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Characterization of novel small RNA based regulatory networks in the opportunistic pathogen *Pseudomonas aeruginosa*

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Contents

Abstract

I piccoli RNA sono corte sequenze di RNA non codificante che regolano l'espressione di numerosi elementi coinvolti nella virulenza di batteri patogeni, in particolar modo quelli coinvolti nell'interazione con l'ospite. In *Pseudomonas aeruginosa*, patogeno opportunista che causa infezioni potenzialmente mortali in soggetti immunodepressi ed in pazienti affetti da fibrosi cistica, i piccoli RNA rivestono un ruolo di grande interesse nello scenario dei regolatori post-trascrizionali, modulando importanti meccanismi regolativi alla base della patogenesi di questo microrganismo.

Il progetto di ricerca discusso in questa tesi di dottorato ha come obiettivo la caratterizzazione di due piccoli RNA di *P. aeruginosa*, denominati ErsA e PesA, conservati sia in ceppi clinici che ambientali.

Come descritto in Ferrara et al. 2015, l'espressione di ErsA è sotto il controllo del fattore sigma "alternativo" σ^{22} (AlgT/U), implicato nella patogenesi in risposta a diversi stress ambientali, e l'espressione di ErsA viene modulata da segnali connessi con la transizione ambiente esterno-ospite, cambiamenti della temperatura oppure modulazione dei livelli di ossigeno, Nel ceppo standard di laboratorio PAO1, ErsA regola negativamente a livello post-trascrizionale l'RNA messaggero (mRNA) di AlgC codificante per un enzima cruciale nella sintesi degli zuccheri utilizzati nella produzione di esopolisaccaridi, componente principale della matrice del biofilm batterico. Partendo da questo scenario, abbiamo usato diverse strategie sperimentali per delineare in modo più dettagliato il contributo di ErsA nella formazione di biofilm come analisi *in silico*, *in vivo* ed *in vitro*, corroborate da studi fenotipici e genetici (RNA-sequencing) eseguiti su un ceppo di PAO1 deleto del gene codificante per ErsA (PAO1 Δ*ersA*).

I risultati ottenuti dall'analisi fenotipica, suggeriscono un'influenza positiva di ErsA sul corretto sviluppo del biofilm di *P. aeruginosa*, presumibilmente modulando a livello post-trascrizionale l'espressione di AmrZ, identificato come nuovo target diretto di ErsA. AmrZ è un regolatore trascrizionale che controlla l'espressione di numerosi geni coinvolti nella produzione di strutture necessarie allo sviluppo del biofilm batterico, alcuni dei quali risultano essere deregolati in assenza di ErsA come emerso dall'analisi del profilo trascrizionale del ceppo PAO1 Δ*ersA*.

In parallelo allo studio su ErsA, abbiamo caratterizzato anche un secondo piccolo RNA, PesA, codificato dall'isola di patogenicità PAPI-1 del ceppo PA14. I nostri risultati mostrano che la delezione di *pesA* in PA14 (PA14 Δ*pesA*) determina una minore virulenza del ceppo rispetto al suo corrispondente wild-type, come evidenziato dall'alta percentuale di sopravvivenza delle cellule

epiteliali bronchiali derivate da pazienti affetti da fibrosi cistica in seguito all'infezione con il ceppo PA14Δ*pesA*. Inoltre, abbiamo evidenziato una regolazione positiva che PesA esercita sull'operone *pyoS3A-I* deputato alla produzione di piocine S3, batteriocine importanti per la virulenza e la colonizzazione di nuove nicchie ecologiche. Infine, la mancanza di PesA contribuisce alla maggiore sensibilità all'antibiotico fluorochinolone ciprofloxacina e al trattamento con raggi UV suggerendo un potenziale coinvolgimento delle piocine S3 nella riparazione dei danni al DNA.

Abstract

In bacterial pathogens, small RNAs (sRNAs) are involved in the coordinate expression of the virulence factors underlying the interaction with host. Several sRNAs have been described in the human pathogen *Pseudomonas aeruginosa*, as integral part of the complex and intricate regulatory networks that represent the bedrock of its pathogenicity. This PhD project aimed to expand the knowledge about the recently described sRNA ErsA and to functional characterize the novel sRNA PesA which is transcribed within the pathogenicity island PAPI-1 of *P. aeruginosa* PA14 strain. ErsA is expressed in all the *P. aeruginosa* sequenced genomes, and in PAO1 it is responsive to infection-relevant host *stimuli* such as oxygen availability and temperature shift and its transcription is under the control of the alternative sigma factor σ^{22} (AlgT/U), a mediator of the stress response implicated in the bacterium pathogenicity. In addition, ErsA has been suggested to directly exert a negative post-transcriptional regulation on the virulence-associated *algC* gene encoding for the bifunctional enzyme AlgC implicated in alginate and exopolysaccharides biosynthesis for biofilm formation. Here, we investigated the multiple aspects of ErsA regulation in AlgC-related pathways, in particular biofilm formation, using a strategy based on *in silico*, *in vivo* and *in vitro* analyses, RNA-seq and phenotypic assays. Our combinatorial approach shows an interesting positive contribution of ErsA in biofilm development, likely by regulating at post-transcriptional level the AmrZ transcriptional regulator and resulting in altered transcriptional levels of genes belonging to the AmrZ regulon and crucial in biofilm formation as *algD*, and *pel* genes.

In this study we characterized also a novel sRNA of *P. aeruginosa* PA14 named PesA. We show that PesA, which is transcribed within the pathogenicity island PAPI-1 of *P. aeruginosa* strain PA14, contributes to *P. aeruginosa* PA14 virulence and pathogenesis. Specifically, *pesA* gene deletion results in a less pathogenic strain, showing higher survival of cystic fibrosis human bronchial epithelial cells after infection compared to PA14 wildtype. Furthermore, Δ*pesA* is more sensitive to fluoroquinolone antibiotic ciprofloxacin and UV irradiation, this last is comparable to that of a strain deleted for *pyoS3A-I*. The *pyoS3A-I* locus encodes for pyocin S3 and we show that PesA influences the production of these bacteriocins positively regulating at post-transcriptional level the *pyoS3A-I* operon. On the whole, our results suggest a positive contribution of PesA in *P. aeruginosa* virulence and niche establishment. Furthermore, we suggest a potential involvement of pyocin S3 in DNA damage repair, introducing a link between DNA damage and repair modulated by PesA that has to be elucidated.

PART I

State of the art

1. *Pseudomonas aeruginosa*

In the genus of *Pseudomonas*, characterized by roughly 200 species widespread in the environment as soil, water, plants and animals, *Pseudomonas aeruginosa* is the type species of the genus, most frequently associated to human infection (Coggan and Wolfgang, 2012).

P. aeruginosa is a Gram-negative, motile and aerobic microorganism, characterized by high metabolic versatility and a remarkable tolerance to a wide variety of physico-chemical conditions, including temperature, high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. Indeed, the emergence of *P. aeruginosa* as a human pathogen in the past century is considered associated to a massive use of antibiotics and disinfectants to eradicate "old" pathogens which caused the availability of new niches (Bjarnsholt and Givskov, 2007).

P. aeruginosa is classified as a human opportunistic pathogen, since not normally part of the microbiota, which can establishes acute and chronic infections in hospitalized patients and immuno-compromised individuals, settling in a multitude of sites and tissues, including: lungs (Fig.1), burns, wounds, eyes, ears, and abiotic surfaces (i.e. catheters and ventilators) (Driscoll et al., 2007).

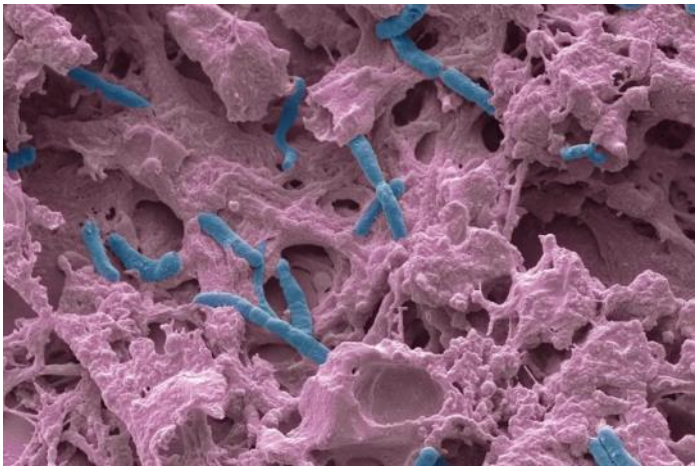

Figure 1 Coloured scanning electron micrograph (SEM) *Pseudomonas aeruginosa* bacteria (blue) in the lung of a burns patient. Photo credit: Steve Gschmeissner/Science photo library, www.sciencephoto.com.

P. *aeruginosa* has been recognized as one of the most encountered bacteria and the predominant cause of mortality in cystic fibrosis (CF) patients (Folkesson et al., 2012). Cystic fibrosis is a genetic disorder caused by a defect in the cystic fibrosis conductance regulator (CFTR) that mainly affects the physiological activity of secretory districts as lungs, digestive and reproductive systems (Kreda et al., 2012). According to the Cystic Fibrosis Foundation Patient Registry, more than 70.000 people are affected by cystic fibrosis worldwide, approximately 1.000 new cases of CF are diagnosed each year and more than 75 percent of people with CF are diagnosed by age 2.

Alteration in CFTR channel in the lungs results in sticky and thick sputum which interfere with the epithelial clearance and innate immune system protection. As a consequence, inflammatory conditions and microbial infections provoke the lung function collapse which is associated to the high mortality rate of CF patients (Folkesson et al., 2012; Ratjen, 2009). The presence and persistence of *P*. *aeruginosa* in CF airways reflects its versatile metabolic capacity and broad potential for adaptation to fluctuating environmental conditions. Indeed, the optimum temperature for *P*. *aeruginosa* growth is 37° C; however, it is able to tolerate temperatures as high as 50°C. Furthermore, the environment in CF lungs is considered microaerophilic or anaerobic and *P*. *aeruginosa* is capable of growing under both aerobic as well as anaerobic conditions using preferentially nitrate as a terminal electron acceptor (Hassett et al., 2002, 2009; Schobert and Jahn, 2010).

All these extraordinary features are provided by the large genome (from 6.22 to 6.91 Mb) and a notable number of regulatory genes (10% of the genome) to control the *P*. *aeruginosa* virulence (Olson et al., 2000). Almost the totality of the genome is characterized by highly conserved sequences. The small percentage of diversity (0.5-0.7%) is similar in the core genome, except for some genes involved in virulence and immunogenicity, as pyoverdine synthesis and uptake, flagella and O-antigen fraction in LPS (lipopolysaccharide) composition, which are under diversifying selection in patients (Klockgether et al., 2011; Smith et al., 2006)

P. *aeruginosa* strains display a huge variability in virulence, ranging from very moderate to highly virulent strains. PAO1 is a moderate virulent strain, isolated the first time from a wound over 50 years ago. In contrast, PA14 is a highly virulent isolate and in laboratory conditions is used as a model for clinical isolates (He et al., 2004; Wiehlmann et al., 2007). Although PA14 and PAO1 present a high degree of genome identity, the PA14 genome is slightly bigger than PAO1 genome and contains two pathogenicity islands that are absent in PAO1, that carry several genes implicated in virulence, as exotoxin gene *exoU* and the Rcs/Pvr two-component systems that control the *cupD*

fimbrial cluster (Mikkelsen et al., 2009; Nicastro et al., 2009). Furthermore the pathogenicity island is involved also in the pathogenicity of *P.aeruginosa* in murine models, although is not possible to predict the precise number of genes involved in this process (Harrison et al., 2010a).

2. *P. aeruginosa* virulence and pathogenesis

The pathogenesis of *P. aeruginosa* is a multifactorial process and characterized by a number of virulence properties that lead to *P. aeruginosa* to colonize, resist and persist in the host. According to Pollack (Pollack, 2000) there are three stages of infection: i) bacterial attachment and colonization, ii) local infection, and iii) bloodstream dissemination and systemic disease. In the first step, flagella, type IV pili and LPS lead the attachment of bacteria to host-cells by binding epithelial gangliosides, asialoGM1 and asialoGM2. All these features are immunogenic and cause the response of the innate immune system and early inflammation state. Then, the invasion of the bacteria is modulated by the type III secretion system (T3SS), which is involved in the injection of cytotoxins into the host cell, and by several proteases which disrupt the epithelial junctions.

