sigma-Aldrich) and retested in the PA14/PA14-R3 co-cultivation system. As expected, a strong inhibition of the $3OC_{12}$-HSL-dependent QS response was observed, with an IC_{50} even lower than that calculated for the compound from the Prestwick library (Fig. 9A). Notably, niclosamide was also able to inhibit luminescence emission by the PA14-R3 reporter strain grown in the presence of exogenously-added synthetic $3OC_{12}$-HSL (3 μM final concentration; Fig. 9B). This result suggests that the QS inhibitory activity of niclosamide relies on its ability to hamper the response of *P. aeruginosa* to the signal molecule rather than to inhibit its synthesis. The possibility that the observed QS-inhibitory activity of niclosamide was due to unspecific inhibition of either bioluminescence-generating enzymes or bacterial transcription was ruled out by the observation that niclosamide had no effect on the bioluminescence emitted by a *P. aeruginosa* strain in which bioluminescence genes where under the control of the promoter region of the QS-independent *cysB* gene (data not shown), involved in cysteine metabolism and iron uptake (Imperi *et al.,* 2010).

The effect of niclosamide on the production of $3OC_{12}$-HSL was then assessed. *P. aeruginosa* PA14 was grown in the absence or in the presence of 20 μM niclosamide, and $3OC_{12}$-HSL levels in culture supernatants were quantitatively determined during the whole growth curve. Niclosamide caused a significant reduction (30-60%) of $3OC_{12}$-HSL production from the late exponential growth to the entry into the stationary phase, after which $3OC_{12}$-HSL concentration fell to almost undetectable levels in both niclosamide-treated and –untreated cultures (Fig. 10A). When $3OC_{12}$-HSL levels were determined in *P. aeruginosa* PA14 cultures grown in the presence of different niclosamide concentrations (0-50 μM), the maximum inhibitory effect on $3OC_{12}$-HSL production (about 60% reduction) was observed at 5 μM, and higher concentrations did not further reduce $3OC_{12}$-HSL production (Fig. 10B).

Since $3OC_{12}$-HSL influences the expression of the other QS systems, the effect of niclosamide on the production of C_4-HSL and PQS was tested. To this aim, C_4-HSL and PQS levels in *P. aeruginosa* PA14 cultures treated or not with 20 μM niclosamide were determined along the growth curve. While niclosamide did not significantly affect PQS levels (data not shown), it considerably delayed the production of C_4-HSL (Fig. 10C). By comparison with untreated cultures, C_4-HSL levels were significantly lower

in niclosamide-treated cultures during the exponential growth, while levels were comparable between treated and untreated cultures in the stationary phase (Fig. 10C). This could be due, at least in part, to the positive effect exerted by $3OC_{12}$-HSL on C_4-HSL production (Williams and Càmara, 2009). However, the niclosamide-induced delay in C_4-HSL production was also evident in a $3OC_{12}$-HSL-defective mutant strain inactivated in the *lasI* gene, encoding the $3OC_{12}$-HSL synthase (Fig. 10C), indicating that niclosamide also affects C_4-HSL production independently of its inhibitory activity on $3OC_{12}$-HSL production.

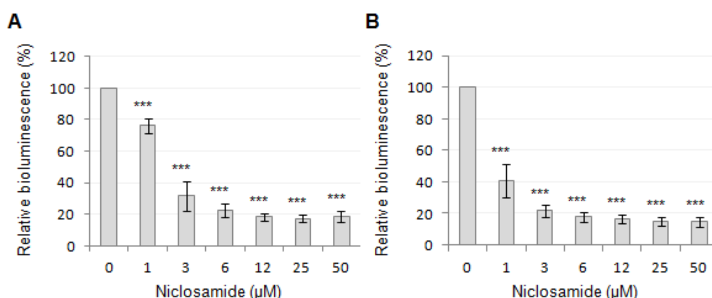

Figure 9. Effect of niclosamide on the $3OC_{12}$-HSL-dependent QS of *P. aeruginosa*. Response to increasing concentrations of niclosamide (0-50 µM) of (A) the PA14/PA14-R3 co-cultivation system and (B) the PA14-R3 biosensor in the presence of 3 µM exogenously-provided $3OC_{12}$-HSL. Bioluminescence emission was normalized to the cell density of the bacterial culture (relative bioluminescence, LCPS/A_{600}), and expressed as percentage relative to untreated controls. Values are the mean (± SD) of at least three independent experiments. *** $p < 0.001$ (ANOVA).

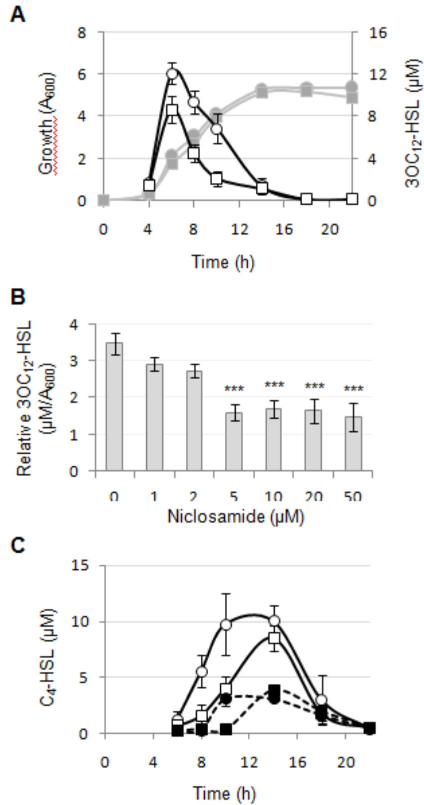

Figure 10. Effect of niclosamide on acyl-HSL production. (A) Growth curve and $3OC_{12}$-HSL production by *P. aeruginosa* PA14 treated (squares) or untreated (circles) with 20 µM niclosamide. Symbols: bacterial growth (left vertical axis), grey lines and filled symbols; $3OC_{12}$-HSL levels in culture supernatants (right vertical axis), black lines and open symbols. (B) Relative $3OC_{12}$-HSL levels (µM/A_{600}) in culture supernatants of *P. aeruginosa* PA14 grown for 8 h in the presence of increasing concentrations of niclosamide (0-50 µM). (C) C_4-HSL levels in culture supernatants of *P. aeruginosa* PA14 (solid lines, white symbols) and PA14 *lasI* mutant (dashed lines, black symbols) grown in the presence (squares) or in the absence (circles) of 20 µM niclosamide. Growth curves of bacterial cultures were comparable to those reported in (A). Values are the mean (± SD) of at least three independent experiments. *** $p < 0.001$ (ANOVA).

3.2.3. Niclosamide represses QS-activated genes.

To investigate the global effect of niclosamide on the *P. aeruginosa* transcriptome, the transcriptional profiles of PA14 grown to A_{600} = 2.5 in the presence or in the absence of 20 µM niclosamide were compared by Affymetrix high-density oligonucleotides microarray analysis. Niclosamide affected the transcription of 258 genes, 73.2% of which were repressed by this drug, including genes involved in the production of important virulence factors, such as phospholipase C, LasA protease, pyocyanin, chitinase, rhamnolipids, and LasB elastase (Tables 2 and S1). Moreover, niclosamide repressed the transcription of genes involved in adhesion and biofilm formation, such as those coding for adhesins (PA0852-*cbpD* and PA2570-*pa1L*) and for cyclic di-GMP turnover or response proteins (PA1120-*tpbB*, PA2572, and PA4781). The transcription of the *mexGHI-opmD* genes, encoding an efflux pump required for full virulence in rat and plant infection models (Aendekerk *et al.,* 2005), was also strongly decreased in the presence of niclosamide (Table 2). Notably, among the 189 genes repressed by niclosamide, 96 have been identified as genes activated by $3OC_{12}$-HSL and/or C_4-HSL in the main reference studies (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003) (Table S1), and additional 25 genes have been suggested to be part of the QS network (Rampioni *et al.,* 2007; Rampioni *et al.,* 2009;Déziel *et al.,* 2005; Bredenbruch *et al.,* 2006; Lequette *et al.,* 2006; Rampioni *et al.,* 2010). Among the core components of the *P. aeruginosa* QS network (*i.e.* signal synthases and signal receptor genes), only the C_4-HSL receptor gene *rhlR* was significantly repressed by niclosamide (Table 2). Similar results have been reported for other QSIs, such as furanone C-30 (Hentzer *et al.,* 2003), iberin (Jakobsen *et al.,* 2012) and ajoene (Jakobsen 2012). In total, 121 out of the 189 genes repressed by niclosamide (64%) can be classified as QS-regulated.

Niclosamide displayed a positive effect on the transcription of 69 genes, including two genes involved in type VI secretion pathway (PA0070-*tagQ1* and PA0085-*hcp1;* Table 2). These genes are the only virulence-related determinants whose transcription is induced by niclosamide. Only four of the niclosamide-activated genes were previously reported to be repressed by $3OC_{12}$-HSL and/or C_4-HSL (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003) (Table S2), suggesting that the majority of the genes induced by niclosamide are affected via QS-independent pathway(s). This observation, together with the finding that 36% of the niclosamide-repressed genes have never been reported as QS-controlled, suggests that this drug may have additional cellular targets besides the QS network.

Notably, a total of 16 putative or confirmed transcriptional regulators were identified among the genes repressed or activated by niclosamide (Tables S1 and S2). Besides *rhlR*, niclosamide decreased the transcription of *pprB*, encoding a transcriptional activator associated with biofilm formation (Giraud *et al.,* 2011). Conversely, it positively affected the transcription of *cifR* (Table 2), encoding the transcriptional repressor of the Cif toxin, responsible for apical membrane down-regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) in epithelial cells (MacEachran *et al.,* 2008). The niclosamide-affected transcriptional factors may act as ancillary regulators, increasing the number of genes whose expression is altered by this drug beyond the QS regulon. However, a complete understanding of the niclosamide impact on *P. aeruginosa* physiology is partially hampered by the high percentage of niclosamide-controlled genes (~ 41%) coding for proteins still classified as hypothetical (Tables S1 and S2).

Table 2. List of selected genes whose transcription is affected by niclosamide.