The attachment and invasion are also supported by swarming, swimming and twitching bacterial movements that are important to form *P. aeruginosa* microcolonies which develop in mature biofilm. The exopolysaccharide matrix composing biofilm protects the bacterium from the host immune system components and antibiotics treatment, leading the chronic infection in the lung. Furthermore, alginate that represents the main component of *P. aeruginosa* biofilm, provides an additional or alternative adhesin which attaches to the tracheobronchial mucin (N-acetylglucosamine) conferring the mucoid phenotype typical of *P. aeruginosa* clinical strains isolated from the lung of CF patients during chronic infection (Fig. 2)

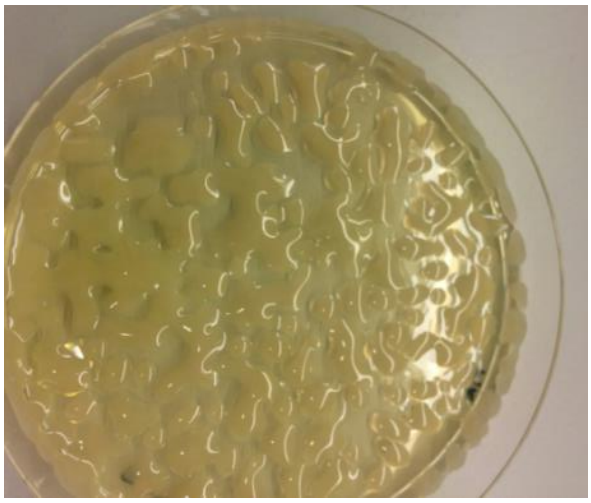

Figure 2. A mucoid phenotype expressed by a *P. aeruginosa* clinical strain isolated from CF sputum sample on *Pseudomonas* isolation agar (PIA).

Once disseminated, *P. aeruginosa* produces pyocyanin and pyoverdine, blue and green pigments respectively, to interfere with host cell electron transport pathways and to captures iron in order to avoid iron starvation during the competition for the niche.

Blood stream invasion and dissemination of *Pseudomonas* is probably due to the same factors involved in local settlement and infection. The resistance of *P. aeruginosa* to phagocytosis and serum bactericidal response is due to its mucoid capsule and LPS. Moreover, the lipidic component of LPS (lipid A, endotoxin) mediates the usual pathologic aspects of Gram-negative septicemia, *e.g.* fever, hypotension and intravascular coagulation. The virulence mechanism of *P. aeruginosa* is summarized in Fig.3.

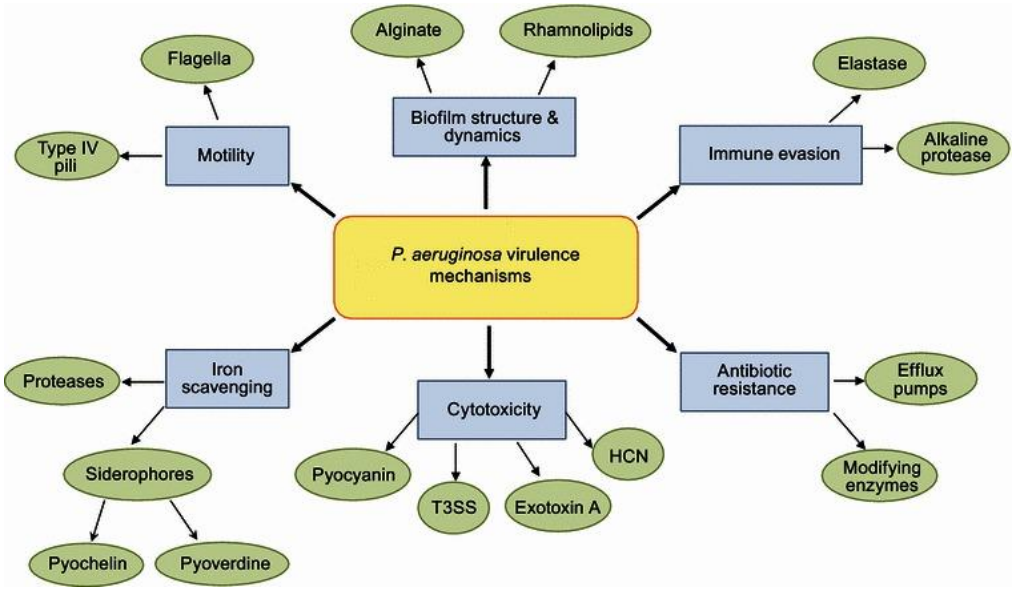

Figure 3. Virulence mechanisms employed during *P. aeruginosa* infections. Modified from Lee and Zhang, 2015.

P. aeruginosa acute infections often spread rapidly and can cause tissue damage and sepsis with high mortality rates; chronic infections can persist for weeks, months, or years in the face of intensive clinical intervention. As mentioned before, acute infections are characterized by the expression of a plethora of secreted and cell-associated virulence factors which worsen the clinical status of immunocompromised patients and are clearly recognizable. Chronic infections are characterized by a permanent inflammatory state due to the recurrent, intermittent bacterial infection. The main example for chronic infection is induced by *P. aeruginosa* in CF lung which is highly different compared to the strains isolated from acute infection site. Indeed, clinical isolates

from chronic infection site accumulated mutations and loss of function in genes involved for example in acute infection traits, as pili, flagella, quorum sensing adopting a more sessile mode of life (Fig. 4) (Gellatly and Hancock, 2013; Smith et al., 2006). The acquisition of the mucoid phenotype is also the result of the accumulation of a mutation, specifically in *mucA* gene, involved in the negative control of the alternative sigma factor AlgU which is activated in stress condition and regulates a number of genes associated to central metabolism, virulence and motility (Ciofu et al., 2010; Jones et al., 2010; Yu et al., 1995). Sigma factors are global regulators of transcription initiation in bacteria, which allow the RNA polymerase binding to a specific promoter. They regulate the expression of a number of genes simultaneously, connected to virulence and stress response.

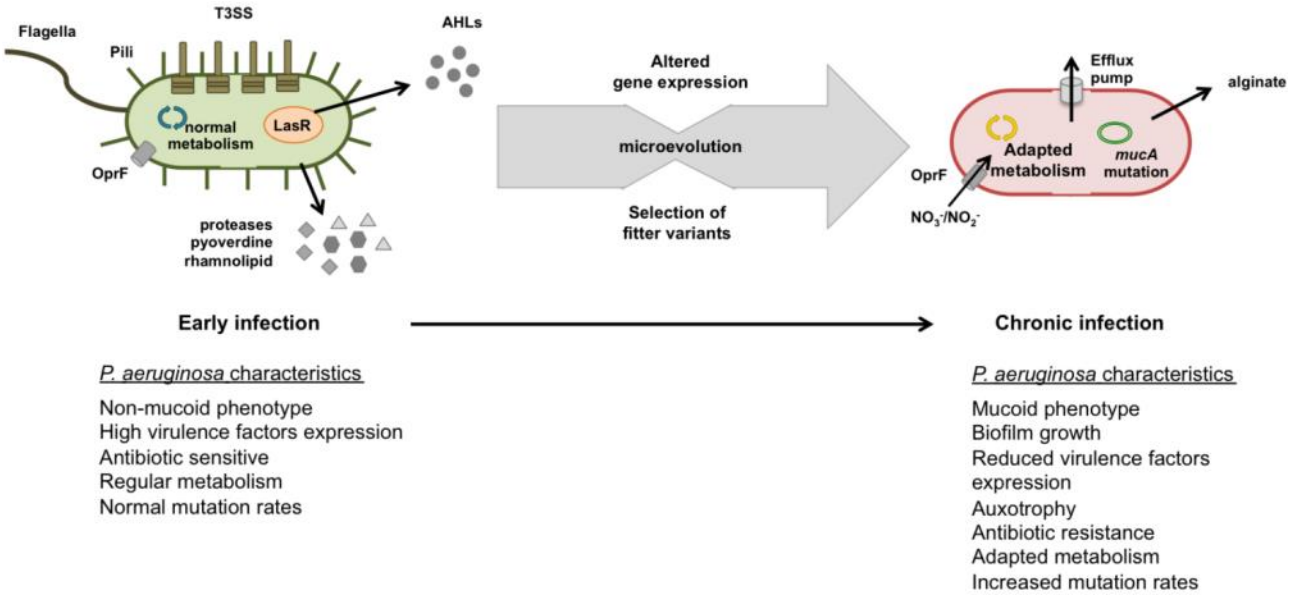

Figure 4. Graphic representation of *P. aeruginosa* changes during infection in CF airways. During the early infection *P. aeruginosa* produces cell-associated virulence factors and secreted factors, and is still antibiotic sensitive. In chronic infection stage *P. aeruginosa* accumulates mutations to be fully adapted to CF environment and is characterized by overproduction of alginate, loss of the appendages and show more resistance to antibiotics. Modified from (Sousa and Pereira, 2014)

2.1 *P. aeruginosa* sigma factors

In *P. aeruginosa*, transcription initiation is the major event in regulation of target genes orchestrated by sigma factors, which confers to the bacterium adaptability and versatily to several environmental conditions, including human host.

Flexible changes in the transcriptional profile of *P. aeruginosa* are crucial in the switch between acute and chronic infections and in modulating all the phenotypes connected to this event, as biofilm formation (Oliver et al., 2000; Sadikot et al., 2005). Alternative sigma factors and extra-cytoplasmatic function factors (ECF) are known to provide fast regulation of gene expression in challenging environmental conditions. *P.aeruginosa* produces 25 sigma factors, including one strain-specific (encoded from PA14_27690 gene in PA14 strain) and 21 ECF sigma factors (Potvin et al., 2008).

The basic initiation of transcription in bacteria is performed by the multisubunit RNA polymerase (RNAP) that binds the sigma factor σ^{70} (Helmann and Chamberlin, 1988) to detect the promoter region. The σ^{70} proteins bind the positions -10 and -35 and allow the melting of double-stranded DNA to interact with the core of the RNAP.

P. aeruginosa produces a major σ^{70} named **RpoD** which recognizes and controls the expression of a large number of housekeeping genes. RpoD has extensive homology with σ^{70} of *E. coli* (Tanaka and Takahashi, 1994) and it exerts is transcriptional activity essentially during the exponential phase of growth (Fujita et al., 1994) RpoD is constitutivelty transcribed from PC promoter under steady-state and upon heat shock, and transiently transcribed from PHS promoter upon heat-shock (Aramaki and Fujita, 1999). RpoD, as all σ^{70}, is characterized by four domains of which domain 2 and 4 are the most conserved and involved in the binding to -10 and -30 regions (Harley and Reynolds, 1987; Zhou and Gross, 1992).

P. aeruginosa and *E.coli* share similarities also for another σ^{70}-like, **RpoH**, which is 61% identical to σ^{H} (σ^{32}) of *E. coli* (Benvenisti et al., 1995). In *P. aeruginosa rpoH* transcription is AlgU-dependent and it is required for positive regulation of heatshock genes as well as for more than 20 heat-shock proteins (HSPs), including DnaK, DnaJ, GrpE, GroEL and GroES and specific proteases (Arsène et al., 2000). Interestingly, the promoter of RpoH was found to be activated in mucoid *mucA* mutants, suggesting a connection between the conversion to mucoid phenotype and the heat-shock response in *P. aeruginosa* (Schurr and Deretic, 1997).

FliA (RpoF or σ^{28}) is an alternative σ factor. The *fliA* gene of *P. aeruginosa* shares up to 67% similarity with the gene of *Salmonella typhimurium* and its major function is the control of flagellin

biosynthesis, since the insertional inactivation of *fliA* results in a non-motile *P. aeruginosa* (Starnbach and Lory, 1992). The molecular mechanism that controls the expression of *fliA* is unclear but its transcription seems to be independent of RpoN or other flagellar regulator such as FleQ or FleR (Dasgupta et al., 2003).

RpoS is one of the best-characterized alternative σ^{70} in *P. aeruginosa* (Schuster et al., 2004; Whiteley et al., 2000) and a homologue of σ^{s} in *E. coli*. RpoS is involved in the regulation of QS via the modulation of the transcriptional regulators RhlR and LasR (Latifi et al., 1996)lately affecting the formation of biofilm (Davies et al., 1998) as described in the following section of this thesis. RpoS is involved in the secretion of extracellular virulence factors such as alginate, exotoxin A, Las A and Las B elastases and exoenzyme S (Hogardt et al., 2004; Sonnleitner et al., 2003; Suh et al., 1999) and its transcription in *Pseudomonas* implicates four genetic loci *psrA, gacA, lasI* and *rhlI*. PsrA has a significant role in *rpoS* transcription during the late-exponential and stationary growth phases, Vfr has a probable role in transcriptional repression of RpoS during the exponential phase and ClpXP protease is involved in degradation of RpoS during the exponential phase (Bertani et al., 2003) RpoS has also been reported to be a general stress regulator in *P. aeruginosa*, *P. fluorescens* and *P. putida* (Venturi, 2003, 2006). In addition, RpoS is known to be involved in the expression of a subset of extracellular toxins that are also regulated by other global regulators in response to cell density and to other environmental factors.