PA number [a]	Gene name [a]	Fold change [b]	Gene product [a]
Virulence factors			
PA0026	*plcB*	-4.15	phospholipase C, PlcB
PA0070	*tagQ1*	2.73	protein secretion apparatus, type VI secretion system
PA0085	*hcp1*	2.99	Type VI protein secretion system component Hcp
PA1871	*lasA*	-10.05	LasA protease precursor
PA1901	*phzC1/C2*	-2.22	phenazine biosynthesis protein PhzC
PA1905	*phzG2*	-2.19	probable pyridoxamine 5'-phosphate oxidase
PA2300	*chiC*	-7.24	Chitinase
PA3478	*rhlB*	-5.99	rhamnosyltransferase chain B
PA3479	*rhlA*	-5.65	rhamnosyltransferase chain A
PA3724	*lasB*	-2.90	elastase LasB
PA4210	*phzA1/A2*	-3.06	probable phenazine biosynthesis protein
PA4211	*phzB1/B2*	-2.42	probable phenazine biosynthesis protein
Adhesion and biofilm formation			
PA0852	*cbpD*	-3.92	chitin-binding protein CbpD precursor
PA1120	*tpbB*	-2.19	diguanylate cyclise
PA2570	*pa1L*	-4.08	PA-I galactophilic lectin
PA2572	---	-3.17	probable two-component response regulator
PA4781	---	-2.50	cyclic di-GMP phosphodiesterase
Gene regulation			
PA2931	*cifR*	2.10	transcriptional regulator
PA3477	*rhlR*	-2.56	transcriptional regulator RhlR
PA4296	*pprB*	-3.21	two-component response regulator
Drug-efflux			
PA4205	*mexG*	-29.17	hypothetical protein
PA4206	*mexH*	-16.94	probable RND efflux membrane fusion protein precursor
PA4207	*mexI*	-13.87	probable RND efflux transporter
PA4208	*opmD*	-9.12	probable outer membrane protein precursor

[a] PA number, gene name and gene product are from the *Pseudomonas* Genome Database (http://www.pseudomonas.com). Genes previously reported to be activated by the $3OC_{12}$-HSL and/or C_4-HSL QS systems are in bold characters (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003). RND, resistance-nodulation-cell division.[b] Fold change in gene expression of *P. aeruginosa* PA14 grown in LB supplemented with 20 µM niclosamide compared to the same strain grown in LB.

3.2.4. Niclosamide strongly reduces the virulence potential of *P. aeruginosa in vitro.*

In order to validate the transcriptomic data at the phenotypic level, we assessed the effect of niclosamide on the production of a set of QS-regulated virulence traits. In particular, we focused on (i) the LasB elastase, which is directly regulated by the $3OC_{12}$-HSL receptor LasR at the transcriptional level (Anderson *et al.,* 1999), (ii) pyocyanin and rhamnolipids, which are regulated by a number of different regulatory pathways and extracellular signals (Lau *et al.,* 2004; Reis *et al.,* 2011), and (iii) multifactorial phenotypes, such as motility and biofilm, which are crucial for the establishment and persistence of *P. aeruginosa* infections (Parsek and Singh, 2003; Zolfaghar *et al.,* 2003; Andrews *et al.,* 1982).

In accordance with microarray analysis, niclosamide had a marked inhibitory effect on the levels of QS-regulated secreted virulence factors of *P. aeruginosa* PA14 (Fig. 11). Production of both pyocyanin and elastase was dramatically reduced (85-90%) by 5-10 µM niclosamide. Likewise, the amount of rhamnolipids in supernatants of niclosamide-treated cultures was about 25% of the niclosamide-untreated control level (Fig. 11).

Regarding bacterial motility, niclosamide only slightly reduced swimming and twitching motilities of *P. aeruginosa* PA14 at high concentrations (= 50-100 µM), while it exerted a dramatic inhibitory effect on swarming motility (Fig. 12A-C). Swarming was completely prevented at 4 µM niclosamide concentrations, although a significant reduction was also observed at lower concentrations (Fig. 12B), which however had no effect on the $3OC_{12}$-HSL-dependent QS system (Figs. 9 and 10).

Niclosamide was also tested for its effect on *P. aeruginosa* biofilm formation using a standard crystal violet binding assay (Merritt *et al.,* 2005). Niclosamide showed a significant biofilm inhibitory activity, resulting in a 2-fold reduction and 3-fold increase in the number of attached and planktonic cells, respectively (Fig. 12D). However, such inhibitory activity was only observed at = 200 µM niclosamide, *i.e.* at concentrations which are exceedingly higher than those active against the QS response and virulence factor production (Figs. 10-12), suggesting that the effect of niclosamide on biofilm formation is independent of its anti-QS activity.

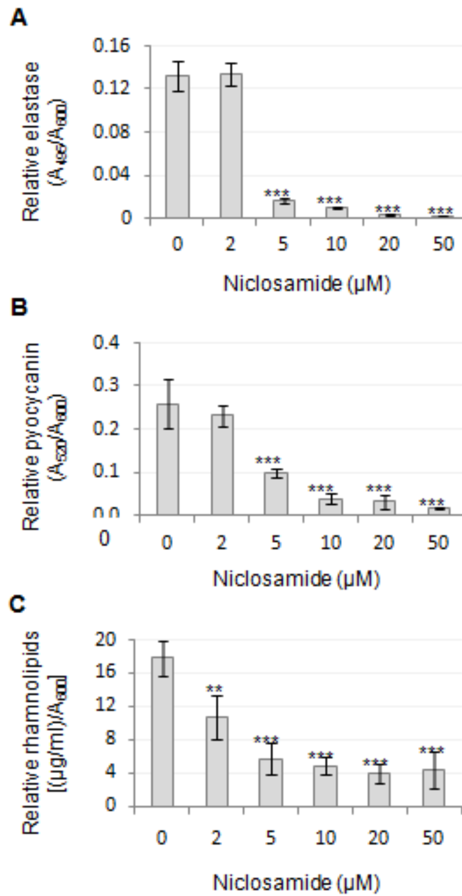

Figure 11. Effect of niclosamide on the production of QS-regulated extracellular virulence factors. (A) LasB elastase, (B) pyocyanin and (C) rhamnolipids levels in culture supernatants of *P. aeruginosa* PA14 grown for 10 h (A and B) and for 24 h (C) in the presence of increasing concentrations of niclosamide (0-50 µM). Values were normalized to the cell density of the bacterial culture (relative values), and are the mean (± SD) of four independent experiments. ** $p < 0.01$; *** $p < 0.001$ (ANOVA).

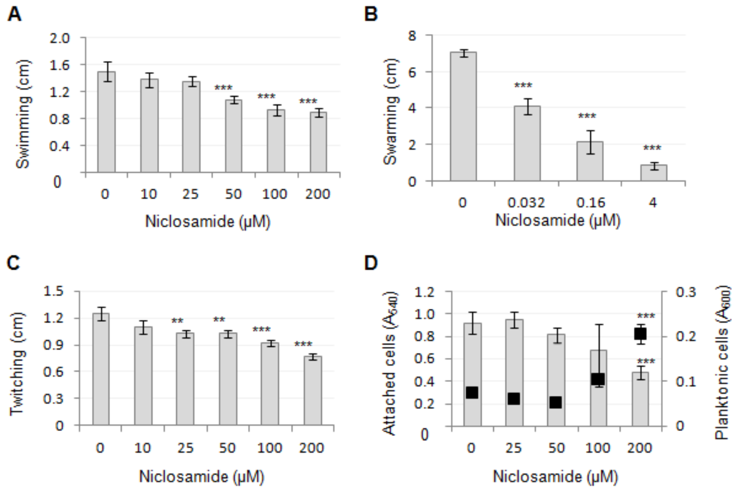

Figure 12. Effect of niclosamide on *P. aeruginosa* motility and biofilm formation. (A) Swimming, (B) swarming and (C) twitching by *P. aeruginosa* PA14 in the presence of increasing concentrations of niclosamide. (D) Biofilm formation by *P. aeruginosa* PA14 in the presence of increasing concentrations of niclosamide, assessed as amount of attached cells (grey histograms, left axis) versus planktonic cells (black squares, right axis). Values are the mean (± SD) of at least three independent experiments. ** $p < 0.01$; *** $p < 0.001$ (ANOVA).

3.2.5. Niclosamide protects *G. mellonella* from *P. aeruginosa* infection.

In order to explore the suitability of niclosamide as an anti-virulence drug against *P. aeruginosa* infections, we assessed the ability of this compound to inhibit the pathogenicity of *P. aeruginosa* in the *G. mellonella* insect model of infection (Jander *et al.,* 2000).

Larvae of the wax moth *G. mellonella* are extremely sensitive to *P. aeruginosa* injected into the hemolymph, and the PA14 strain was found to be highly virulent in this model, with an LD_{50} of about one bacterial cell (Jander *et al.,* 2000). In our study, *G. mellonella* larvae were inoculated with 10 µl of saline solution containing a lethal dose of *P. aeruginosa* PA14 (10 ± 4 cells from exponential cultures) and containing or not 750 µM niclosamide ethanolamine salt, and then incubated at 28°C for up to one week. Niclosamide ethanolamine salt was used because of its higher solubility in aqueous solutions compared with niclosamide (Andrews *et al.,* 1982). Notably, the two niclosamide formulations displayed comparable inhibitory effects on virulence factors production, motility and QS signal molecule production (data not shown). Considering that the average weight of *G. mellonella* larvae was about 500 mg (see Material and Methods), and arbitrarily assuming 500 µl as the hemolymph volume of the larva, the final concentration of niclosamide in each larva was estimated to be approximately 15 µM. We have shown above that such niclosamide concentration inhibits $3OC_{12}$-HSL production and expression of $3OC_{12}$-HSL-dependent virulence factors (Figs. 10 and 11), without affecting bacterial growth (Fig. 10A). While 100% of the larvae untreated with niclosamide died within 60 hours post-infection, niclosamide almost completely protected *G. mellonella* larvae from the lethal challenge with *P. aeruginosa* (Fig. 13), even if incubation was prolonged for a week (data not shown). To monitor the presence of PA14 in niclosamide-treated and -untreated larvae, at 60 h post-infection five larvae per group were homogenated in saline solution and serial dilutions of the resulting homogenates were plated on *Pseudomonas* isolation agar. Dead larvae contained about 7 (± 5) × 10^8 *P. aeruginosa* cells per larva, while no bacterial cells were detectable in niclosamide-treated larvae. Notably, niclosamide-treated larval hemolymph had no effect on *P. aeruginosa* PA14 growth *in vitro* (details in Materials and Methods), further confirming that the observed effect of niclosamide on *P. aeruginosa* pathogenicity is due to virulence inhibition rather than to growth-inhibitory effect. Overall, these findings indicate that niclosamide allowed the innate immune response of *G. mellonella* to efficiently counteract the infection.

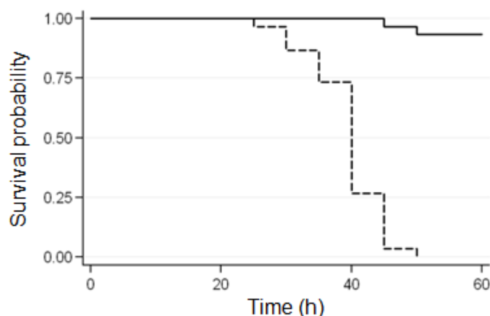

Figure 13. Efficacy of niclosamide in protecting *G. mellonella* larvae from *P. aeruginosa* killing. Kaplan-Meier plot showing the survival of *G. mellonella* larvae inoculated with a lethal dose of *P. aeruginosa* PA14 (10 ± 4 exponentially-growing cells) in 10 µl of saline supplemented or not with 750 µM niclosamide ethanolamine salt, and then incubated at 28°C. All untreated larvae died within 60 h post-infection, while *G. mellonella* killing was almost completely prevented upon treatment with the niclosamide ethanolamine salt. $\chi^2_{(1)} = 61.07$; $p = 0.0000$ (Log-rank).

3.3. Discussion

The need for new anti-infective strategies based on non-antibiotic compounds together with the growing awareness of QS importance in bacterial infections has raised the interest towards the identification of QSIs endowed with anti-virulence properties (reviewed in Galloway *et al.,* 2012).

The relationships among QS, virulence regulation and biofilm formation has most extensively been studied in *P. aeruginosa*. Therefore, it is not surprising that most of research on QS inhibition has been centred on this bacterium as a model system (Galloway *et al.,* 2012).

Research on QS inhibition in Gram-negative bacteria has largely been focused on structural homologues of QS signal molecules, targeting the site of the signal receptor protein that is occupied by the natural ligand (Galloway *et al.,* 2012). Alternatively, promising QSIs belonging to different chemical classes have been discovered, by screening random libraries of synthetic and natural compounds, and some of them proved to be effective in preventing *P. aeruginosa* infection in animal models (Galloway *et al.,* 2012; Hentzer 2003; Rasmussen *et al.,* 2005). Probably due to the high toxicity of the majority of the QSIs identified to date, garlic extract is the only QSI that has been tested in humans. Although the results were not statistically significant, a trend towards improvement of the clinical outcome of *P. aeruginosa*-infected CF patients after oral garlic

extract administration was observed (Smyth *et al.,* 2010). A recent study identified ajoene as the most active QSI compound in garlic extract. However, synthesized ajoene was less active than the crude garlic extract *in vitro*, and was very poorly effective in an *in vivo* murine model of infection (Jakobsen *et al.,* 2012). Thus, despite the huge efforts made to date in the field of anti-QS research, clinical applications remain far away (reviewed in Galloway *et al.,* 2012).

The main aim of this work has been to validate a new strategy for the identification of QSIs shortly deliverable to clinical use, by proving that a lateral anti-QS activity can be identified in drugs already used in humans.

By screening a library of FDA-approved chemicals, we identified some hit compounds disclosing relevant QSI activity at concentrations that did not cause substantial inhibition of *P. aeruginosa* growth. The salicylanilide compound niclosamide, a cestocide already approved for use in humans (Andrews *et al.,* 1982), was characterised in detail for its anti-QS activity. At micromolar concentrations niclosamide strongly inhibited both $3OC_{12}$-HSL and C_4-HSL production, as well as production of several secreted virulence factors, such as pyocyanin, elastase and rhamnolipids (Figs. 10 and 11). As a comparative example, the previously-described QSI ajoene only inhibits C_4-HSL production at very high concentrations (> 300 μM) while has no effect on $3OC_{12}$-HSL, the major QS signal produced by *P. aeruginosa* (Jakobsen *et al.,* 2012).

The large percentage of QS-regulated genes repressed by niclosamide (64%) highlights a high degree of target specificity towards C_4-HSL- and $3OC_{12}$-HSL-dependent regulons and is comparable to or even higher than that disclosed by other QSIs identified so far (Hentzer 2003; Jakobsen *et al.,* 2012; Jakobsen *et al.,* 2012 ;Muh *et al.,* 2006). Overall, niclosamide strongly decreased the transcription of multiple genes involved in *P. aeruginosa* pathogenicity, corroborating its potential as an anti-virulence drug.

Consistent with the strong anti-virulence activity *in vitro*, niclosamide suppressed *P. aeruginosa* pathogenicity in an acute infection model based on *G. mellonella* larvae (Fig. 13). *P. aeruginosa* can also cause chronic infections characterized by a biofilm mode of growth. Several studies using cell-flow chambers for biofilm formation coupled with confocal scanner microscopy observations have shown that a proficient QS system is required for optimal biofilm shaping and development (Kirisits and Parsek, 2006). Accordingly, biofilms treated with QSIs showed specific structural features and decreased resistance to antibiotics (Bjarnsholt *et al.,* 2010). In this work we performed a pilot experiment using a simple biofilm model, showing

that niclosamide is able to reduce cell attachment to a plastic surface, while increasing the number of planktonic cells (Fig. 12D). Although this effect was only observed at high niclosamide concentrations, this preliminary result should encourage further characterization of the effect of niclosamide on biofilm development and resistance to antibiotics using advanced biofilm models.

Concerning future developments of niclosamide as an anti-*P. aeruginosa* drug, there are some issues that need to be addressed. First of all, the effect of niclosamide on a wide panel of clinical *P. aeruginosa* strains isolated from different infection sites should be assessed, including CF cronically-infected lungs. Moreover, even if niclosamide is currently used as an antihelmintic drug to treat intestinal infections and displays overall low toxicity (Andrews *et al.,* 1982), it is poorly soluble in water, shows low intestinal absorption and, once in the bloodstream, it is quickly cleared via the urinary tract or by enzymatic modification in the liver (Andrews *et al.,* 1982). Although these features could represent drawbacks to the systemic administration of niclosamide, they could be advantageous in the local treatment of wound infections, burns, otitis, gastrointestinal infections and other external *P. aeruginosa* infections. It is also worth mentioning that the toxicity of inhaled niclosamide powder is quite low for mammals (Andrews *et al.,* 1982), opening new perspectives for aerosol treatment of *P. aeruginosa* lung infections. Additional studies in different mammalian models of both acute and chronic infections are required to assess the suitability of niclosamide as an anti-*P. aeruginosa* drug, prior to move into clinical trials. However, in accordance with the SOSA approach (Wermuth, 2006), niclosamide could also be used as a promising scaffold for the design of structural analogues endowed with improved activity and pharmacokinetic properties. The hypothesis that some niclosamide-derivatives may retain anti-QS activity is strengthened by our preliminary observation that other compounds belonging to the same structural class of niclosamide, *i.e.* the salicylanilides rafoxanide and oxyclozanide, showed *in vitro* the same anti-QS activity as niclosamide (data not shown).

The development of niclosamide-based QSIs could be pursued either by screening random chemical modifications introduced within the salicylanilides structure or by rational drug design. The latter approach requires detailed information about the anti-QS mechanism of action of niclosamide. Unfortunately, despite niclosamide is in use since the 1960s, its mechanism of action remains elusive. The antihelmintic activity of niclosamide seems to rely on its ability to uncouple mitochondrial oxidative phosphorylation (Weinbach and Garbus 1969). More recently, it has been

found to inhibit proliferation of some tumour cells by hampering different regulatory pathways, without relevant effects on normal non-tumour cells (Jin *et al.,* 2010; Sun and Zhang, 1999). Notably, niclosamide has also been reported to act as an anti-mycobacterial agent (Sun and Zhang, 1999), plausibly disrupting *Mycobacterium* membrane potential and pH homeostasis (de Carvalho *et al.,* 2011). Although the characterization of the molecular targets of niclosamide was not the aim of this study, some of our observations might be the first steps towards the comprehension of the niclosamide mechanism of action in *P. aeruginosa*. First, niclosamide is likely to target the $3OC_{12}$-HSL reception process rather than signal biosynthesis (Fig. 9). Second, the maximum inhibitory effect disclosed by niclosamide on $3OC_{12}$-HSL production (60%) is reached at 5 μM concentration, and increases in concentration did not reduce $3OC_{12}$-HSL production (Fig. 10), ruling out the possibility that niclosamide competes with $3OC_{12}$-HSL for receptor binding. Third, microarray analysis showed that niclosamide also affects the expression of genes not controlled by QS, including some putative or confirmed transcriptional regulators. Fourth, niclosamide represses C_4-HSL production both dependently and independently from its action on $3OC_{12}$-HSL (Fig. 10). Finally, the repressive effect disclosed by niclosamide is higher on swarming motility and on the production of virulence factors than on $3OC_{12}$-HSL production (Figs. 10, 11 and 12). Although this could be due to the combined effect of this QSI on $3OC_{12}$-HSL and C_4-HSL production, it cannot be excluded that niclosamide also influences some QS-independent cellular process involved in virulence gene regulation.

Putting together our observations and available literature data on the cellular processes affected by niclosamide in other organisms, a very preliminary hypothesis about the mechanism of action of niclosamide in *P. aeruginosa* could be that this molecule targets some regulatory pathway(s) responsive to the energetic/metabolic status of the cell and required for full activity of the QS signalling network. Studies on the mechanism of action of niclosamide in *P. aeruginosa* are therefore in progress in our laboratory.

In conclusion, the major outcome of this study is the identification of a strong anti-QS activity in a compound already approved for use in humans. Our findings provide a new promising drug candidate against *P. aeruginosa* and a proof of concept that FDA-approved drugs could be endowed with anti-virulence properties that are worthy to be explored.

4. CHAPTER IV

Beyond antibiotics: efflux pumps inhibition as anti-virulence strategy against *Pseudomonas aeruginosa*.

4.1. Background

As mentioned in the introduction, a major reason why *P. aeruginosa* infections are hard to eradicate is that this bacterium is resistant to a wide spectrum of anti-microbials and toxic compounds (Lister *et al.,* 2009). An important *P. aeruginosa* resistance mechanism is the production of efflux pumps of the Resistance-Nodulation-cell Division (RND) family. Such pumps play an essential role in the so called "adaptive resistance" to antibiotics (Askoura *et al.,* 2011). Since no close human homologues of RND transporters have been described, efflux pumps inhibitors (EPI) are considered promising scaffolds and lead compounds for the development of drugs aimed at potentiating antibiotic activity (Zechini and Versace 2009). However, the notion is emerging that, besides exogenous toxic compounds, RND efflux pumps are also involved in bacterial processes important for virulence (Piddock, 2006).

In *P. aeruginosa* the MexAB-OprM RND efflux pump provides natural resistance to a broad spectrum of antibiotics, including (fluoroquinolones, β-lactams, tetracyclins, chloramphenicol, sulphonamides etc, Lister *et al.,* 2009) and it was shown to be the unique RND-type efflux pump expressed by this bacterium under standard laboratory growth conditions (*i.e.* LB medium; Poole *et al.*, 1996). Besides its important role in multi drug resistance, evidences are also available suggesting that MexAB–OprM exports virulence determinants that allow *P. aeruginosa* to cause infection (Hirakata *et al.,* 2002; Join-Lambert *et al.,* 2001). Moreover, it was also reported that MexAB-OprM actively participates in the efflux of $3OC_{12}$-HSL (Evans *et al.*, 1998; Pearson *et al.,* 1999).

Phenyl-arginine β-naphthylamide hydrochloride (PAβN) is the most active and well-known EPI against *P. aeruginosa* RND efflux pumps, and it is particularly efficacious against MexAB-OprM (Davin-Regli *et al.,* 2006; Lee *et al.,* 2001; Lomovskaya *et al.,* 2001; Lomovskaya and Bostian, 2006). In addition, Molin and co-workers recently showed that, at very high concentration (about 200 μM) PAβN, in association with an iron-chelating agent, inhibited *P. aeruginosa* growth and biofilm formation *in vitro* (Liu *et al.*, 2010). However, to our knowledge there is no information in the literature concerning the possible anti-virulence activity of PAβN or other EPIs.