RpoN (σ^{54} or σ^{N}, formerly NtrA) was initially linked to nitrogen assimilation only. However, RpoN has been found to play important roles in motility, in the transport of nutrients, in the formation of pili, in mucoidy and in cell-to-cell signalling (Dasgupta et al., 2003; Heurlier et al., 2003; Ishimoto and Lory, 1989; Mattick et al., 1996; Strom and Lory, 1993; Thompson et al., 2003; Totten et al., 1990). In addition, there are 22 proteins annotated known to have a binding site for σ^{54}, FleQ and FleR involved in the biosynthesis of flagellin in *P. aeruginosa* (Dasgupta et al., 2003), PilR for pilin biosynthesis (Mattick et al., 1996) and AlgB, which regulates alginate exopolysaccharide production. RpoN is hence involved in the expression of different virulence determinants of *P. aeruginosa* including cell-to-cell communication pathways (Hendrickson et al., 2001). All RpoN-regulated genes have the consensus promoter sequence -24(GG)/-12(GC) and their expressions required at least one transcriptional activator (Thöny and Hennecke, 1989).

Alternative σ factors are involved in the regulation of gene expression in response to various extracellular changes, and they are designed as σ-ECF. Despite similarities in the genome size of *Pseudomonas* species (6–7 Mbp), only 10 ECF have been identified in the genome of *P. syringae*;

19 ECF in *P. aeruginosa* and in *P. putida*, 28 ECF predicted in *P. fluorescens* Pf-5. Based on their sequence conservation and function across bacterial species, ECF are classified as σ^{70} and the consensus sequence recognized is highly conserved. In most cases, the activity is modulated by an anti-σ, an inner membrane protein present in both *E. coli* and in *P. aeruginosa*. The anti-σ acts as a sensor in signalling, allowing an adaptive response to specific environmental changes to co-ordinate transcription of a specific regulon. There are three common characteristics shared among all ECFs, i) promoter sequence recognized contains an (AAC) motif in the 35 region; ii) ECF is cotranscribed with an anti-σ factor having an extracytoplasmic sensory domain and an intracellular inhibitory domain; ii) ECF are involved in regulating extracellular pathways as periplasmic stress and heatshock (σ^{E}), iron transport (σ^{FecI}), metal ion efflux systems (CnrH), alginate secretion (AlgU) and synthesis of membrane-localized carotenoids (CarQ) (Raivio and Silhavy, 2001).

Some ECF have been characterized and have a defined function in *P. aeruginosa*: AlgU was described to be involved in alginate biosynthesis (Schurr et al., 1996). PvdS is implicated in iron regulation responsible for the biosynthesis of pyoverdine (Cunliffe et al., 1995). SigX was shown to control the expression of the major outer membrane protein OprF (Brinkman et al., 1999)

AlgU shares a 79% amino acid sequence similarity to the *E. coli* σ^{E} heat-shock produced in response to elevated temperatures, suggesting similar functions in response to stress. In *P. aeruginosa*, AlgU has been firstly identified to be linked in the switch betweend non-mucoid to mucoid phenotype in cystic fibrosis (CF) patients interacting with the promoter of *algD*, the key enzyme in the alginate biosynthesis pathway (Schurr et al., 1996). AlgU was also described to be involved in the regulation of resistance to oxidative and heat-shock stress (Schurr and Deretic, 1997). AlgU is transcribed, together with its regulators from *algTmucABCD* operon. MucA represses AlgU activity acting as an anti-σ and its inactivation results in constitutive expression of the alginate pathway dependent on *algU* transcription. MucB is another negative regulator of AlgU localized in the periplasm, and a deletion of the leader peptide and the cytoplasmic location of MucB abrogated its ability to inhibit mucoidy (Schurr et al., 1996). As described in the following sections, AlgU is crucial in regulating exopolysaccharide production through the activation of transcriptional regulators, as AmrZ, and post-transcriptional regulator as the small RNA ErsA, which is one of the main topics of this thesis.

PvdS controls the production of the siderophore pyoverdine and the expression of two *P. aeruginosa* extracellular virulence factors: the endoprotease PrpL (Wilderman et al., 2001) and exotoxin A (Helmann, 2002; Wilson and Lamont, 2000). The regulation of pyoverdine production

and uptake depends also to another ECF-σ, FpvI, together with one anti-σ, FpvR. PvdS shows considerable similarity to σ^{FecI} of *E. coli*, a positive regulator for transcription of *fec* implicated in ferric citrate transport (Miyazaki et al., 1995). The *pvdS* gene is expressed only in iron-limiting conditions and it is regulated by Fur (Ferric uptake regulator) repressor (Cunliffe et al., 1995). Fur interacts with the DNA operator sequences (Fur box) to regulate the expression of a subset of genes iron-regulated (Ochsner et al., 1995). It was been described that in the presence of iron, Fur binds the *pvdS* promoter to repress the transcription of *pvdS*, affecting the expression of *pvdA* encoding a key enzyme in the pyoverdine biosynthetic pathway (Leoni et al., 1996). Exotoxin A is also secreted under iron-limiting conditions. The structural gene *toxA* is regulated at the transcriptional level by the products of *regAB*. The expression of both *toxA* and *regAB* is repressed under iron-deficient conditions, suggesting a role for Fur in the regulation of *toxA* expression. In addition exotoxin A and regA are also positively regulated by PtxR Lys-R-type transcriptional regulator (Hamood et al., 1996). PtxR and PvdS were also shown to be implicated in the synthesis of pyoverdine via a positive regulation of the *pvdABCD* operon, which is negatively regulated under high-iron conditions (Stintzi et al., 1999).

SigX is the most recent σ^{70} ECF with a function demonstrated in *P. aeruginosa* and it shares 49% similarity to σ^{w} of *Bacillus subtilis* induced by alkaline shock, salt, phage infection and antibiotics that affect cell-wall biosynthesis (Schöbel et al., 2004). In *P. aeruginosa* and in *P. fluorescence*, *sigX* is located upstream of *oprF* and the disruption of *sigX* in both affects *oprF* expression (Brinkman et al., 1999).

2.2 Secreted virulence factors

Secreted virulence factors, as exotoxin A, proteases and pyocyanin determine the peculiar acute traits of *P. aeruginosa* infections and their expression represents the cross point between colonization and acute infection establishment (Moradali et al., 2017).

2.2.1 Exotoxins and proteases

Exotoxin A (ExoA), proteases and elastases are the main cause of local tissue damage, bacterial invasion (Woods and Iglewski), alteration of immune system and cell death due to inhibition of protein synthesis (Steadman et al., 1993).

In particular, ExoA is an ADP-ribosyl transferase secreted into the extracellular space, which inhibits the elongation factor-2 (EF-2) and it has been shown to depress the host response to

infection (Michalska and Wolf, 2015). Furthermore, the injection of ExoA in mice induces hepatocyte apoptosis, the release of TNF, IFN-γ, IL-2, and IL-6 into the circulation and an increase in plasma transaminase activities (Mühlen et al., 2004).

The most studied and relevant proteases in *P*. *aeruginosa* infection are elastase LasA and LasB, and alkaline protease. Alkaline protease, AprA is a type I secreted zinc protease required for degradation of host immune system components and host fibronectin (Hong and Ghebrehiwet, 1992) and is involved also in degrading free flagellin to prevent Toll-Like receptor 5 activation (Bardoel et al., 2011).

LasA and LasB are regulated by the *las* quorum-sensing system and secreted via Type II secretion machinery (De Kievit and Iglewski, 2000). LasA is a 20 kDa serine protease and has been described to cleave the pentaglycine bonds in the peptidoglycan of *Staphylococcus aureus* (Vessillier et al., 2001). LasB degrades elastin, fibrin, and collagen (Cotar et al., 2010). All these proteases are associated to tissue invasion, damage and necrosis and the pathogenic role of alkaline proteases, beside lung infections, has been shown also in corneal infections (Galloway, 1991; Guzzo et al., 1991). Other proteases, classified as endoproteases, cleave lactoferrin and transferrin altering the iron-caption in the host (Wilderman et al., 2001).

P. *aeruginosa* produces also a number of hemolysins as the phospholipase C, which is secreted in the extracellular compartment and contributes to acute infection and inflammation, inducing cell death in animal models (Wieland et al., 2002).

2.2.2 Pigments

One of the peculiar traits of *P*. *aeruginosa* is the production of pigments that confers different colorations associated to precise physiological processes.

The phenazine-based pyocyanin compound is a non-fluorescent blue pigment, particularly interesting for its capability to generate reactive oxygen species (ROS) and thereby its application is very common as a redox compound in biosensors (Priyaja et al., 2016). Pyocyanin is present in large amount in CF sputum and since it can cross the biological membrane, *P*. *aeruginosa* uses this compound in oxygen-limited condition, to transport electrons produced by respiration to remote electron acceptors (Jennings et al., 2015; Lau et al., 2004).

Pyoverdine (fluorescins or pseudobactins) and pyochelin are the two small molecules produced by *P*. *aeruginosa* in iron-limited condition. In the host *P*. *aeruginosa* produces pyoverdine and pyochelin to chelate extracellular Fe^{3+} since the iron supply is limited and almost all sequestered in heme molecules or combined to lactoferrin and transferrin proteins (Cornelissen and Sparling, 1994).

The expression of pyoverdine and pyochelin genes is modulated by the repressor Fur (ferric uptake regulator). In normal condition, Fur is complexed with Fe^{2+} and this conformation is suitable to bind DNA region (named Fur box) within promoters of iron uptake genes repressing their expression. More than 200 genes are known to be involved in iron metabolism in *P*. *aeruginosa*, but only a few of them present a Fur box (Cornelis et al., 2009). One example is the sigma factor PvdS which regulates a number of genes in iron-deplete conditions, including those encoding for pyoverdine, but is known to be also involved indirectly or directly in virulence factors production, such as exotoxin A (Leoni et al., 2000; Wilderman et al., 2001).

However, beyond the role of siderophores, pyoverdine and pyochelin are both involved in acute and chronic infections, increasing inflammation and causing tissue damages as shown in a burned mouse model or in case of mouse pulmonary infections (Cornelis et al., 2013; Lyczak et al., 2002; Meyer et al., 1996; Takase et al., 2000)

2.2.3 Type III and type VI secretion system

The injection of toxic compounds into the host cell cytosol, or their secretion in the extracellular compartment, is provided by a number of secretion systems. Type III secretion system is a complex machinery attached to the bacterial surface which is designated to deliver toxic proteins, named

effectors, directly in the host cell through a pore formed in the membrane. So far, four effectors have been described in *P. aeruginosa*, ExoY, ExoS, ExoT, ExoU (Galle et al., 2012; Hauser, 2009). ExoS and ExoT are bifunctional, with GTPase activating protein (GAP) and ADP-ribosyltransferase activity, while ExoY is an adenylate cyclase. The pathological role of these exotoxins is to disrupt epithelial junctions, to alter the cytoskeletal organization and to modify the host cell membrane permeability in the lung leading to the symptoms of bacterial pneumonia (Hauser, 2009).

Type VI secretion system (T6SS) is most recent in characterization compared to T3SS. Its function is to inject virulence factors, as Hcp1 and VgrG proteins in the host cell lumen by a needle-like structure and the Hcp1 protein can be recovered directly from the CF sputum with *P. aeruginosa* infection (Filloux, 2011). *P. aeruginosa* encodes three T6SS clusters, H1, H2 and H3-T6SS. As recently described in Allsopp et al. (Allsopp et al., 2017), the T6SS is fine tune regulated at both transcriptional and post-transcriptional levels, by the pleiotropic transcriptional regulator AmrZ and by the small RNA RsmA. This regulation is pivotal for *P. aeruginosa* since the T6SS is built to kill bacterial competitors during the settlement of the infection and prevail in specific niches.

2.2.4 Pyocins

Pyocins were described for the first time by François Jacob as bacteriocins produced by *P. aeruginosa* when treated with a mutagen agent as ultraviolet irradiation. These inducible compounds when absorbed by the cell surface of other bacteria cause their death.

After this first evidence, other studies increased the knowledge about pyocins and in particular the classification in R-pyocins, F-pyocins and S-pyocins (Michel-Briand and Baysse, 2002; Takeya et al., 1967).

Although almost all the *P. aeruginosa* strains can produce pyocins, the spontaneous production level is low and could increase if induced by mutagen agents. The mechanism of action of pyocins is based on a hit mechanism to kill the target (Kuroda and Kageyama, 1979; Okawa et al., 1973). However, not all the pyocins secreted are active, but one of them is enough to exert the killing activity in the host. Furthermore, strains producing pyocins are insensitive to their own pyocins, although one exception has been reported (Goodwin et al., 1972).