The main aim of this study has been to provide a first proof of concept that, apart from their role in antibiotic resistance, EPIs may act as anti-virulence drugs against *P. aeruginosa,* by using PAβN as model compound.

4.2. Results

4.2.1. *In vitro* anti-virulence activity of PAβN.

A major requirement for anti-virulence drugs is their ability of inhibiting virulence traits without affecting growth (Cegelski *et al.,* 2008), hence in this study we used concentrations of PAβN not affecting growth, at least under our experimental conditions (*i.e.* ≤ 50 μM; Fig. 14A).

Since PAβN inhibits the MexAB-OprM efflux pump (Lomovskaya *et al.,* 2001) that exports $3OC_{12}$-HSL outside the cell (Evans *et al*., 1998; Pearson *et al*., 1999), we measured $3OC_{12}$-HSL, C_4-HSL and PQS levels in the supernatants collected from cultures of *P. aeruginosa* PAO1 grown in LB supplemented with increasing concentrations of PAβN, by using specific biosensors (Duan and Surette, 2007; Fletcher *et al*., 2007; Massai *et al*., 2011).

Results showed that the production of $3OC_{12}$-HSL increased with concentration of PAβN ≥ 9 μM (Fig. 14B), while PAβN did not affect C_4-HSL and PQS production (data not shown).

Since the $3OC_{12}$-HSL-dependent QS response activates the production of secreted factors important for *P. aeruginosa* pathogenicity, such as pyocyanin, proteases and elastase, we tested the effect of PAβN on these exoproducts. As shown in fig. 15, pyocyanin concentration in supernatants of *P. aeruginosa* cultures increased in the presence of 9 and 27 μM PAβN in comparison with the untreated control (considered as 100% of pyocyanin production). Conversely, PAβN did not affect the production of proteases and elastase (data not shown).

Besides extracellular virulence factors, the three types of motility displayed by *P. aeruginosa* (*i.e.* swimming, twitching and swarming are very important for acute infections (Furukawa *et al*., 2006), and are positively influenced by QS (Shrout *et al.,* 2006). As shown in Figure 16, the addition of 50 μM PAβN caused a eight-fold and two-fold reduction of twitching and swimming motility compared to the untreated control, respectively, while swarming motility was completely abrogated in the presence of 6.25 μM PAβN.

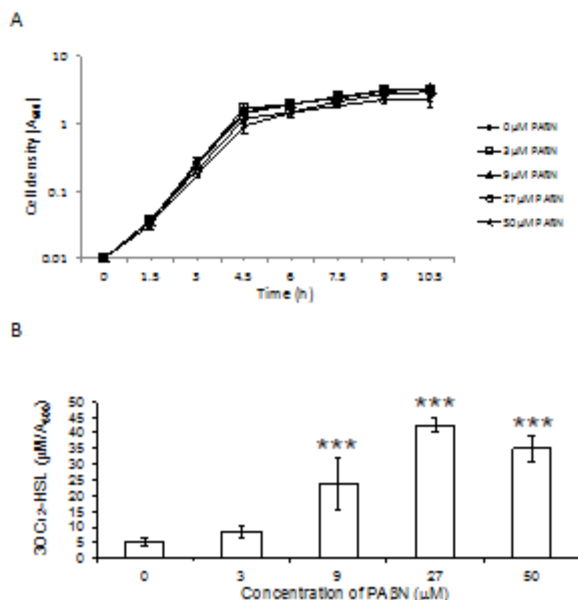

Figure 14. Effect of PAβN on *P. aeruginosa* growth and $3OC_{12}$-HSL levels in culture supernatants. *P. aeruginosa* PAO1 growth curves (A) and $3OC_{12}$-HSL levels in samples withdrawn at 8 h of growth ($A_{600} \approx 0.3$) (B) in LB supplemented with the indicated concentrations of PAβN. Values are the mean (± SD) of at least three independent experiments. * $p < 0.01$ (ANOVA). The 3OC12-HSL quantification was performed by means of the PA14-R3 biosensor as described in Massai *et al.,* 2011.

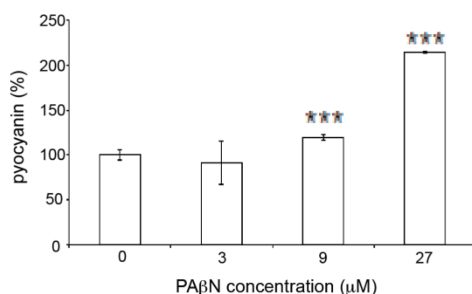

Figure 15. Effect of PAβN on pyocyanin levels in *P. aeruginosa* culture supernatants. Pyocyanin production measured in *P. aeruginosa* PAO1 cultures grown 8 h in LB ($A_{600} \approx 0.3$; see Fig. 14A) in the absence or in the presence of different PAβN concentrations, indicated below the histogram. * $p < 0.01$ (ANOVA).

Overall, the above results indicate that PAβN has incongruous effects on different virulence-related phenotypes. In particular, the PAβN-induced increase in $3OC_{12}$-HSL levels does not correspond to a coordinate change in the levels of important $3OC_{12}$-HSL-dependent phenotypes such as proteases, elastases and motility. Moreover, since PAβN inhibits the MexAB-OprM efflux pump, that exports $3OC_{12}$-HSL outside the cell, it is not clear how this EPI can lead to an increased extracellular concentration of $3OC_{12}$-HSL.

To gain more insights about the effect of PAβN on the overall *P. aeruginosa* physiology, we compared the transcriptional profiles of PAO1 grown in LB to an A_{600} of ≈ 3.0 in the presence or in the absence of 27 μM PAβN by Affymetrix high-density oligonucleotides microarray analysis.

The transcription of 109 genes was affected by PAβN, corresponding to about 2% of *P. aeruginosa* genes (Winsor *et al.*, 2011). Of these, 70 genes were down-regulated and 39 genes were up-regulated in the presence of PAβN. Among the 70 down-regulated genes (Table 3), 47 genes (67.1%) were previously reported to be repressed by iron (Ochsner *et al.*, 2002; Palma *et al.*, 2003) or phosphate (Romanowski *et al.*, 2011), including many genes important for *P. aeruginosa* pathogenesis *in vitro* and *in vivo* (Jansen *et al.,* 2006; Ochsner *et al.,* 2002; Romanowski *et al.*, 2011; Wagner *et al.,* 2004; Zaborina *et al.,* 2008).

Among the 39 genes up-regulated by PAβN (Table 4), the most represented categories (43.5 %) were genes involved in nitrogen and phenazines metabolism. The latter constitute a group of nitrogen-containing heterocyclic compounds, including pyocyanin (Parson *et al.*, 2007). The positive effect of PAβN on the transcription of genes involved in phenazines metabolism is in line with the increased pyocyanin production previously observed in the presence of PAβN (Fig. 15) and validates the microarray analysis.

Although PAβN increases $3OC_{12}$-HSL levels in *P. aeruginosa* supernatants, this compound affected very few genes activated by this signal in our microarray analysis (highlighted in bold character in Tables 3 and 4; Schuster *et al.*, 2003, Wagner *et al.*, 2003; Hentzer *et al.*, 2003). In particular, PAβN had no effect on the transcription of *lasI* and *lasR* genes, involved in the synthesis and response to $3OC_{12}$-HSL, respectively. This result was confirmed by testing the effect of different PAβN concentrations (9, 27 and 50 μM) on *lasI* and *lasR* promoter activity, by means of transcriptional fusions (data not shown).

Furthermore, despite PAβN showed a strong negative effect on *P.*

aeruginosa motility (Fig. 16), we did not detect an alteration of genes in the expression of pili, flagella, or surfactants (*i.e.* rhamnolipids) biosynthetic genes.

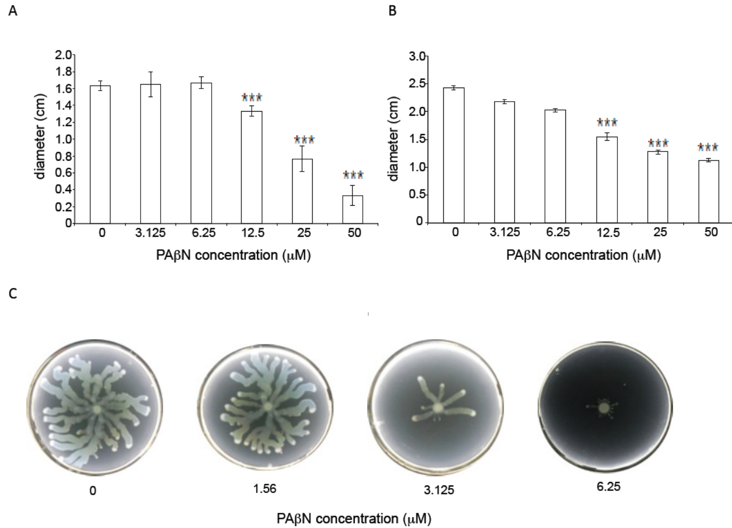

Figure 16. Effect of PAβN on *P. aeruginosa* motility. Histogram representing the average diameter of five *P. aeruginosa* twitching (A) and swimming (B) zones at different PAβN concentration, indicated below the bars. * $p < 0.01$ (ANOVA). (C) Images of *P. aeruginosa* swarming plates supplemented with the indicated concentration of PAβN. One representative experiment is shown.

Table 3. Genes whose transcription is repressed by PAβN.

PA number [a]	Gene [a]	Fold change [b]	Gene product [a]
PA0672	*hemO*	-4.81	heme oxygenase
PA0676	*vreR*	-4.85	sigma factor, Fe(II)-dicitrate sensor
PA0688	---	-3.16	---
PA0697	---	-3.74	---
PA0698	---	-3.03	---
PA0707	*toxR*	-2.12	transcriptional regulator ToxR
PA0842	---	-4.32	---
PA1116	---	-3.13	---
PA1245	***aprX***	**-3.44**	**AprX**
PA1300	---	-4.92	probable σ_{70} factor, ECF subfamily, homologous to FecI
PA1301	---	-3.43	probable transmembrane sensor, homologous to FecR
PA1355	---	-2.99	---
PA1877	---	-2.16	probable secretion protein
PA1912	*femI*	-2.35	ECF sigma factor, FemI
PA2033	---	-4.72	---
PA2034	---	-3.92	---
PA2143	**---**	**-2.20**	**---**
PA2384	---	-4.71	---
PA2385	*pvdQ*	-2.98	PvdQ
PA2386	*pvdA*	-3.97	L-ornithine N^5-oxygenase
PA2393	---	-3.09	probable dipeptidase precursor
PA2394	*pvdN*	-2.85	PvdN
PA2395	*pvdO*	-2.32	PvdO
PA2396	*pvdF*	-3.22	pyoverdine synthetase F
PA2397	*pvdE*	-3.16	pyoverdine biosynthesis protein PvdE
PA2398	*fpvA*	-2.03	ferripyoverdine receptor
PA2399	*pvdD*	-3.32	pyoverdine synthetase D
PA2400	*pvdJ*	-3.36	PvdJ
PA2401	---	-2.92	Not found
PA2402	---	-3.43	probable non-ribosomal peptide synthetase
PA2405	---	-2.42	---
PA2411	---	-4.31	probable thioesterase
PA2412	---	-6.16	---
PA2413	*pvdH*	-3.58	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase
PA2424	*pvdL*	-3.73	PvdL

PA number [a]	Gene [a]	Fold change [b]	Gene product [a]
PA2425	*pvdG*	-2.58	PvdG
PA2426	*pvdS*	-10.48	σ factor PvdS
PA2427	---	-3.62	---
PA2428	---	-18.06	---
PA2570	***PaIL***	**-2.42**	**PA-I galactophilic lectin**
PA3377	*phnJ*	-21.10	Uncharacterized enzyme of phosphonate metabolism
PA3407	*hasAp*	-4.15	heme acquisition protein HasAp
PA3410	---	-2.03	probable σ_{70} factor, ECF subfamily
PA3530	*Bfd*	-2.54	Bacterioferritin-associated ferredoxin
PA3581	*glpF*	-2.06	glycerol uptake facilitator protein
PA3586	---	-2.20	probable hydrolase
PA3899	---	-2.25	probable σ_{70} factor, ECF subfamily
PA4220	*fptB*	-2.48	---
PA4221	*fptA*	-2.52	Fe(III)-pyochelin outer membrane receptor precursor
PA4224	*pchG*	-2.05	pyochelin biosynthetic protein PchG
PA4225	*pchF*	-2.26	pyochelin synthetase
PA4226	*pchE*	-2.06	dihydroaeruginoic acid synthetase
PA4228	*pchD*	-2.09	pyochelin biosynthesis protein PchD
PA4230	*pchB*	-2.69	salicylate biosynthesis protein PchB
PA4467	---	-3.70	---
PA4468	*sodM*	-11.65	superoxide dismutase
PA4469	---	-10.96	---
PA4470	*fumC1*	-8.96	fumarate hydratase
PA4471	*fagA*	-7.96	---
PA4515	*piuC*	-2.48	---
PA4570	---	-5.53	---
PA4703	**---**	**-2.04**	**---**
PA4708	*phuT*	-3.17	Heme-transport protein, PhuT
PA4709	*phuS*	-3.37	probable hemin degrading factor
PA4710	*phuR*	-4.65	Heme/Hemoglobin uptake outer membrane receptor PhuR precursor
PA5360	*phoB*	-15.28	two-component response regulator PhoB
PA5365	*phoU*	-9.40	phosphate uptake regulatory protein PhoU
PA5366	*pstB*	-14.02	ATP-binding component of ABC phosphate transporter
PA5367	*pstA*	-14.17	membrane protein component of ABC phosphate transporter

PA number [a]	Gene [a]	Fold change [b]	Gene product [a]
PA5369	*pstS*	-23.49	phosphate ABC transporter, periplasmic phosphate-binding protein, PstS

[a] PA number, gene name and gene product are from the *Pseudomonas* Genome Database (http://www.pseudomonas.com). Genes previously reported to be activated by the $3OC_{12}$-HSL and/or C_4-HSL QS systems are in bold characters (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). [b] Fold change in gene expression of *P. aeruginosa* PAO1 grown in LB supplemented with 27 µM PAβN compared to the same strain grown in LB. Genes in light-yellow belong to the iron starvation response. Genes in light-green belong to the phosphate-limitation response.

Table 4. Genes whose transcription is activated by PAβN.

PA number [a]	Gene [a]	Fold change [b]	Gene product [a]
PA0509	*nirN*	2.27	probable *c*-type cytochrome
PA0510	*nirE*	2.33	---
PA0511	*nirJ*	2.23	heme d_1 biosynthesis protein NirJ
PA0514	*nirL*	2.30	heme d_1 biosynthesis protein NirL
PA0516	*nirF*	2.30	heme d_1 biosynthesis protein NirF
PA0517	*nirC*	3.53	probable *c*-type cytochrome precursor
PA0518	*nirM*	3.32	cytochrome c_{551} precursor
PA0519	*nirS*	4.48	nitrite reductase precursor
PA0523	*norC*	2.87	nitric-oxide reductase subunit C
PA0525	*norD*	2.19	---
PA0526	---	2.54	---
PA0918	---	2.26	cytochrome b_{561}
PA1168	**---**	**2.94**	**---**
PA1169	---	2.41	probable lipoxygenase
PA1213	---	2.03	---
PA1215	**---**	**2.64**	**---**
PA1217	**---**	**2.38**	**probable 2-isopropylmalate synthase**
PA1221	**---**	**2.01**	**---**
PA1901	***phzC1/C2***	**2.24**	**phenazine biosynthesis protein PhzC**
PA1902	***phzD1/D2***	**2.26**	**phenazine biosynthesis protein PhzD**
PA1903	***phzE1/E2***	**2.22**	**phenazine biosynthesis protein PhzE**
PA1904	***phzF1/F2***	**2.11**	**probable phenazine biosynthesis protein**
PA2953	*qteE*	2.06	quorum threshold expression element, QteE
PA3195	***gapA***	**2.14**	**glyceraldehyde 3-phosphate dehydrogenase**
PA3392	*nosZ*	2.16	nitrous-oxide reductase precursor

PA number [a]	Gene [a]	Fold change [b]	Gene product [a]
PA3602	*yerD*	2.07	---
PA3720	---	2.87	---
PA3880	---	2.63	---
PA4139	**---**	**2.01**	**---**
PA4333	*fumA*	2.01	probable fumarase
PA4430	---	2.28	probable cytochrome *b*
PA4504	*dppC*	2.18	probable permease of ABC transporter
PA4587	*ccpR*	3.20	cytochrome c_{551} peroxidase precursor
PA4810	*fdnI*	2.22	nitrate-inducible formate dehydrogenase, γ subunit
PA5091	*hutG*	2.11	*N*-formylglutamate amidohydrolase
PA5098	*hutH*	3.16	histidine ammonia-lyase
PA5100	*hutU*	2.95	Urocanase
PA5410	*gbcA*	2.14	GbcA, glycine betaine catabolism protein
PA0524	*norB*	5.51	nitric-oxide reductase subunit B

[a] PA number, gene name and gene product are from the *Pseudomonas* Genome Database (http://www.pseudomonas.com). Genes previously reported to be activated by the 3OC$_{12}$-HSL and/or C$_4$-HSL QS systems are in bold characters (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003). [b] Fold change in gene expression of *P. aeruginosa* PAO1 grown in LB supplemented with 27 µM PAβN compared to the same strain grown in LB. Genes in pale-blue are related to the nitrogen metabolism. Genes in rose are involved in phenazines (pyocyanin) production.

4.2.2. *In vivo* anti-virulence activity of PAβN.

The above results show that PAβN (≤ 50 µM) inhibits *P. aeruginosa* processes related to motility and micronutrients acquisition (*i.e.* phosphate and iron), relevant for pathogenesis in several models of acute infection. On the other hand, this compound stimulates the production of pyocyanin, playing a positive role in *P. aeruginosa* virulence.

The various effect of PAβN on *P. aeruginosa* virulence-related traits *in vitro* raises the question of what kind of activity this compound would have on *P. aeruginosa* virulence *in vivo*. To tackle this issue, we tested the effect of PAβN on *P. aeruginosa* virulence in *Galleria mellonella*, a simple model of infection that well correlates with murine acute infection models (Jander *et al*., 2000).

Since the average weight of *G. mellonella* larvae is about 500 mg, and arbitrarily assuming 500 µl as the hemolymph volume of the larva, we calculated that to reach 50 µM final concentration of PAβN, each larva

should be injected with 25 µl of saline containing 1 mM PAβN. As a preliminary control experiment we verified that the survival of not-infected larvae was not affected by injection with 25 µl of saline containing 1 mM PAβN (data not shown). An additional control aimed at ruling out the possibility that PAβN could kill *P. aeruginosa* before the inoculum in *G. mellonella*, confirmed that incubation of *P. aeruginosa* PAO1 cells in 1 mM PAβN in saline for 90 minutes did not significantly affect bacterial cell viability (data not shown; further details in Materials and Methods).

Finally, *G. mellonella* larvae were inoculated with 25 µl of saline containing 8 ± 3 *P. aeruginosa* cells, and containing or not 1 mM PAβN. While 75% of the untreated larvae died within 24 hours post-infection, 70% of the larvae treated with 50 µM PAβN survived the lethal challenge with *P. aeruginosa*, even if incubation was prolonged for three days (Fig. 17).

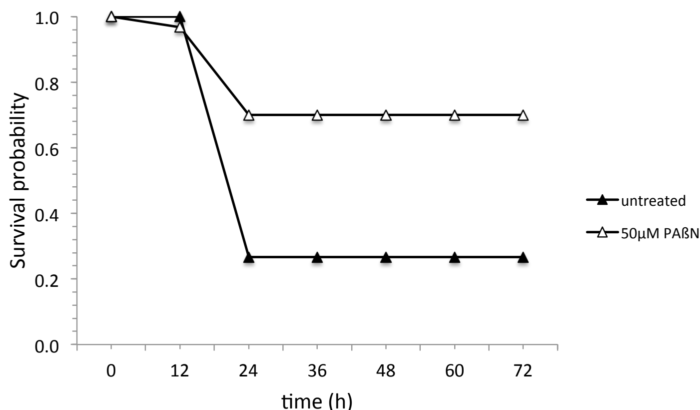

Figure 17. Efficacy of PAβN in protecting *G. mellonella* larvae from *P. aeruginosa* infection. Plot showing the survival of *G. mellonella* larvae inoculated with *P. aeruginosa* PAO1 (8 ± 3 exponentially-growing cells) in 25 µl of saline supplemented (open triangle) or not (solid triangle) with PAβN, and then incubated at 30°C. The final concentration of PAβN in each larva was estimated to be approximately 50 µM. The average of three independent experiments performed on 10 larvae per sample is reported. Differences between treated and untreated samples after 24h are statistically significant $p<0.01$ (ANOVA).

To rule out any growth inhibitory effect of PAβN due to conversion into toxic compound(s) in the larval hemolymph, additional larvae were inoculated with PAβN or saline as a control. 5 μl aliquots of the larval hemolymph recovered 2, 4 or 6 h after treatment were spotted on *Pseudomonas* Isolation Agar plates previously inoculated with *P. aeruginosa* PAO1 cells to produce a lawn of confluent growth. No inhibition halos were detected after 14 h of incubation at 37°C (data not shown).

Overall, the above results indicate that PAβN decreased *P. aeruginosa* pathogenic potential in this virulence model of infection, likely allowing the innate immune response of *G. mellonella* to efficiently counteract the infection.