Three types of pyocins have been described as produced by *P. aeruginosa*. The first pyocin identified and called R1, is synthetized in rod-like particles, and is nuclease and protease resistant (Takeya et al., 1967)

The second type of pyocin is called F-pyocin and occurs as a flexuous rod-like structure, and is usually produced associated to R-type pyocins. R-type pyocins are contractile but non flexible, while the F-type pyocins are flexible but non contractile (Govan, 1974). The R-pyocins are characterized by a double hollow cylinder, constituted by a sheath and a core. The sheath is the contractile unit; the core is located in the sheath and is composed by ≈180 subunits. The distal portion of the pyocin particle is accompanied by six tail fibers constituted by a subunit protein in which the subgroup specificity could be located at the C-terminal end (five subgroups: R1–R5) (Yui Furihata, 1972). These structural features share similarities with bacteriophage tail (Fig. 5).

The fibers are basically recognized by the LPS of Gram negative bacteria and several strains are sensitive to the R-type pyocin of *P. aeruginosa*, such as *Neisseria gonorrhea*, *Neisseria meningitides*, *Haemophilus ducreyi*, and *Haemophilus influenzae* (Andersen et al., 1995; Blackwell et al., 1979; Filiatrault et al., 2001; Levin and Stein, 1996).

After the interaction with the receptor, the contraction of the R-pyocin sheath allows the penetration of the core in the outer membrane for the killing activity. The release of intracellular material is followed by cell death for depolarization of the cytoplasmic membrane within 20 min (Uratani and Hoshino, 1984; Yui Furihata, 1972).

Concerning F-type pyocins, the structure is composed by one squared end and the other with a fiber-like structure which resemble flexible phage tails (Kuroda and Kageyama, 1979; Takeya et al., 1967).

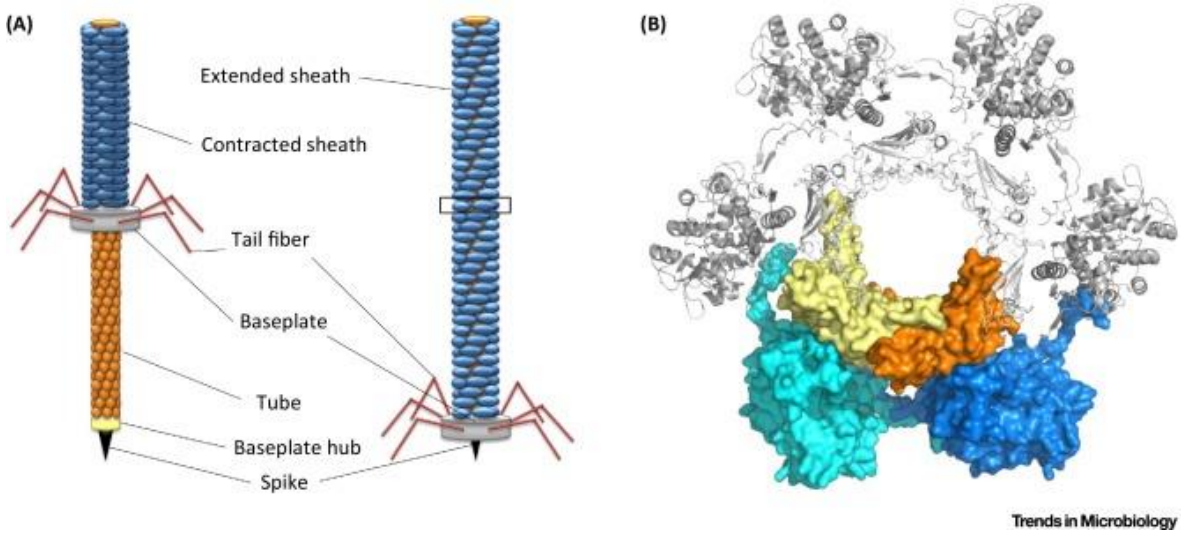

Figure 5. Structure of pyocin. (A) The structure consists of a rigid tube (orange subunits), contractible sheath (blue subunits), lipopolysaccharide(LPS)-targeting tail fibers (red) attached to the baseplate (gray), and spike (black) connected via the baseplate hub (pale yellow) to the central tube. (B) Tail-tube architecture of pyocin R2 in extended conformation. Modified from (Ghequire and De Mot, 2015).

Another large group of pyocins are the protease-sensitive "soluble" (S-type) pyocin. The first S1-pyocin was identified treating a strain producing R-type pyocin by an anti-R serum. Other S-type pyocins as Sa and S3, were been isolated from a *P. aeruginosa* strain isolated from cystic fibrosis patient sputum. Two other soluble pyocins, S4 and S5, have been predicted bioinformatically in 2000 and they have a tRNase structure and a pore-forming activity, respectively (Duport et al., 1995; Elfarash et al., 2012, 2014; Parret and De Mot, 2000).

S-type pyocins AP41, S1, S2, and S3 are constituted of two protein components in which the large component carries an endonuclease C-terminal domain responsible for the killing activity that causes cell death by DNA cleavage (Sano et al., 1993; Sano and Kageyama, 1984).

The activity of S-type pyocins is also based on the interaction with the outer membrane of Gram-negative bacteria. The N-terminal portion of the larger protein corresponds to the receptor binding domain (domain I). The second domain is not present in S2 and its role is not completely understood. The third domain (domain III) is involved in the translocation across the outer membrane, and the C-terminal end (domain IV) is crucial for the DNase activity (Michel-Briand and Baysse, 2002).

Furthermore, a majority of S-type pyocins have been found to invade the cells using a TonB-dependent outer membrane receptor involved in ferri-siderophore uptake. In particular, S2 and S4 use the ferripyoverdine receptor FpvAI (Denayer et al., 2007; Elfarash et al., 2012), pyocin S3 targets FpvAII (Baysse et al., 1999) (Baysse et al., 1999), while pyocin S5 recognizes the FptA ferripyochelin receptor (Elfarash et al., 2014).

AP41 pyocin causes induction of synthesis of other pyocins (R2 and S2 pyocins), in the sensitive target cell PAO1 and the receptor of AP41 pyocin seems to belong to another system where iron is involved (Michel-Briand and Baysse, 2002).

Other pyocin families, comparable in size to the S pyocins, are M-type and L-type pyocins. M-type pyocins provides a module that cleaves lipid II peptidoglycan precursors (Barreteau et al., 2009). L-type pyocins have two mannose-binding lectin domains which kill target cells via an unknown mechanism (Ghequire et al., 2013, 2014; McCaughey et al., 2014).

Recently, bacterial bacteriocins are considered a valid alternative therapeutic strategy to treat both multidrug-resistant and chronic bacterial infections (Behrens et al., 2017). The use of bacteriocins could target a particular microorganism closely related to the bacteriocin-producers, not affecting the rest of microflora (Francino, 2016). In addition, the "selective killing action" strongly reduces the selective pressure for resistance among strains sharing the same niches. *In vitro* studies have

already demonstrated the activity of bacteriocins against both planktonic and biofilm-bacteria (Smith et al., 2012). Furthermore they have also key properties *in vivo*, as specificity and high activity that suggest they may be excellent candidates for further therapeutic development (Behrens et al., 2017).

2.3 Cell surface virulence factor

The first stages of *P. aeruginosa* infections rely on both attachment and adhesion to the host-cell surface. The expression of pili and flagella, as well as LPS, is pivotal for the human pathogen to find a niche and colonize the host inducing at once the inflammatory and immunogenic response typical of *P. aeruginosa* acute infection.

2.3.1 Type IV pili and flagella

Several bacteria, characterized by motile phenotype, use either flagella or type IV pili (TFP) to spread in the environment. *P. aeruginosa* possess and uses both of these motile appendage types implementing multiple motile styles as swarming, swimming, twitching, crawling, and walking (Kazmierczak et al., 2015). Type IV pili are long, thin (5–8 nm diameter) appendages present also on archaeal cell surfaces (Makarova et al., 2016). They are involved not only in bacterial motility, but also in cell-cell interaction and adhesion. Indeed, mutations in TFP production, result in less virulent strains, since lacking of colonization machinery (Bradley, 1980; Burrows, 2005; Mattick, 2002; Pelicic, 2008).

Two major TFP families have been described in *P. aeruginosa*, type IVa and Type IVb both characterized by high dynamicity. The retraction and extension movements of TFP in combination with other appendages, confer the force for the different types of locomotion, in particular for twitching motility (Gibiansky et al., 2010; Mattick, 2002).

However, the main structure for swimming and swarming motilities is flagellum. *P. aeruginosa* possess a single polar flagellum which, through a rotating motion, contributes to swimming motility in aqueous environment and swarming on semi-solid surface (Kazmierczak et al., 2015; Kohler et al., 2000). The structural protein forming the body of flagellum is the called flagellin and the flagellar biosynthesis is based on the hierarchical expression of specific genes: i) *fleQ* gene, ii) basal-body genes and iii) flagellin and chemotaxis gene. All these genes are fine regulated by *fleQ* and the stationary-phase sigma factor *rpoS* (Dasgupta et al., 2004; Garrett et al., 1999; Wolfgang et al., 2004). The flagellar master regulator FleQ is inhibited by c-di-GMP binding or by sequestration from FleN which also modulates swarming motility regulating the flagella number (Dasgupta et al., 2000; Dasgupta and Ramphal, 2001; Hickman and Harwood, 2008). In addition, the transcriptional regulator AmrZ is known to regulate flagellar expression repressing *fleQ* and inhibiting flagellar-dependent motility (Tart et al., 2006).

Clinical isolates from CF lungs are non-motile due to the high production of mucin secreted in the airways of CF patients and the suppression of motility results in enhanced biofilm formation (Landry et al., 2006). The molecular reason for the inhibition of motility is that FliD flagellar cap protein is attached to the mucin and their interaction interferes with the rotation of the flagellar machinery (Arora et al., 1998; Landry et al., 2006). The genetic reason is the mutation in the *mucA* gene present in CF clinical isolates, which reduces flagellar expression and promotes biofilm formation (Poschet et al., 2001). The *mucA* mutation causes a cascade effect (Fig. 6), starting with the unadjusted expression of AlgU which triggers the expression of alginate genes and AmrZ (Tart et al., 2005). As mentioned before, the transcriptional regulator AmrZ inhibits the flagellar expression repressing FleQ (Garrett et al., 1999; Tart et al., 2005, 2006). FleQ represses also *pel* operon expression, thus its inactivation results in more Pel production and consequently more biofilm production (Hickman and Harwood, 2008). In latest biofilm stages of maturation, the motility is important to permit cell dispersal. Thus, swimming motility is re-activated. Biofilm dispersal is associated to the release of a chemical signal, *cis*-2-decenoic acid (Davies and Marques, 2009), but its role in activating motility is unknown.

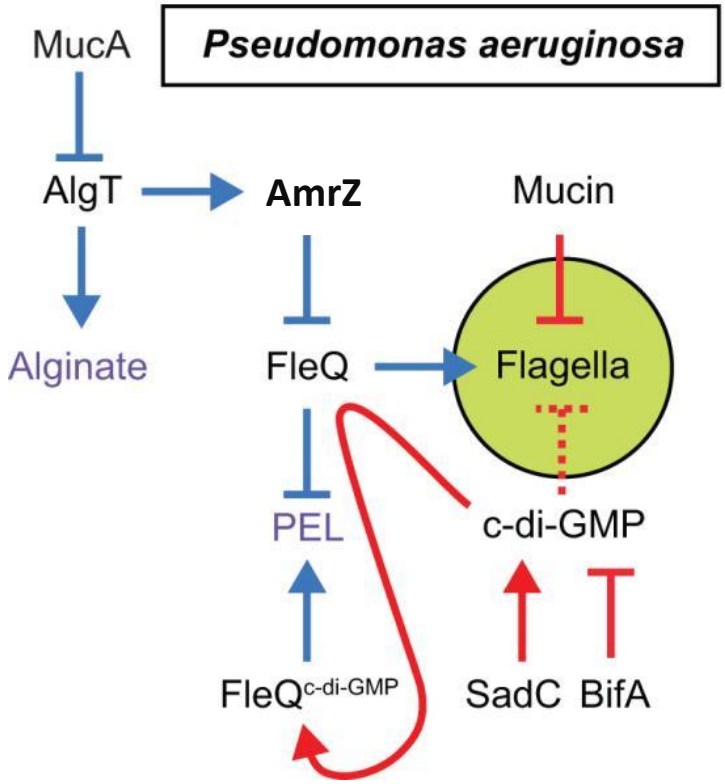

Figure 6. *P. aeruginosa* biofilm-related motility regulation. Transcriptional regulation is labeled in blue, functional regulation labeled in red, and the biofilm matrix products in purple. Modified from (Guttenplan and Kearns, 2013)

The rotator movement is provided by the rotor component of the flagellum, while the attachment to the membrane of *P*. *aeruginosa* is allocated in the static component named stator. The rotor is composed by 25 copies of FliG, 34 of FliM, >100 of FliN proteins all arranged in a C-ring structure, while the stator proteins Mot-A,B,C,D,Y are associated in two sets to facilitate the flagellar movement (Fig. 7).

The flagellum is also highly immunogenic, due to the flagellin which causes the release of cytokines and neutrophils in the infection site (Hayashi et al., 2001; Smith et al., 2003). Interestingly, CF clinical isolates show the absence of appendages during chronic infection, suggesting a strategy to escape from immune system response (Wolfgang et al., 2004).

The inverse relation biofilm-flagellar motility rely also in the repression of flagellar genes during the last stages of biofilm maturation (O'Toole and Kolter, 1998; Sauer et al., 2002; Wolfgang et al., 2004)

Considering the important role of flagella and pili in *P*. *aeruginosa* virulence, they are studied as main components of new vaccines in burned mouse model (Korpi et al., 2016) and in the recent era of increased drug resistance for the majority pathogenic human bugs, including *P*. *aeruginosa*, the immunotherapy's field is representing a valid alternative to antibiotics to prevent and fight bacterial infection.

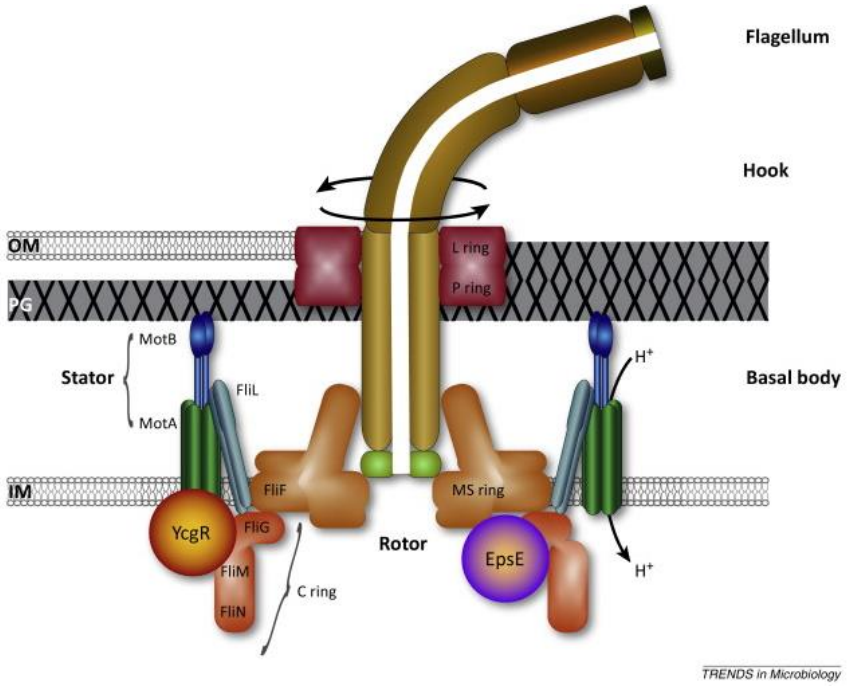

Figure 7. Flagellar structure. Simplified diagram showing the main components of the flagellum of Gram negative (left side) bacteria: the basal body, hook, and flagellar filament. The motor is composed of the stator (MotA and MotB proteins) and the rotor (C ring, composed of FliG, FliM, and FliN).. Modified from (Belas, 2014)

2.3.2 Swarming and twitching motility regulation

As previously described, swarming in *P*. *aeruginosa* requires a complete flagellum and twitching is a type IV pili-dependent movement. Interestingly, the presence of type IV pili has been described as important for swarming motility in *P*. *aeruginosa* PAO1 strain (Kohler et al., 2000), in addition to rhamnolipids (RLs) and 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), which act as wetting agents and chemotactic-like *stimuli* (Caiazza et al., 2007; Tremblay et al., 2007).

P. *aeruginosa* can synthesize maximum two flagella, thus the retractable type IV pili could provide and additional feature in viscous surface propagation.

Swarming motility is characterized by a fast migration of bacteria and occurs in presence of viscous surface and in laboratory condition is generally used a range of 0.5-0.8% of agar concentration.

Several genes connected to *P*. *aeruginosa* virulence are differentially expressed in swarming condition, including type III secretion system genes, protease and iron transport (Wang et al., 2014). Furthermore, swarming cells have been described as more resistant to some antibiotics, as gentamicin, polymyxin B and ciprofloxin compared to strains with a planktonic lifestyle (Gooderham et al., 2008).

The presence of rhamnolipids is a clear evidence of the involvement of cell to cell signaling in swarming motility regulation. In particular, mutant in *rhl* system are unable to swarm since deficient in flagellum production, pilus assembly and rhamnolipid production. Moreover, the production of rhamnolipids is also reduced in the *lasI* and *lasR* mutants, which if mutated show a delayed swarming behavior (Shrout et al., 2006).

Several roles have been attributed to rhamnolipids in *Pseudomonas genus* (Soberón-Chávez et al., 2005). Beside the main role of these biosurfactants in solubilizing and promoting the uptake of hydrophobic substrates, *P*. *aeruginosa* produces rhamnolipids as toxic compounds against both Gram positives and Gram negatives microorganism to prevail in niche colonization (El-Sheshtawy and Doheim, 2014). In addition, the production of rhamnolipids in swarming motility has been shown to contribute to the correct shaping of biofilm development (Lequette and Greenberg, 2005).

Due to their pleiotropic role in *P*. *aeruginosa* pathogenicity, as hemolytic, phospholipidic and immunogenic activity, rhamnolipids are considered secreted virulence factor (Reis et al., 2011), however the biological meaning *in vivo* has not been fully understood.

Twitching motility occurs on solid or with moderate viscosity surface, equivalent to a media with 1% agar. Retraction and rotating movement given by TFP, allow to bacterial cells a fast migration along a surface from the initial point of adhesion. This movement impact on cells disposition in

biofilm differentiation, and help cells to escape when necessary during host colonization (Alarcon et al., 2009). The regulation of twitching motility is basically associated to the expression of pilin subunit, PilA, controlled by the sigma factor RpoN (σ^{54}) and the two component system PilR-PilS (Mikkelsen et al., 2011b; Strom and Lory, 1993). The autophosphorylation of the sensor kinase PilS and the subsequent phosphorylation of its response regulator PilR, trigger the PilA expression which is sensed as a signal from PilS in the inner membrane in a feed-forward manner (Giltner et al., 2011).

A minor pilin operon, controlled by AlgR-FimS system and the alternative sigma factor AlgU (σ^{22}/AlgT), has been described as an indirect regulator of twitching motility controlling the expression of lectin LecB, which triggers the PilJ expression, important for pilus biogenesis (Nguyen et al., 2015a, 2015b).

2.3.3. Lipopolysaccharide

Lipopolysaccharide is one of the main components of the virulence factors arsenal in *P. aeruginosa*. It is composed by a glycolipid matrix and three different regions: LipidA, anchored to the membrane, the oligosaccharide core divided into the inner and outer core, and the highly variable O-specific polysaccharide region (named also O- antigen) (King et al., 2009).

Lipid A backbone is composed by diglucosamine biphosphate anchored to the bacterial membrane by O- and N-linked primary and secondary fatty acids. Lipid A induces endocytotoxicity in the host but the inflammatory response induced by lipid A of *P. aeruginosa* is less than other enterobacteria. This is probably the reason why clinical isolates from CF patients present mutated form of lipid A in particular in acyl chains composition. These changes result in more severe inflammatory response to lipid A, contributing to increase the lung damage. Furthermore, modifications in lipid A are associated also to environmental changes, conferring more resistance to antibiotics treatment and immune system (Lam et al., 2011; Maeshima and Fernandez, 2013).

Bound to the lipid A there is the inner-core structure which contains two D-manno-oct-2-ulosonic acid residues and two L-glycero-D-manno-heptose residues. The outer core of the *P. aeruginosa* LPS is usually present in 2 different isoforms or glycoforms and the glycoform 2 is the structure to which the O-antigen is attached (Bystrova et al., 2003).

The O-polysaccharide region is the hydrophilic and immunogenic portion of LPS characterized by polysaccharide repeated units that protrude from the core to the exterior of the bacterial surface. The O-antigen portion confers the serogroup specificity of *P. aeruginosa* and is recognized by the innate

immune response by antibodies specific for each variants of the O-antigen. There are at least 11 O-antigen structural variants (Pier, 2007) with minor differences among related structures in properties such as side-group substituents, linkages between sugars or conformations of different monosaccharide. The sugar composition of *P*. *aeruginosa* O-side chains is strain dependent and in *P*. *aeruginosa* is basically composed by rhamnose (Bystrova et al., 2006; Knirel et al., 2006).

2.4 Quorum sensing

Multiple virulence factors and pathogenesis traits in *P. aeruginosa* are regulated by cell to cell signaling mechanism known as quorum sensing (QS). It is a widespread communication network based on sensing and exchanging of small signal molecules which provide information about the entire dynamic of population in that particular timeframe and environment. The concentration of the signal molecule is cell density-dependent and only when a certain threshold is reached the signal molecule binds and activates a cognate receptor (Papenfort and Bassler, 2016) .

Currently, three inter-linked QS systems have been characterized in *P. aeruginosa*: *las*, *rhl* and *pqs* (Papenfort and Bassler, 2016; Rampioni et al., 2016). A fourth molecule, IQS, was recently proposed to work as and additional communication system active during phosphate limiting conditions (Lee et al., 2013; Papenfort and Bassler, 2016). In contrast, however, other works suggested that IQS is aeruginaldehyde, a byproduct produced during pyochelin siderophore biosynthesis both in *P. aeruginosa* and *P. fluorescens*, rather than a *bona fide* QS system (Murcia et al., 2015; Ye et al., 2014).

The *las* and *rhl* systems are based on *N*-acylhomoserine lactones, while *pqs* employs 2-alkyl-4-quinolones (AQs) as QS signal molecules. Briefly, as schematized in Fig.8, the *las* system produces by LasI synthase the N-3-oxo-dodecanoyl-homoserin lactone (3-oxo-C12-HSL) signal molecule (Lee and Zhang, 2015; Smith and Iglewski, 2003). When the cell density increases and 3-oxo-C12 reaches the critical threshold, it binds to the transcriptional regulator LasR, encoded by *lasR* gene. The 3-oxo-C12-HSL–LasR complex binds to the promoter regions of multiple genes, activating or repressing their transcription. Among the genes upregulated there is *lasI*, to increase the production of 3-oxo-C12-HSL with an auto-induction effect, and *rhlR*, which increases the production of the *rhl* response regulator RhlR, activating the second QS system (de Kievit et al., 2002).

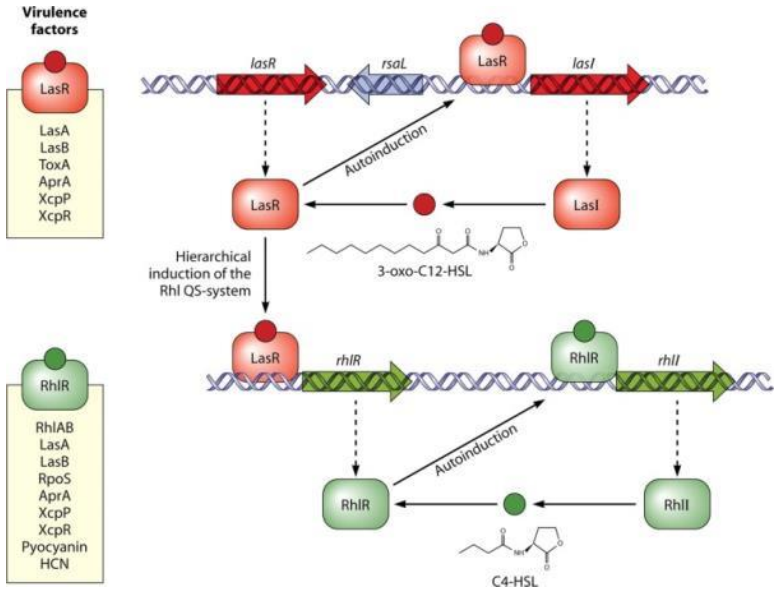

Figure 8. *Las* and *rhl* inter-linked QS systems. The activation of *las* system by 3-oxo-C12 triggers the expression of the transcriptional regulator RhlR involved in the activation of *rhl* QS system. Genes controlled by each Qs system are listed on the left side. Modified from (Jimenez et al., 2012).

The transcriptional unit for the third QS system *pqs* is composed by the *pqsABCDE-phnAB* operon and the *pqsR* gene. The *pqsABCDE-phnAB* operon synthesizes proteins (or enzymes) involved in the synthesis of the first signal molecule 2-heptyl-4-quinolone (HHQ), precursor of 2-heptyl-3-hydroxy-4-quinolone (also known as *Pseudomonas* Quinolone Signal, PQS), and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO).

The first four genes of *pqsABCDE* operon are involved only in signal molecules production, while *pqsE* is known to be an important effector involved in *P*. *aeruginosa* virulence (Deziel et al., 2004).