4.3. Discussion

Efflux pumps inhibition is considered one of the most promising strategies to overcome the problem of bacterial resistance to antibiotic. Indeed, several research groups and biotech companies are developing compounds with this activity and the literature concerning the development of EPIs as antibiotic adjuvants is steadily increasing over years (Pagès and Amaral, 2009; Van Bambeke *et al.,* 2006).

To the best of our knowledge, here we demonstrate for the first time that the chemical inactivation of RND efflux pumps in *P. aeruginosa* can have pleiotropic effects that go far beyond the increased susceptibility to antibiotics. In particular, PAβN 6.5 μM abrogates swarming motility, an *in vitro* phenotype strictly related to virulence *in vivo* (Overhage *et al.,* 2008). Moreover, the microarray analysis showed that the effect of PAβN on *P. aeruginosa* physiology is overall specific, since it affects particular groups of genes, mainly related to iron and phosphate acquisition, and nitrogen metabolism. The microarray results showing that PAβN inhibits the transcription of the master iron-starvation regulator *pvdS* gene were also validated by real-time PCR (Fig. S1). This is relevant, considering that *pvdS*-controlled processes are crucial for the establishment of the *P. aeruginosa* infection (Beare *et al.,* 2003; Vasil and Ochsner, 1999; Xiong *et al.,* 2000).

Overall, with the only exception of pyocyanin, the inhibitory effect exerted by PAβN *in vitro* against virulence-related phenotypes well correlates with the ability of this compound to protect *G. mellonella* larvae from *P. aeruginosa* infection.

Since PAβN inhibits the MexAB-OprM efflux pump (Lomovskaya *et*

al., 2001), that exports $3OC_{12}$-HSL outside the cell Evans *et al.,* 1998; Pearson *et al.,* 1999), we cannot explain how this EPI can lead to increased extracellular concentration of $3OC_{12}$-HSL. However, despite this unsolved issue, our findings could give some hints for future researches concerning PAβN mechanism of action as an anti-virulence compound. In particular, we showed that, despite its positive effect on extracellular $3OC_{12}$-HSL levels, PAβN does not affect the transcription of $3OC_{12}$-HSL-dependent genes and the expression of $3OC_{12}$-HSL-dependent phenotypes (*e.g.* proteases, elastase). This apparent contradiction could be explained by some microarray results. Indeed, PAβN reduced the expression of *pvdQ,* a gene encoding an acylase responsible for the degradation of $3OC_{12}$-HSL (Huang *et al*., 2003). Therefore, the PAβN-mediated increase of $3OC_{12}$-HSL extracellular levels could be due, at least in part, to a decreased degradation of this molecule by PvdQ. Conversely, PAβN enhanced the transcription of *qteE*, a gene coding for a protein that decreases LasR protein stability at low cell density (Siehnel *et al.,* 2010). It is tempting to speculate that, in the cytoplasm, the reduced level of LasR caused by the increased expression of QteE could counterbalance the accumulation of $3OC_{12}$-HSL due to the reduced transcription of *pvdQ*. The effect of PAβN on *pvdQ* and *qteE* transcription has been confirmed by real-time PCR analysis (Fig. S1), however, further studies with *qteE* and *pvdQ P. aeruginosa* mutants will be required to strengthen this hypothesis.

Another observation worth of discussion is that, despite the strong inhibitory effect of PAβN on motility, we did not detect an alteration of genes in the expression of pili, flagella, or surfactants biosynthetic genes. However, motility is a pleiotropic and a high energetically demanding process, strongly affected by metabolic factors (Shrout *et al.,* 2006). In this context, the metabolic alteration caused by PAβN (*e.g.* up-regulation of nitrogen metabolism genes) could account for the effect of this EPI on *P. aeruginosa* motility. Moreover, it is well documented that *pvdQ* is up-regulated in swarming cells, while its deletion abrogates swarming motility in *P. aeruginosa* (Overhage *et al.,* 2008). Thus, the PAβN-mediated reduction of *pvdQ* expression is in line with the strong inhibitory effect of this compound on swarming motility.

Unfortunately, the toxic properties of PAβN (Askoura *et al.,* 2011) hinder further experiments in murine models of infection. However, PAβN-derived compounds are the most studied and in-development family of EPI against *P. aeruginosa,* and the identification of EPI candidates with

improved pharmacological properties with respect to PAβN could be achieved in a near future (Askoura *et al.,* 2011).

In conclusion, this study provides the first proof of concept that efflux pump inhibitors, besides enhancing antibiotic activity, could be endowed with anti-virulence properties that are worthy to be explored.

5. REFERENCES

Adamo R, Sokol S, Soong G, Gomez MI, Prince A. 2004. *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. Am J Respir Cell Mol Biol. **30:**627-634.

Aendekerk S, Diggle SP, Song Z, Høiby N, Cornelis P, Williams P, Cámara M. 2005. The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. Microbiology. **151:**1113-1125.

Allen L, Dockrell DH, Pattery T, Lee DG, Cornelis P, Hellewell PG, Whyte MK. 2005. Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. J Immunol.**15:**3643-3649.

Amara N, Krom BP, Kaufmann GF, Meijler MM. 2011. Macromolecular inhibition of quorum sensing: enzymes, antibodies, and beyond. Chem. Rev. **111:**195-208.

Anderson RM, Zimprich CA, Rust L. 1999. A second operator is involved in *Pseudomonas aeruginosa* elastase (*lasB*) activation. J. Bacteriol. **181:**6264-6270.

Andrews P, Thyssen J, Lorke D. 1982. The biology and toxicology of molluscicides, Bayluscide. Pharmacol. Ther. **19:**245-295.

Antoniani D, Bocci P, Maciag A, Raffaelli N, Landini P. 2010. Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. Appl Microbiol Biotechnol. **85:**1095-104.

Askoura M, Mottawea W, Abujamel T, Taher I. 2011. Efflux pump inhibitors (EPIs) as new antimicrobial agents against *Pseudomonas aeruginosa*. Libyan J Med. **13:**6.

Atkinson S, Williams P. 2009. Quorum sensing and social networking in the microbial world. J. R. Soc. Interface. **6:**959-978.

Babić F, Venturi V, Maravić-Vlahovicek G. 2010. Tobramycin at subinhibitory concentration inhibits the RhlI/R quorum sensing system in a *Pseudomonas aeruginosa* environmental isolate. BMC Infect. Dis. **10:**148.

Baucheron S, Mouline C, Praud K, Chaslus-Dancla E, Cloeckaert A. 2005. TolC but not AcrB is essential for multidrug-resistant *Salmonella enterica* serotype *Typhimurium* colonization of chicks. J Antimicrob Chemother. **55:**707-712.

Beare PA, For RJ, Martin LW, Lamont IL. 2003. Siderophore-mediated cell signalling in Pseudomonas aeruginosa: divergent pathways regulate virulence factor production and siderophore receptor synthesis. Mol Microbiol. **47:**195-207.

Bjarnsholt T, Tolker-Nielsen T, Høiby N, Givskov M. 2010. Interference of *Pseudomonas aeruginosa* signalling and biofilm formation for infection control. Expert Rev. Mol. Med. **12:**e11.

Bjarnsholt T, Tolker-Nielsen T, Høiby N, Givskov M. 2010. Interference of Pseudomonas aeruginosa signalling and biofilm formation for infection control. Expert Rev Mol Med. **7:**12:e11

Bredenbruch F, Geffers R, Nimtz M, Buer J, Häussler S. 2006. The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. Environ. Microbiol. **8:**1318-1329.

Breidenstein EB, de la Fuente-Núñez C, Hancock RE. 2011. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol. **19:**419-426.

Buckley AM, Webber MA, Cooles S, Randall LP, La Ragione RM, Woodward MJ, Piddock LJ. 2006. The AcrAB-TolC efflux system of *Salmonella enterica* serovar *Typhimurium* plays a role in pathogenesis. Cell Microbiol. **8:**847-856.

Buckling A, Harrison F, Vos M, Brockhurst MA, Gardner A, West SA, Griffin A. 2007. Siderophore-mediated cooperation and virulence in *Pseudomonas aeruginosa*. FEMS Microbiol Ecol. **62:**135-141.

Burse A, Weingart H, Ullrich MS. 2004 [b]. The phytoalexin-inducible multidrug efflux pump AcrAB contributes to virulence in the fire blight pathogen, *Erwinia amylovora.* Mol Plant Microbe Interact. **17:**43-54.

Burse A, Weingart H, Ullrich MS. 2004[a]. NorM, an *Erwinia amylovora* multidrug efflux pump involved in in vitro competition with other epiphytic bacteria. Appl Environ Microbiol. **70:**693-703.

Cámara M, Williams P, Hardman A. 2002. Controlling infection by tuning in and turning down the volume of bacterial small-talk. Lancet Infect Dis. **2:**667-676.

Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ. 2008. The biology and future prospects of antivirulence therapies. Nat Rev Microbiol. **6:**17-27.

Collier DN, Anderson L, McKnight SL, Noah TL, Knowles M, Boucher R, Schwab U, Gilligan P, Pesci EC. 2002. A bacterial cell to cell signal in the lungs of cystic fibrosis patients. FEMS Microbiol Lett. **215:**41-46.

Cosson P, Zulianello L, Join-Lambert O, Faurisson F, Gebbie L, Benghezal M, Van Delden C, Curty LK, Köhler T. 2002. *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. J Bacteriol. **184:**3027-3033.

Darch SE, West SA, Winzer K, Diggle SP. 2012. Density-dependent fitness benefits in quorum-sensing bacterial populations. Proc Natl Acad Sci U S A. **109:**8259-8263.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. **280:**295-298.

Davies JC. 2002. *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. Paediatr Respir Rev. **3:**128-134.

Davin-Regli A, Bolla JM, James CE, Lavigne JP, Chevalier J, Garnotel E, Molitor A, Pagès JM. 2008. Membrane permeability and regulation of drug "influx and efflux" in enterobacterial pathogens. Curr Drug Targets. **9:**750-759.

de Carvalho LP, Darby CM, Rhee KY, Nathan C. 2011. Nitazoxanide Disrupts Membrane Potential and Intrabacterial pH Homeostasis of *Mycobacterium tuberculosis*. ACS Med. Chem. Lett. **2:**849-854.

Denning GM, Wollenweber LA, Railsback MA, Cox CD, Stoll LL, Britigan BE. 1998. *Pseudomonas* pyocyanin increases interleukin-8 expression by human airway epithelial cells. Infect Immun. **66:**5777-5784.

Déziel E, Gopalan S, Tampakaki AP, Lépine F, Padfield KE, Saucier M, Xiao G, Rahme LG. 2005. The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI, rhlRI* or the production of *N*-acyl-L-homoserine lactones. Mol. Microbiol. **55:**998-1014.

Diggle SP, Winzer K, Chhabra SR, Worrall KE, Cámara M, Williams P. 2003. The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol Microbiol. **50:**29-43.

DiMango E, Zar HJ, Bryan R, Prince A.1995. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. J Clin Invest.**96:**2204-2210.

Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 1**5:**167-193.

Drenkard E. 2003. Antimicrobial resistance of Pseudomonas aeruginosa biofilms. Microbes Infect. **5:**1213-1219.

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

Duan K, Surette MG. 2007. Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. J. Bacteriol. **189:**4827-4836.

Ejim L, Farha MA, Falconer SB, Wildenhain J, Coombes BK, Tyers M, Brown ED, Wright GD. 2011. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. Nat. Chem. Biol. **7:**348-350.

Erickson DL, Endersby R, Kirkham A, Stuber K, Vollman DD, Rabin HR, Mitchell I, Storey DG. 2002. *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. Infect Immun.**70:**1783-1790.

Essar DW, Eberly L, Hadero A, Crawford IP. 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. J. Bacteriol. **172:**884-900.

Evans K, Passador L, Srikumar R, Tsang E, Nezezon J, Poole K. 1998. Influence of the MexAB-OprM multidrug efflux system on quorum sensing in Pseudomonas aeruginosa. J Bacteriol. **180:**5443-5447.

Falagas ME, Bliziotis IA. 2007. Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? Int J Antimicrob Agents. **29:**630-636.

Fletcher MP, Diggle SP, Crusz SA, Chhabra SR, Cámara M, Williams P. 2007. A dual biosensor for 2-alkyl-4-quinolone quorum-sensing signal molecules. Environ. Microbiol. **9:**2683-2693.

Fuqua C, Parsek MR, Greenberg EP. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu Rev Genet. **35:**439-468.

Furukawa S, Kuchma SL, O'Toole GA. 2006. Keeping their options open: acute versus persistent infections. J Bacteriol. **188:**1211-1217.

Gallagher LA, Manoil C. 2001. *Pseudomonas aeruginosa* PAO1 kills Caenorhabditis elegans by cyanide poisoning. J Bacteriol. **183:**6207-6214.

Galloway WR, Hodgkinson JT, Bowden S, Welch M, Spring DR. 2012. Applications of small molecule activators and inhibitors of quorum sensing in Gram-negative bacteria. Trends Microbiol. **20:**449-458.

Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, van Soolingen D, Jensen P, Bayona J. 2010. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet. **375:**1830-1843.

Gilbert N. 2010. Universities shun Europe's drug initiative. Nature. **466:**306-307.

Giraud C, Bernard CS, Calderon V, Yang L, Filloux A, Molin S, Fichant G, Bordi C, de Bentzmann S. 2011. The PprA-PprB two-component system activates CupE, the first non-archetypal *Pseudomonas aeruginosa* chaperone-usher pathway system assembling fimbriae. Environ. Microbiol. **13:**666-683.

Hancock RE, Speert DP. 2000. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. Drug Resist Updat. **3:**247-255.

Hentzer M, Givskov M. 2003. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin Invest. **112:**1300-1307.

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J. **22:**3803-3815.

Hirakata Y, Srikumar R, Poole K, Gotoh N, Suematsu T, Kohno S, Kamihira S, Hancock RE, Speert DP. 2002. Multidrug efflux systems play an important role in the invasiveness of Pseudomonas aeruginosa. J Exp Med. **196:**109-118.

Huang JJ, Han JI, Zhang LH, Leadbetter JR. 2003. Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and Pseudomonas aeruginosa PAO1. Appl Environ Microbiol. **69:**5941-5949.

Iino R, Nishino K, Noji H, Yamaguchi A, Matsumoto Y. 2012. A microfluidic device for simple and rapid evaluation of multidrug efflux pump inhibitors. Front Microbiol. **3:**40.

Imperi F, Massai F, Ramachandran Pillai C, Longo F, Zennaro E, Rampioni G, Visca P, Leoni L. 2013. New Life for an Old Drug: the Anthelmintic Drug Niclosamide Inhibits Pseudomonas aeruginosa Quorum Sensing. Antimicrob Agents Chemother. **57:**996-1005.

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

Jakobsen TH, Bragason SK, Phipps RK, Christensen LD, van Gennip M, Alhede M, Skindersoe M, Larsen TO, Høiby N, Bjarnsholt T, Givskov M. 2012. Food as a source for quorum sensing inhibitors: iberin from horseradish revealed as a quorum sensing inhibitor of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **78:**2410-2421.

Jakobsen TH, van Gennip M, Phipps RK, Shanmugham MS, Christensen LD, Alhede M, Skindersoe ME, Rasmussen TB, Friedrich K, Uthe F, Jensen PØ, Moser C, Nielsen KF, Eberl L, Larsen TO, Tanner D, Høiby N, Bjarnsholt T, Givskov M. 2012. Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. Antimicrob. Agents Chemother. **56:**2314-2325.

Jander G, Rahme LG, Ausubel FM. 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. **182:**3843-3845.

Jerse AE, Sharma ND, Simms AN, Crow ET, Snyder LA, Shafer WM. 2003. A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. Infect Immun. **71:**5576-5582.

Jin Y, Lu Z, Ding K, Li J, Du X, Chen C, Sun X, Wu Y, Zhou J, Pan J. 2010. Antineoplastic mechanisms of niclosamide in acute myelogenous leukemia stem cells: inactivation of the NF-kappaB pathway and generation of reactive oxygen species. Cancer Res. **70:**2516-2527.

Join-Lambert OF, Michéa-Hamzehpour M, Köhler T, Chau F, Faurisson F, Dautrey S, Vissuzaine C, Carbon C, Pechère J. 2001. Differential selection of multidrug efflux mutants by trovafloxacin and ciprofloxacin in an experimental model of Pseudomonas aeruginosa acute pneumonia in rats. Antimicrob Agents Chemother. **45:**571-576.

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

Kirisits MJ, Parsek MR. 2006. Does *Pseudomonas aeruginosa* use intercellular signalling to build biofilm communities? Cell. Microbiol. **8:**1841-1849.

Lacroix FJ, Cloeckaert A, Grépinet O, Pinault C, Popoff MY, Waxin H, Pardon P. 1996. *Salmonella typhimurium* acrB-like gene: identification and role in resistance to biliary salts and detergents and in murine infection. FEMS Microbiol Lett. **135:**161-167.

Lamarche MG, Déziel E. 2011. MexEF-OprN efflux pump exports the Pseudomonas quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). PLoS One. **6:**e24310.

Latifi A, Winson MK, Foglino M, Bycroft BW, Stewart GS, Lazdunski A, Williams P. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol Microbiol. **17:**333-343.

Lau GW, Hassett DJ, Ran H, Kong F. 2004. The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol. Med. **10:**599-606.

Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D. 2004. *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. Infect Immun. **72:**4275-4278.

Lazdunski AM, Ventre I, Sturgis JN. 2004. Regulatory circuits and communication in Gram-negative bacteria. Nat Rev Microbiol. **2:**581-592.

Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, Miyata S, Diggins LT, He J, Saucier M, Déziel E, Friedman L, Li L, Grills G, Montgomery K, Kucherlapati R, Rahme LG, Ausubel FM. 2006. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol. **7:**R90.

Lee MD, Galazzo JL, Staley AL, Lee JC, Warren MS, Fuernkranz H, Chamberland S, Lomovskaya O, Miller GH. 2001. Microbial fermentation-derived inhibitors of efflux-pump-mediated drug resistance. Farmaco. **56:**81-85.

Lépine F, Milot S, Déziel E, He J, Rahme LG. 2004. Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by *Pseudomonas aeruginosa*. J Am Soc Mass Spectrom. **15:**862-869.

Lequette Y, Lee JH, Ledgham F, Lazdunski A, Greenberg EP. 2006. A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit. J. Bacteriol. **188:**3365-3370.

Lin J, Sahin O, Michel LO, Zhang Q. 2003. Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of Campylobacter jejuni. Infect Immun. **71:**4250-4259.

Lister PD, Wolter DJ, Hanson ND. 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev. **22:**582-610.

Lomovskaya O, Bostian KA. 2006. Practical applications and feasibility of efflux pump inhibitors in the clinic--a vision for applied use. Biochem Pharmacol. **71:**910-918.

Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshino K, Ishida H, Lee VJ. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob Agents Chemother. **45:**105-16.

MacEachran DP, Stanton BA, O'Toole GA. 2008. Cif is negatively regulated by the TetR family repressor CifR. Infect. Immun. **76:**3197-3206.

Maeda T, García-Contreras R, Pu M, Sheng L, Garcia LR, Tomás M, Wood TK. 2012. Quorum quenching quandary: resistance to antivirulence compounds. ISME J. **6:**493-501.

Martinez JL, Sánchez MB, Martínez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C. 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev. **33:**430-449.

Massai F, Imperi F, Quattrucci S, Zennaro E, Visca P, Leoni L. 2011. A multitask biosensor for micro-volumetric detection of *N*-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal. Biosens. Bioelectron. **26:**3444-3449.

McGowan JE Jr. 2006. Resistance in nonfermenting gram-negative bacteria: multidrug resistance to the maximum. Am J Infect Control. **34:**29-37.

Mellbye B, Schuster M. 2011. The sociomicrobiology of antivirulence drug resistance: a proof of concept. MBio. **2:**131-111.

Middleton B, Rodgers HC, Cámara M, Knox AJ, Williams P, Hardman A. 2002. Direct detection of N-acylhomoserine lactones in cystic fibrosis sputum. FEMS Microbiol Lett. **207:**1-7.

Minagawa S, Inami H, Kato T, Sawada S, Yasuki T, Miyairi S, Horikawa M, Okuda J, Gotoh N. 2012. RND type efflux pump system MexAB-OprM of *Pseudomonas aeruginosa* selects bacterial languages, 3-oxo-acyl-homoserine lactones, for cell-to-cell communication. BMC Microbiol. **12:**70.

Müh U, Hare BJ, Duerkop BA, Schuster M, Hanzelka BL, Heim R, Olson ER, Greenberg EP. 2006. A structurally unrelated mimic of a *Pseudomonas aeruginosa* acyl-homoserine lactone quorum-sensing signal. Proc. Natl. Acad. Sci. USA. **103:**16948-16952.

Nikaido H. 1996. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol. **178**:5853-5859.

Nikaido H. 2009. Multidrug resistance in bacteria. Annu Rev Biochem. **78:**119-146.

Njoroge J, Sperandio V. 2009. Jamming bacterial communication: new approaches for the treatment of infectious diseases. EMBO Mol. Med. **1:**201-210.

Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML. 2002. GeneChip expression analysis of the iron starvation response in Pseudomonas aeruginosa: identification of novel pyoverdine biosynthesis genes. Mol Microbiol. **45:**1277-1287.

Ohman DE, Burns RP, Iglewski BH. 1980. Corneal infections in mice with toxin A and elastase mutants of *Pseudomonas aeruginosa*. J. Infect. Dis. **142:**547–555.

O'Malley YQ, Reszka KJ, Spitz DR, Denning GM, Britigan BE. 2004. *Pseudomonas aeruginosa* pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. Am J Physiol Lung Cell Mol Physiol. **287:**L94-103.