The *pqs* system is the most complex *P*. *aeruginosa* QS system and the physiological role of its component has been recently addressed (Rampioni et al., 2016). As summarized in Fig. 9, both HHQ and PQS binds to and activates the PqsR transcriptional regulator; indeed, the PqsR-HHQ/PQS complex promotes the expression of *pqsABCDE* modulating the activity of *pqsA* promoter, thus increasing HHQ and PqsE levels. HHQ is involved only in PqsR-dependent P*pqsA* regulation, while the authors show that PQS exerts its regulatory role also in a PqsR-independent manner affecting the transcriptional levels of genes coding for virulence factors such as pyoverdine, ExoS toxin and AprX protease. In addition, PqsR-independent PQS regulation is linked to its iron chelator activity involved in iron starvation response and PQS may work as an ion-trap (Diggle et al., 2007). However, the regulation of PQS on P*pqsA* not dependent to PqsR is unrelated to iron starvation response but inhibited in high-iron concentration.

Notably, PqsE down-regulates P*pqsA* activity, AQs production and the expression of genes involved in denitrification and T6SS. Conversely, PqsE upregulates genes involved in biofilm formation, virulence factors production and antibiotic resistance.

HQNO, synthesized by the contribution of other two genes *pqsH* and *pqsL*, does not affect the *P. aeruginosa* transcriptome, and its role seems to be linked to environmental competition due to its cytochrome inhibitory activity.

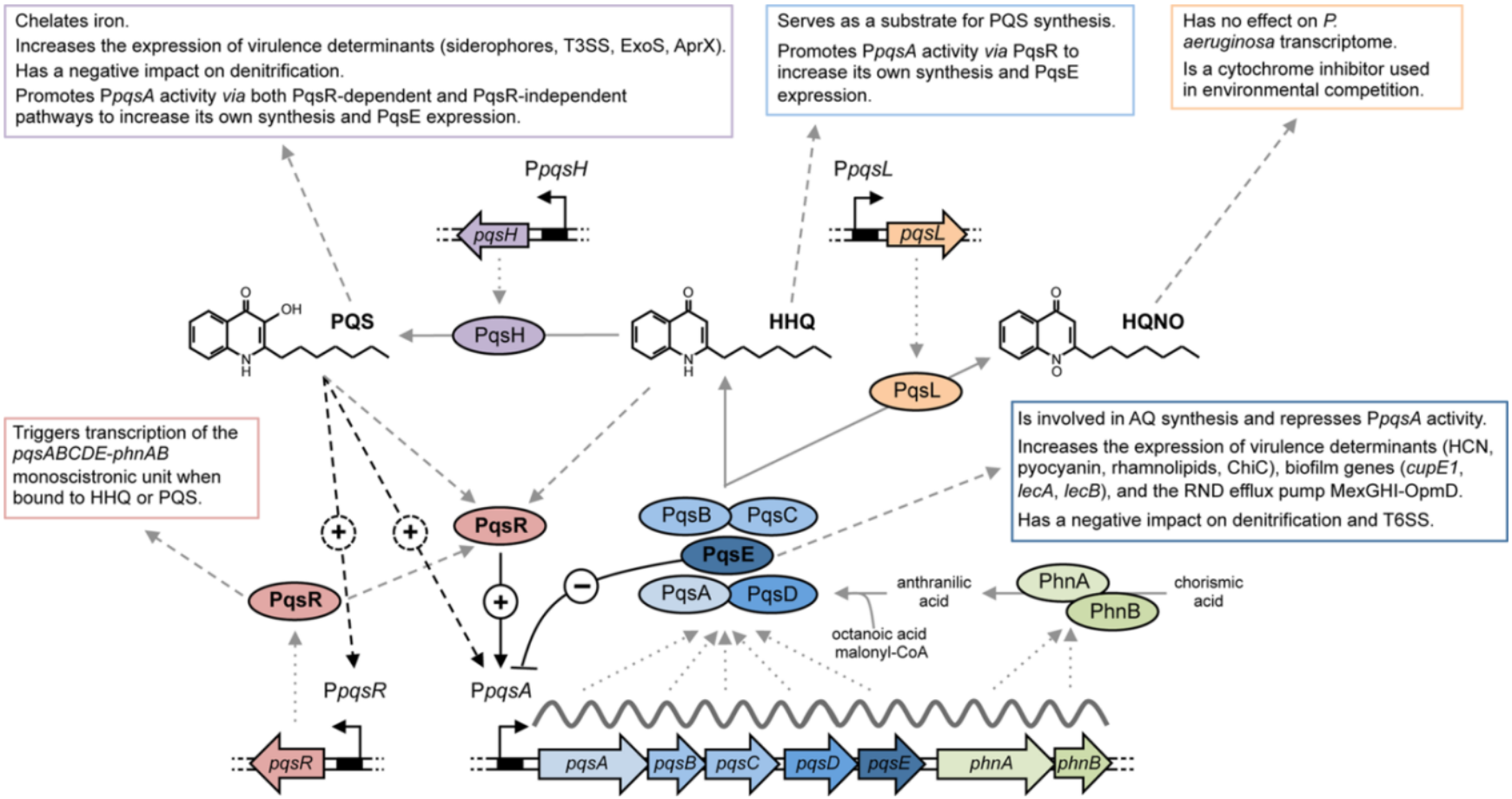

Figure 9. Detailed representation of pqs QS network. Modified from Rampioni et al., 2016. Dotted grey arrows indicate gene expression; solid grey arrows represent biosynthesis; solid black arrow indicates PqsR-dependent activation (+); dashed black arrows indicate PqsR-independent activation (+); black T-line indicates negative regulation (-); dashed grey arrows represent information flow.

3. Biofilm in bacteria

Biofilms have been defined as microbial communities, aggregated and embedded in a self-produced extracellular matrix. The matrix is basically composed by exopolysaccharide polymers which allow cell to cell contact and the attachment to a surface. The term "aggregate" refers to groups of bacteria adherent to each other and disposed in layers which compose the complex architecture of mature biofilms (Hall-Stoodley et al., 2004).

Biofilm formation relies on both social and physical organization into bacterial communities and it represents without any doubt the most spread and coordinated microbial lifestyle in nature (Vlamakis et al., 2013). Interestingly, despite biofilm grown in lab condition are characterized by a single microbial species, natural biofilms present polymicrobial communities organized in stable bacterial consortia (Flemming et al., 2007; Vlamakis et al., 2013). The spatial distribution of the species in biofilm is also driven by oxygen concentration, with decrease from the external structure into the biofilm center until is completely depleted. Thus, microorganisms with aerobic metabolism are located in the upper layers of the biofilm, while anaerobic bacteria find favorable niches deeply in the biofilm (Schramm et al., 1996).

In addition, biofilms drive a number of biogeochemical processes in different environments, as soil and water, earning an important position in biotechnological applications, as the production of fine chemicals and the degradation of wastewater and solid waste (McDougald et al., 2011). However, biofilm developed on abiotic surface, as medical devices or implants, leads the propagation of bacterial infection in humans contributing to the outbreak of resistant bacteria in nosocomial environments.

Biofilm formation is a vantage for the bacteria population in terms of resistance to antibiotic treatment and environmental stress (Stewart, 2014) and a common mechanism that occurs in biofilm is the acquisition of resistance genes by horizontal transfer, which is facilitated by the high cell density and mobile genetic elements into the matrix (Mah, 2012). The horizontal transfer take place in several ways, for example by plasmid conjugation, as the case of *P. putida* and *Escherichia coli* (Van Meervenne et al., 2014), or using type IV secretion system as occur in *Vibrio cholerae* biofilm (Borgeaud et al., 2015).

In addition, in biofilm structures, bacteria develop complex interaction between individual cells, providing a coordinate strategy to survive in disadvantageous conditions, for example nutrients limitation, in oxygen-limiting conditions or low pH (Rinaudi and Giordano, 2010). Thus, several

environmental and intracellular signals are important for biofilm development, as well as transcriptional and post-transcriptional regulators.

For example, products of primary and secondary metabolism function are considered intracellular signals, as in the case of indole, which if accumulated into the cells promotes biofilm formation in a number of microorganism, as *E.coli*, *Klebsiella oxytoca, Providencia stuartii*, *Citrobacter koseri*, *Morganella morganii*, and *Haemophilus influenzae* (Hu et al., 2010).

Oxygen is one of the most described environmental cues that influence cellular adhesion and biofilm formation. In oxygen-starvation *P*. *aeruginosa* produces more biofilm, with increased antibiotic tolerance and alginate biosynthesis (Schobert and Tielen, 2010). However, *E*. *coli* adherence is reduced in a microaerophilic environment (Landini and Zehnder, 2002).

Iron is another essential nutrient for bacteria but difficult to trace since most of the iron in the environment is stable in inorganic complexes or tightly bound to siderophores (Miethke and Marahiel, 2007). The effect of iron as environmental cue on biofilm formation varies in different bacteria. In *P*. *aeruginosa*, for example, high concentrations of iron induce expression of adhesion factors, while poor iron in the environment inhibit biofilm formation process (Bollinger et al., 2001) and this is also the case of *V*. *cholerae* biofilm formation which is reduced upon iron-limiting conditions (Mey et al., 2005).

Temperature is another crucial signal, especially for pathogens or commensal bacteria, since the host-colonization relies on temperature switches, usually from lower to higher temperature. Concerning *E*. *coli*, temperatures higher than 32°C, typical of the host, inhibit the synthesis of curli adhesins (Olsèn et al., 1993), however at the same temperature, curli are stimulated in pathogenic enteroinvasive (EIEC) or enteroemorragic (EHEC) strains of *E*. *coli* (Cookson et al., 2002). A number of regulators are also employed in biofilm development and sigma factors represent one of the most common classes. They direct RNA polymerase to specific DNA sequences in gene promoter regions, thus allowing correct transcription initiation. RpoS (σ^S) is the key regulator of general stress response and, in *E*. *coli*, it also affects the expression of genes involved in biofilm formation such as curli fibres and cellulose (Gerstel et al., 2003), and genes connected to the biosynthesis of the signal molecule c-di-GMP, which mediates the transition from planktonic to sessile communities (Sommerfeldt et al., 2009). In *P*. *aeruginosa*, the RpoS expression is related to quorum-sensing system. During biofilm formation, expression of RpoS is repressed and *rpoS*-deficient mutants form biofilm better than wild type cells (Whiteley et al., 2000).

Concerning biofilm post-transcriptional regulation, small noncoding RNAs (sRNAs) are the major players. A sRNA can bind and regulate a multitude of target mRNAs and mechanism of action is discussed in details in section 4.1 of this thesis. One example of sRNA involved in biofilm regulation is MtvR, discovered in the highly epidemic clinical isolates *Burkholderia cenocepacia* J2315, *E.coli* and *P. aeruginosa*. MtvR is involved in a number of phenotypes, typical of global regulators, related to growth and survival after several stresses, swimming and swarming motility, resistance to antibiotics but in particular biofilm formation (Ramos et al., 2014).

Furthermore, there are sRNAs which modulate the production of other global regulators; for example, the DsrA sRNA regulates RpoS and H-NS (heath-stable nucleoid-structuring protein) expression, promoting the production of the former and inhibiting the synthesis of the latter (Lease and Belfort, 2000), lately affecting biofilm development in *E. coli*.

Another interesting group is characterized by sRNAs that bind proteins. The best-studied example in *E. coli* is CsrB, a sRNA that interacts and modulates the activity of the post-transcriptional global regulator CsrA. CsrA is a small protein of 61 amino acids that binds to mRNAs and regulates their stability and translation. Although it is commonly a repressor, CsrA is a positive regulator of flagella biosynthesis (Wei et al., 2001) while it represses c-di-GMP synthesis and exopolysaccharide poly-N-acetylglucosamine (PNAG) production, playing a negative role in biofilm formation (Jonas et al., 2008; Wang et al., 2005).

4. *P. aeruginosa* biofilm

P. aeruginosa is considered a model organism to study Gram negative biofilms. This human pathogen can form biofilm on biotic and abiotic surface such as the mucus plugs of the CF lung, catheters and contact lenses, increasing the severity of infections which result in increased difficulty to eradicate the bacterium.

P. aeruginosa biofilm matrix is composed mainly by EPS, proteins and extracellular DNA (eDNA), holding the bacterial cells together and shaping the tridimensional structure necessary for nutrients exchanging, protection and resistance (Ghafoor et al., 2011).

There are several examples about the role of *P. aeruginosa* biofilm in infectious disease, and these include cystic fibrosis pneumonia, chronic wound infections and chronic otitis media. Concerning cystic fibrosis, the presence of biofilm in the lung is considered a clear sign of *P. aeruginosa* chronic infection. The characteristic of this biofilm is the presence of polymorphonuclear leukocytes (PMNs) surrounding the biofilm structures which causes a constant inflammatory state, a massive immune response and tissue damage, ultimately resulting in respiratory failure. However, antibiotic treatment and immune response are not enough to disrupt biofilm formations in the CF lung. In addition, activated PMNs and hydrogen peroxide contribute to induce mutation in the *P. aeruginosa* genome, in particular in *mucA* gene causing the conversion in mucoid phenotype and the overproduction of alginate is an advantage for the entire population since it protects bacteria from phagocytosis and clearance from the lung (Boucher et al., 1997; Mulchay et al., 2014).

Interestingly, biofilms of chronic wound infections and otitis media are also characterized by an excess of PMNs which causes an extreme immune-inflammatory response and tissue damage, but if the infection is eradicated and biofilm removed, tissue recovery is possible.

4.1 Biofilm development

Two models of biofilms development have been described in *P. aeruginosa* (Fig. 10). The first type is based on precise stages and describes the planktonic state *in vitro* and the initial phases of CF lung colonization by *P. aeruginosa* (Fig. 10A). When the infection switches from acute to chronic, and the CF airways are covered by a thick layer of mucus, the biofilm model 2 (Fig. 10B) predominates on model 1 (Hall-Stoodley et al., 2004; Lau et al., 2005).

Based on the first model, the biofilm development in *P. aeruginosa* follows three main stages which are common to other Gram negative bacteria: i) attachment, ii) maturation, iii) dispersion.

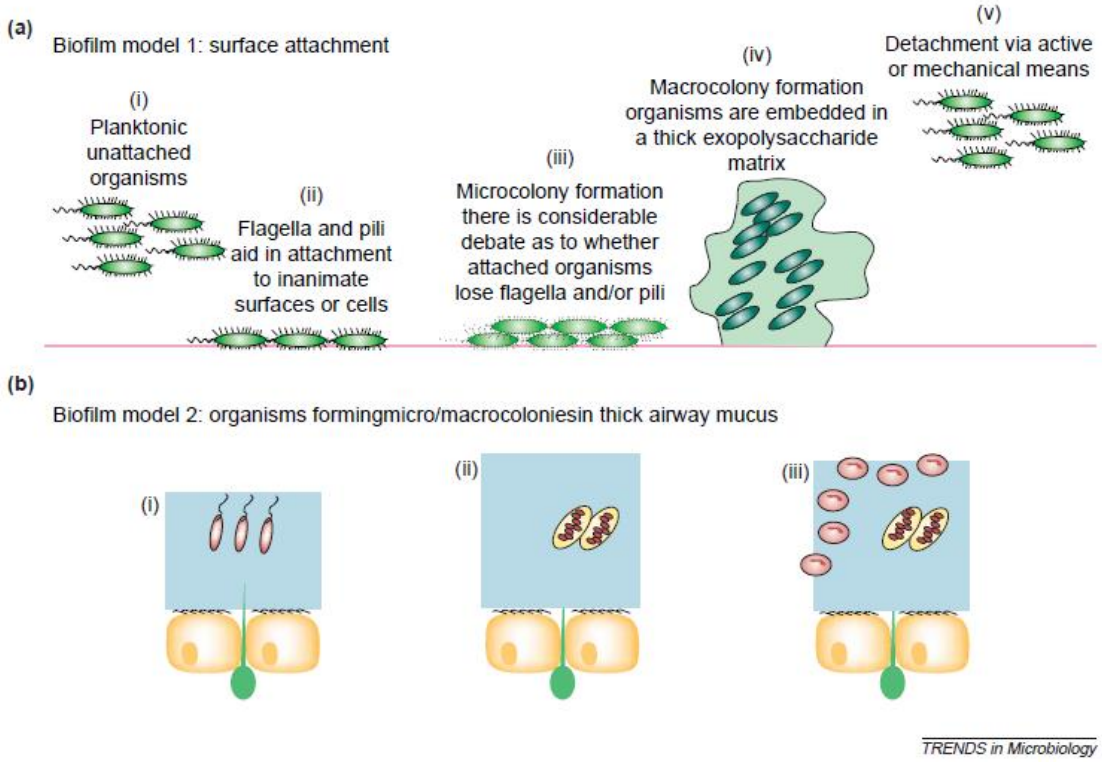

Figure 10. Models of biofilm formation. A) Biofilm model 1 describing the *P. aeruginosa* planktonic state B) Biofilm model 2 occurring in *P. aeruginosa* chronic infection in CF airways. Modified from (Lau et al., 2005).

In the first step, cells are attached to the surface with a reversible adhesion by Van der Waals interactions and are controlled by physiochemical properties as hydrophobicity and electrical charge (Hall-Stoodley et al., 2004; Landini et al., 2010; Sauer et al., 2002; Stewart, 2014).

The initial attachment is followed by population growth and the subpopulations which are irreversible attached to the surface start to form the substrate for microcolonies (Stewart, 2014; Zhao et al., 2013). Specific genetic programs are now activated and stronger cell-cell interactions allow the three-dimensional structures formation, commonly named as “mushrooms”. The surface of these structures is composed by bacteria which move using twitching motility, and at this stage the differentiation from early biofilm to mature biofilm is determined by the EPS production which surrounds the bacterial colonies. In the last stages of biofilm the variety of thickness of the tridimensional structures is important to generate specific physiochemical conditions pivotal for the survival of the all microbial communities (Hall-Stoodley et al., 2004).

In the last step of biofilm life-cycle, a fragmentation of the structure occurs, mainly due to environmental changes as accumulation of oxygen, nitrogen reactive species, and loss of nutrients (Gjermansen et al., 2010; Sauer et al., 2004). Bacterial dispersal represents and effective way to escape from a disadvantageous situation in the host and it relies on the production of enzymes to degrade the EPS matrix (Abee et al., 2011; Nijland et al., 2010).

In biofilm model 2, which describe the cystic fibrosis (CF) airways infected by *P. aeruginosa*, the bacterial cells are embedded in a layer of thickened mucus and rarely come into indirect contact with epithelial surface. Bacteria lose flagella and pili, and convert to a mucoidy phenotype with a massive production of alginate, forming biofilm macrocolonies. Biofilm structures and thick mucus determine a strong protection for *P. aeruginosa* from clearance by neutrophils and macrophages, allowing the establishment of chronic infections (Hassett et al., 2003; Lau et al., 2005).

4.2 Biofilm composition

Bacteria with a planktonic lifestyle and able to form biofilm, present peculiar traits in gene expression and morphology, completely different from motile bacteria (Landini et al., 2010) and as well as in other Gram negative bacteria, fundamental is the role played by the self-produced EPS matrix which represent the major component (from 50 to 90%) of the biofilm biomass compared to microbial cell density.

The EPS components present intermolecular interactions, which determine also the physiology of the population in the biofilm. Moreover, EPS fill the spaces without cells in biofilm resulting in more stable structure (Flemming et al., 2007; Ryder et al., 2007).

P. aeruginosa produces at least three exopolysaccharides: alginate, Psl and Pel, each of them plays an important role in biofilm shaping and stability. *P. aeruginosa* PAO1 strain produces both Pel and Psl, while PA14 synthesizes only Pel (Friedman and Kolter, 2004a, 2004b).

Alginate, which is overproduced by clinical isolates in CF patients, is a linear polyanionic exopolysaccharide composed by uronic acids. Alginate production requires a number of enzymes and precursor substrates with a fine control on its production. Its production involves 11 operons and the *algC* gene that is located elsewhere in the genome (Gacesa, 1998; Ramsey and Wozniak, 2005). The coordinated expression of these genes is due to several transcriptional regulators, including AlgR, Vfr, AlgB, AlgP (Hp-1), IHF, CysB and AmrZ (Baynham et al., 1999; Leech et al., 2008; Pritchett et al., 2015; Whitchurch et al., 2005; Wozniak and Ohman, 1994) whose expression

is controlled by the alternative sigma factor AlgU which also directs the transcription of the alginate biosynthesis operon (Damron and Goldberg, 2012; Jones et al., 2010; Wozniak and Ohman, 1994).
Although alginate is considered the main component of *P. aeruginosa* biofilm, the presence of the two others important EPS, Pel and Psl, allows to non-mucoid strains lacking alginate biosynthesis machinery to form biofilm (Mann and Wozniak, 2012; Wozniak et al., 2003). Pel is a glucose-rich polymer synthesized by the *pelA-G* operon (Jennings et al., 2015) essential for the formation of a pellicle at the air-liquid interface and recently it has been described as important for *P. aeruginosa* PA14 biofilm scaffold, in particular in the early stages of formation (Jennings et al., 2015).
Psl is a mannose and galactose polymer, produced by *pslA-O* operon, important for cell-cell interaction and for cell-surface adhesion in the first stages of biofilm development. It also contributes to maintain the stability in mature biofilms, covering the mushroom surfaces (Ma et al., 2007; Zhao et al., 2013). Furthermore, Psl acts also as a signal to stimulate biofilm formation and in non-mucoid strains is the main component which confers stability and promotes cell-cell interaction (Colvin et al., 2012; Ghafoor et al., 2011; Ma et al., 2012; Yang et al., 2011).
Since Psl is known to promote adhesion and cellular aggregation, recent studies suggests its role in promoting immune evasion and effectively antibodies generated against Psl can provide protection against *P. aeruginosa* (DiGiandomenico et al., 2012; Mishra et al., 2012). The role of Pel and Psl in biofilm formation is strain specific (Ghafoor et al., 2011), since PAO1 biofilm relies mainly on Psl (Yang et al., 2011), while Pel is important for mature biofilm in PA14 (Colvin et al., 2011). However Pel and Psl are both required to have a proper biofilm development in PAO1 (Colvin et al., 2012).
Beside the important role of EPS, *P. aeruginosa* produces extracellular DNA that is an important biofilm determinant in several bacterial biofilm, for nutrient diffusion, antibiotic resistance and structural stability. Indeed, eDNA interacts with psl fibers forming a web which constitute a scaffold for the entire biofilm structure (Wang et al., 2015). Furthermore, eDNA facilitates twitching motility by maintaining the right cell alignments (Gloag et al., 2013)

4.3. Gene regulation in biofilm formation

Generally, microorganisms form biofilm in response to several factors including nutritional cues and biochemical changes or, in some cases, to sub-inhibitory concentrations of antibiotics. The switch from planktonic to sessile lifestyle undergoes genetic rearrangement resulting in specific phenotypic expression and requires secondary messengers, transcriptional and post-transcriptional regulators (Moradali et al., 2017).

C-di-GMP: The secondary messenger c-di-GMP stimulates the biosynthesis of adhesins and exopolysaccharide in biofilm formation, negatively affecting the *P*. *aeruginosa* motility. This regulation contributes to determine the conversion into sessile phenotype.

C-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGC) and is processed by phosphodiesterases (PDE). The regulatory role of c-di-GMP is exerted by its binding to effectors (c-di-GMP receptors) (Hengge, 2009). In *P*. *aeruginosa*, at least four c-di-GMP effectors are present including FleQ, PelD, Alg44, and PilZ (Boyd and O'Toole, 2012).

FleQ is a c-di-GMP-binding transcription factor, which generally activates flagella biosynthesis but it has been described also as a repressor for the transcription of the *pel* operon in the absence of c-di-GMP and an activator when is bound to c-di-GMP (Baraquet et al., 2012; Hickman and Harwood, 2008; Matsuyama et al., 2016).

PilZ is described as a c-di-GMP binding protein and is involved in twitching motility regulating type IV pili biogenesis (Alm et al., 1996).

PelD, which belong to PilZ family protein, contributes to Pel biosynthesis in *P*. *aeruginosa*. It is also described as a c-di-GMP binding protein and it presumably requires activities of DGCs and PDEs as a means to ensure the regulated production of Pel (Baker et al., 2016; Ha and O'Toole, 2015; Yang et al., 2011).

Alg44 also belongs to the PilZ family protein and is involved in alginate biosynthesis regulation (Remminghorst and Rehm, 2006; Whitney et al., 2015).

P. *aeruginosa* PAO1 and PA14 strains produce roughly 40 DCGs and PDEs, to modulate the c-di-GMP levels in the cell and regulate the EPS production during the biofilm formation (Hickman et al., 2005; Kuchma et al., 2007; Kulasakara et al., 2006). There are currently several examples of DGC and PDE in regulating motility and biofilm formation affecting the local concentration of the secondary messenger c-di-GMP, as the DGCs RoeA and SadC. RoeA is involved in biofilm

formation regulating the polysaccharides production, SadC is mainly involved in modulation of flagellar motility (Bernier et al., 2011; Merritt et al., 2010).

Two-component systems (TCS): Expression of the *pel* and *psl* genes for exopolysaccharide production in *P. aeruginosa* can be also regulated by GacA/GacS two-component system (TCS). GacA/GacS TCS is a global signal transduction system highly conserved in Gram-negative bacteria (Heeb and Haas, 2001). GacS is a sensing transmembrane protein that can respond to environmental signals and activate the cognate response regulator GacA by phosphorylation, as reported in Fig. 11 (Pernestig et al., 2001; Rich et al., 1994). GacA in turn triggers the expression of two small regulatory RNA molecules RsmY/RsmZ, which allow the translation of mRNAs target, as those involved in biofilm formation, antagonizing the repressors RsmA/RsmE (Lapouge et al., 2007; Valverde et al., 2003). Furthermore, RsmA binds *pslA* in the region spanning the ribosome binding site and forming a secondary structure which prevent the ribosome binding (Irie et al., 2010).