Orsi GB, Raponi M, Franchi C, Rocco M, Mancini C, Venditti M. 2005. Surveillance and infection control in an intensive care unit. Infect Control Hosp Epidemiol **26:** 321-325.

Overhage J, Bains M, Brazas MD, Hancock RE. 2008. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J Bacteriol. **190:**2671-2679.

Page MG, Heim J. 2009. Prospects for the next anti-*Pseudomonas* drug. Curr. Opin. Pharmacol. **9:**558-565.

Pagès JM, Amaral L. 2009. Mechanisms of drug efflux and strategies to combat them:challenging the efflux pump of Gram-negative bacteria. Biochim Biophys Acta.**1794:**826-833.

Palma M, Worgall S, Quadri LE. 2003. Transcriptome analysis of the Pseudomonas aeruginosa response to iron. Arch Microbiol. **180:**374-379.

Parsek MR, Singh PK. 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu. Rev. Microbiol. **57:**677-701.

Parsons JF, Greenhagen BT, Shi K, Calabrese K, Robinson H, Ladner JE. 2007. Structural and functional analysis of the pyocyanin biosynthetic protein PhzM from Pseudomonas aeruginosa. Biochemistry. **46(7):**1821-1828.

Pearson JP, Van Delden C, Iglewski BH. 1999. Active efflux and diffusion are involved in transport of Pseudomonas aeruginosa cell-to-cell signals. J Bacteriol. **181:**1203-1210.

Piddock LJ. 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol. **4:**629-636.

Poole K. 2007. Efflux pumps as antimicrobial resistance mechanisms. Ann Med. **39:**162-176.

Poole RK, Anjum MF, Membrillo-Hernández J, Kim SO, Hughes MN, Stewart V. 1996. Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12. J Bacteriol. **178:**5487-5492.

Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science. **268:**1899-1902.

Rampioni G, Pustelny C, Fletcher MP, Wright VJ, Bruce M, Rumbaugh KP, Heeb S, Cámara M, Williams P. 2010. Transcriptomic analysis reveals a global alkyl-quinolone-independent regulatory role for PqsE in facilitating the environmental adaptation of *Pseudomonas aeruginosa* to plant and animal hosts. Environ. Microbiol. **12:**1659-1673.

Rampioni G, Schuster M, Greenberg EP, Bertani I, Grasso M, Venturi V, Zennaro E, Leoni L. 2007. RsaL provides quorum sensing homeostasis and functions as a global regulator of gene expression in *Pseudomonas aeruginosa*. Mol. Microbiol. **66:**1557-1565.

Rampioni G, Schuster M, Greenberg EP, Zennaro E, Leoni L. 2009. Contribution of the RsaL global regulator to *Pseudomonas aeruginosa* virulence and biofilm formation. FEMS Microbiol. Lett. **301:**210-217.

Rasko DA, Sperandio V. 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nat. Rev. Drug. Discov. **9**:117-128.

Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Köte M, Nielsen J, Eberl L, Givskov M. 2005. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J. Bacteriol. **187:**1799-1814.

Romanowski K, Zaborin A, Fernandez H, Poroyko V, Valuckaite V, Gerdes S, Liu DC, Zaborina OY, Alverdy JC. 2011. Prevention of siderophore- mediated gut-derived sepsis due to P. aeruginosa can be achieved without iron provision by maintaining local phosphate abundance: role of pH. BMC Microbiol. **11:**212.

Rosenthal VD, Bijie H, Maki DG, Mehta Y, Apisarnthanarak A, Medeiros EA, Leblebicioglu H, Fisher D, Álvarez-Moreno C, Khader IA, Del Rocío González Martínez M, Cuellar LE, Navoa-Ng JA, Abouqal R, Guanche Garcell H, Mitrev Z, Pirez García MC, Hamdi A, Dueñas L, Cancel E, Gurskis V, Rasslan O, Ahmed A, Kanj SS, Ugalde OC, Mapp T, Raka L, Yuet Meng C, Thu le TA, Ghazal S, Gikas A, Narváez LP, Mejía N, Hadjieva N, Gamar Elanbya MO, Guzmán Siritt ME, Jayatilleke K; INICC members. 2012. International Nosocomial Infection Control Consortium (INICC) report, data summary of 36 countries, for 2004-2009. Am. J. Infect. Control. **40:**396-407.

Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN. 1999. Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections.Infect Immun. **67:**5854-5862.

Rumbaugh KP. 2007. Convergence of hormones and autoinducers at the host/pathogen interface. Anal Bioanal Chem. **387:**425-435.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: A laboratory manual. 2nd edition. New York: Cold Spring Harbor Laboratory Press.

Schuster M, Lohstroh CP, Ogi T, Greemberg EP. 2003. Identification, timing and signal specifity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066-2079.

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

Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol Microbiol. **62:**1264-1277.

Smith RS, Iglewski BH. 2003. *P. aeruginosa* quorum-sensing systems and virulence.Curr Opin Microbiol. **6:**56-60.

Smith RS, Kelly R, Iglewski BH, Phipps RP. 2002. The *Pseudomonas* autoinducer N-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts: implications for inflammation. J Immunol. **169:**2636-2642.

Smyth AR, Cifelli PM, Ortori CA, Righetti K, Lewis S, Erskine P, Holland ED, Givskov M, Williams P, Cámara M, Barrett DA, Knox A. 2010. Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis - a pilot randomized controlled trial. Pediatr. Pulmonol. **45:**356-362.

Stone BJ, Miller VL. 1995. *Salmonella enteritidis* has a homologue of tolC that is required for virulence in BALB/c mice. Mol Microbiol. **17:**701-712.

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

Sun Z, Zhang Y. 1999. Antituberculosis activity of certain antifungal and antihelmintic drugs. Tuber. Lung. Dis. **79:**319-320.

Talbot GH, Bradley J, Edwards JE Jr, Gilbert D, Scheld M, Bartlett JG; Antimicrobial Availability Task Force of the Infectious Diseases Society of America. 2006. Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Bad bugs need drugs: an update on the

development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin. Infect. Dis. **42:**657-668.

Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM. 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proc Natl Acad Sci U S A. **96:**2408-2413.

Tang HB, DiMango E, Bryan R, Gambello M, Iglewski BH, Goldberg JB, Prince A. 1996. Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. Infect Immun. **64:**37-43.

Van Delden C, Iglewski BH. 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis. **4:**551-560.

Vasil ML, Ochsner UA. 1999. The response of Pseudomonas aeruginosa to iron: genetics, biochemistry and virulence. Mol Microbiol. 1999 **34:**399-413.

Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewsky BH. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. **185:**2080-2095.

Wagner VE, Gillis RJ, Iglewski BH. 2004. Transcriptome analysis of quorum-sensing regulation and virulence factor expression in Pseudomonas aeruginosa. Vaccine. **6:**15-20.

Wang L, Dong Y, Zhang L. 2008. Quorum quenching: impact and mechanisms. In Chemical communication among bacteria. S.C. Winans and B.L. Bassler (Eds). **24:**379-392.

Warren JJ, Winkler JR, Gray HB. 2012. Redox properties of tyrosine and related molecules. FEBS Lett. **586:**596-602.

Weinbach EC, Garbus J. 1969. Mechanism of action of reagents that uncouple oxidative phosphorylation. Nature. **221:**1016-1018.

Wermuth CG. 2006. Selective optimization of side activities: the SOSA approach. Drug Discov. Today. **11**:160-164.

Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP. 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev. **25:**365-404.

Williams P, Cámara M. 2009. Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol.**12:**182-191.

Winstanley C, Fothergill JL. 2009. The role of quorum sensing in chronic cystic fibrosis *Pseudomonas aeruginosa* infections. FEMS Microbiol. Lett. **290:**1-9.

Wu H, Song Z, Hentzer M, Andersen JB, Heydorn A, Mathee K, Moser C, Eberl L, Molin S, Høiby N, Givskov M. 2000. Detection of N-

acylhomoserine lactones in lung tissues of mice infected with Pseudomonas aeruginosa. Microbiology. **146:**2481-2493.

Xiong YQ, Vasil ML, Johnson Z, Ochsner UA, Bayer AS. 2000. The oxygen- and iron-dependent sigma factor pvdS of *Pseudomonas aeruginosa* is an important virulence factor in experimental infective endocarditis. J Infect Dis. **181:**1020-1026.

Zaborina O, Holbrook C, Chen Y, Long J, Zaborin A, Morozova I, Fernandez H, Wang Y, Turner JR, Alverdy JC. 2008. Structure-function aspects of PstS in multi-drug-resistant Pseudomonas aeruginosa. PLoS Pathog. **4:**43.

Zechini B, Versace. 2009. I. Inhibitors of multidrug resistant efflux systems in bacteria. Recent Pat Antiinfect Drug Discov. **4:**37-50.

Zhang L, Mah TF. 2008. Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. J Bacteriol. **190:**4447-4452.

Zolfaghar I, Evans DJ, Fleiszig SM. 2003. Twitching motility contributes to the role of pili in corneal infection caused by *Pseudomonas aeruginosa*. Infect. Immun. **71:**5389-5393.

6. LIST OF ABBREVIATIONS

$3OC_{12}$-HSL	3-oxo-docecanoyl homoserine lactone
ABC	ATP-binding cassette
acyl-HSL	acyl homoserine lactones
AIDS	acquired immunodeficiency syndrome
ATP	adenosine triphosphate
C_4-HSL	butanoyl homoserine lactone
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
EPI	efflux pump inhibitor
LPS	lipopolysaccharide
MATE	multidrug and toxic compound extrusion family
MDR	multidrug-resistance
MFP	membrane fusion protein
MFS	major facilitator superfamily
OMF	outer membrane factor
PAβN	phenyl-arginine β-naphthylamide hydrochloride
PQS	2-heptyl-3-hydroxy-4-quinolone
QS	quorum sensing
RND	resistance-nodulation-division family
SMR	small multidrug resistance family
SOSA	Selective Optimization of Side Activities

7. ACKNOWLEDGEMENTS

It is with immense gratitude that I acknowledge the support and help of all my teachers who helped me till this date, with an emphasis to Professor Elisaberra Zennaro, Professor Livia Leoni and Dr. Giordano Rampioni for their immense help without them this thesis would not have been possible. I consider it an honour to work with them during the past three years.

I am indebted to my many colleagues who supported me during the period of my research in the laboratory especially Dr. Francesca Longo and Dr. Marco Messina. I wish all the very best to all my colleagues who have worked with me during my PhD research.

Thanks to all the researchers who contributed to produce the data presented in this thesis, especially Prof. Paolo Visca, Dr. Francesco Massai and Dr.Francesco Imperi (University La Sapienza, Rome, Italy) for their contribution to the work concerning niclosamide.

Many thanks to all the researchers of the lab 2.1, 2.4, 2.5 and 3.1 for their kind help and support.

Thanks to all my friends (Ravi, Sandip, Deepu ...) for their help, care and support

Last but not least I would like thank my family especially my father for their wonderful support, care and love.