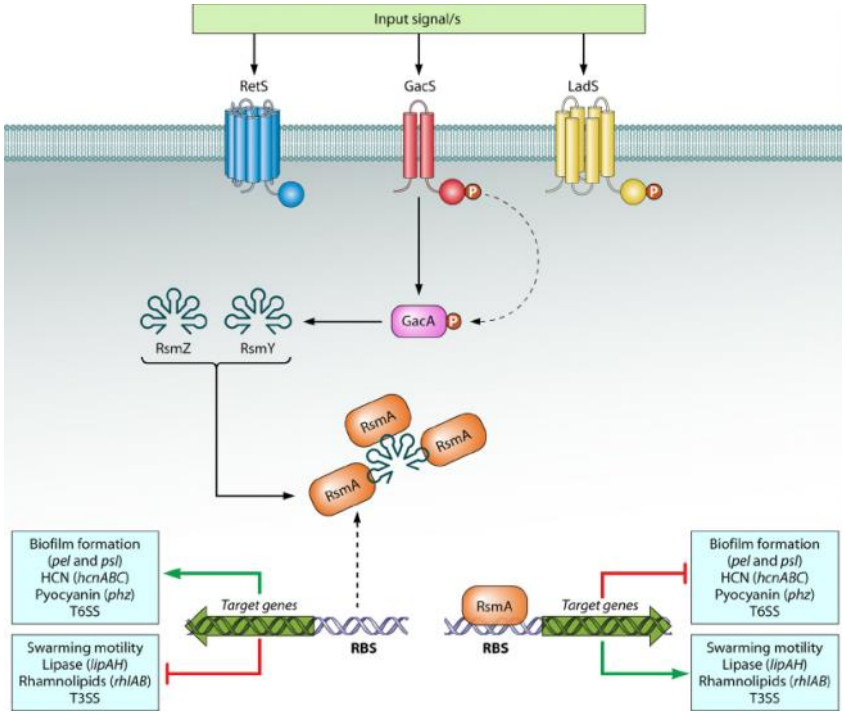

Figure 11. Schematic diagram of the two-component system regulation of biofilm formation and virulence in *P. aeruginosa*. Unknown environmental cues received by the input domains of the three membrane-associated sensor kinases (GacS, LadS and RetS) activate or repress the expression of genes necessary for acute or chronic infection. Modified from (Jimenez et al., 2012)

GacA/GacS activity is in turn inversely controlled by the TCS hybrids RetS and LadS (Sonnleitner and Haas, 2011a; Ventre et al., 2006; Wolfgang et al., 2004). RetS negatively regulates *rsmYZ* expression and its inactivation results in higher *psl* expression levels and hyper attachment, and suppression of the type III secretion system (TTSS). On the contrary, LadS positively controls sRNA levels, and its mutation results in decreased attachment due to reduced Psl production, and elevated TTSS expression. These evidences suggest that LadS may function to balance the RetS regulatory effect on genes important for *P. aeruginosa* acute infection (Heeb and Haas, 2001; Jimenez et al., 2012; Ventre et al., 2006).
Two distinct structurally RsmA homologues, named RsmN/RsmF, if expressed *in trans* could complement an *rsmA* mutant and the relative phenotypes as swarming and pyocyanin production. Furthermore, the deletion of *rsmA, rsmN, rsmF,* results in a massive biofilm production (Marden et al., 2013; Morris et al., 2013).

AlgC: Biofilm formation is also metabolically regulated, as for the example by AlgC, a crucial enzyme involved in biosynthesis of sugar precursors for Pel, Psl, alginate and LPS production. AlgC is a phosphoglucomutase (PGM) which is required for the synthesis of mannose 1-phosphate and glucose 1-phosphate (Coyne et al., 1994; Davies and Geesey, 1995; Olvera et al., 1999). AlgC is a checkpoint enzyme since the overproduction of one exopolysaccharide precludes the expression of the others in particular environmental conditions. Indeed, the expression of *algC* is under the transcriptional control of the alternative sigma factor AlgU (σ^{22}), homologue of the σ^{E} in *E. coli*, which drives and coordinates the stress response (Jones et al., 2010; Yu et al., 1995). Furthermore, in *P. aeruginosa algC* gene is regulated at the post-transcriptional level by the sRNA ErsA, one of the main topics of this thesis. Specifically in PAO1, as shown in Ferrara et al., 2015, ErsA directly exerts a negative post-transcriptional regulation on *algC*. Furthermore, since ErsA is also transcriptionally regulated by AlgU, it has been proposed an incoherent feed forward loop implemented by AlgU for the AlgC regulation.

AmrZ: The ribbon-helix-helix transcription factor AmrZ is part of the AlgU regulon and modulates several pathways important for biofilm formation. AmrZ is composed by three segments: the flexible N-terminal motif (Baynham et al., 1999), the ribbon-helix-helix domain which is involved in the interaction AmrZ-DNA (Pryor et al., 2012), and the C-Terminal domain which is essential for efficient AmrZ-mediated activation and repression of targets (Xu et al., 2016).

AmrZ functions as both an activator and repressor; in particular it activates transcription of the alginate biosynthesis operon and represses flagella-mediated motility, Psl production and c-di-GMP synthesis (Baynham et al., 2006; Baynham and Wozniak, 1996; Jones et al., 2014; Ramsey and Wozniak, 2005; Tart et al., 2006). Conversely, the absence or lower concentration of AmrZ, as in non-mucoid strains, *P. aeruginosa* develops a Psl-based biofilm, enhances the expression of c-di-GMP and increases the twitching motility by expressing flagellar proteins (Jones et al., 2014; Petrova et al., 2014). Although AmrZ has been described as a pleiotropic regulator of biofilm and motility, its direct impact on biofilm has not been elucidated yet.

Quorum sensing: Several works highlighted the role of the three QS systems in motility and biofilm formation. Specifically, Davies et al. (Davies et al., 1998) have discussed the role of the *las* system, showing that the deletion of *lasI* results in a flat biofilm and less resistant to sodium dodecyl sulfate compared to *P. aeruginosa* wild-type. Furthermore LasR binds the promoter region of *psl* operon affecting Psl synthesis (Gilbert et al., 2009). The *rhl* system is also linked to exopolysaccharide production since has been reported to enhancing Pel polysaccharide biosynthesis (Sakuragi and Kolter, 2007). It controls swarming and twitching motility (Daniels et al., 2004; Patriquin et al., 2008), and is essential for rhamnolipids production (Dusane et al., 2010). All these phenotypes are important in microcolonies formation, cell-cell interaction and 3D-shaped biofilm development (Pamp and Tolker-Nielsen, 2007; Van Gennip et al., 2009).

Finally, the cytotoxic virulence factors, lectins LecA and LecB, has been proposed to contribute to biofilm development in *P. aeruginosa*, since LecA and LecB mutants form thin biofilms as compared to the wild-type bacteria (Diggle et al., 2006; Tielker et al., 2005). Both LecA and LecB expressions are regulated by the *rhl* QS system (Winzer et al., 2000).

Concerning the third QS system, *pqs*, there are evidences of increased biofilm formation in presence of exogenous PQS, however the molecular basis of this phenomenon have been not totally established (Diggle et al., 2003).

5. RNA-based mechanisms of post-transcriptional regulation

The dynamic and intricate regulatory network which modulates the *P. aeruginosa* virulence is fine-tuned regulated at every stage in the processing of genetic information. This strategy is important to respond quickly and accurately to environmental cues, avoiding high energy waste which could affect the whole population fitness, resulting in adaptation. The adaptation process frequently affects the proteome profile and this mechanism is controlled by the transcriptional regulation, which ultimately leads to the production of different subsets of proteins, and it is enriched by post-transcriptional regulation (Romeo et al., 2013).

Post-transcriptional regulators are in general designated to modulate RNA decay, translation initiation efficiency or transcript elongation. In bacteria, these regulators include sRNAs and RNA-binding proteins (RBPs). Other RNA regulatory elements found in bacteria include riboswitches encoded in untranslated regions (UTRs) of mRNAs and CRISPR elements (Breaker, 2011; Karginov and Hannon, 2010).

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas (CRISPR associated genes) systems, for example, exist in bacteria and archaea to provide protection from invading viruses and mobile elements. Specifically, CRISPR-Cas9 uses RNA-guided nucleases to cleave foreign genetic elements. There are three types of CRISPR-Cas systems but the main characterized is type II. It consists of the nuclease Cas9, which processes the CRISPR transcript in CRISPR RNA (crRNA). The crRNA use sequences of complementarity to guide the nuclease Cas to target DNA for degradation, currently used in biotechnology applications (Garneau et al., 2010; Gasiunas et al., 2012). Interestingly, it was also revealed a role for a Cas9-based typeII CRISPR-Cas system as a post-transcriptional regulator (reviewed in (Sampson and Weiss, 2014) and there are example of CRISPR-Cas system, as Type I-F in *P. aeruginosa* PA14, involved in host-defence targeting bacterial endogenous genes. Type I-F CRISPR-Cas system degrades mRNA of the QS regulator *LasR*, thus allowing the bacteria evasion and host pro-inflammatory response (Li et al., 2016).

Recently, in addition to these post-transcriptional mechanisms, the specific alteration of ribosome function has been discovered as a novel post-transcriptional regulatory element, not completely explored yet (Little et al., 2016). Several ribosomal proteins are subject of modification as methylation and acetylation or changes in number of C-terminal amino acid residues. Although the physiological meaning of the ribosomal post-transcriptional modification is still unknown, it has

been suggested a role in ribosomal specialization which would result in altered ribosome-mRNA binding or in translational efficiency, ultimately resulting in a changed protein profile (Nesterchuk et al., 2011).

5.1 Regulation by non-coding small RNAs

RNA-mediated regulation has been described in all kingdoms of life. In eukaryotes, microRNAs (miRNAs) or small interfering RNAs (siRNA) are a large class of very short (21-25 nt) non-coding RNAs that affect translation and degradation of target mRNAs by antisense base pairing and may fine-tune the expression of about 30% of all mammalian protein-encoding genes (Wahid et al., 2010). MicroRNA genes are transcribed by RNA polymerase II as large primary transcripts (pri-microRNA) and processed by a protein complex containing the RNase III enzyme (Drosha), in a shorter fragment named precursor microRNA (pre-microRNA). This precursor is subsequently transported to the cytoplasm where it is processed by a second RNase III enzyme, DICER, to form a mature microRNA of approximately 22 nucleotides. Mature microRNAs are assembled into ribonucleoprotein complexes known as RNA-induced silencing complex (RISC) which can modulate stability and/or translation of multiple target mRNAs. RISCs are composed of a variety of proteins such as RNA-binding proteins, RNA helicases, and nucleases (Hesse and Arenz, 2014; Lee et al., 2002; Shukla et al., 2011).

The first miRNA was discovered in *C. elegans* in 1993 and reported in mammalian systems in 2001. Nowadays, miRNAs are known to be pivotal regulators of processes such as cell cycle control, apoptosis and several developmental and physiological processes including stem cell differentiation, hematopoiesis, hypoxia, cardiac and skeletal muscle development, neurogenesis, insulin secretion, cholesterol metabolism, aging, immune responses and viral replication. In addition since their role in several disease, as cancers, heart and neurological disease, they are intensely studied as candidates for diagnostic and prognostic biomarkers and predictors of drug response (Hesse and Arenz, 2014; MacFarlane and Murphy, 2010).

Bacterial sRNAs are typically defined as non-coding RNA molecules (50-500 nt), either generated through processing or synthesized as primary transcripts, which act as regulators that can affect translation initiation, transcription termination or stability of one or more target mRNAs. Although sRNAs are non-coding, there are some example of sRNAs that can encode for polypeptides and concomitantly act as riboregulators, as *S. aureus* RNAIII and *E. coli* SgrS which produce the δ-

haemolysin and the SgrT protein, respectively (Janzon et al., 1989; Wadler and Vanderpool, 2007). In pathogenic bacteria, as *P. aeruginosa*, sRNAs are involved in regulation of virulence gene expression (Heurlier et al., 2004; Kay et al., 2006; Oglesby et al., 2008).

The sRNA regulation takes place after the binding with complete or incomplete complementarity to the target mRNA. In addition, sRNAs can modulate protein activity, in some cases by mimicking other nucleic acids.

Bacteria take advantage in using sRNAs-mediated regulation in term of reduced metabolic cost, multiple interconnected levels of regulation, faster regulation and unique regulatory properties (reviewed in (Beisel and Storz, 2010)). Furthermore the coupled transcriptional and post-transcriptional regulation has been shown to increase the dynamics of gene expression, avoiding the loss of genetic information (Beisel and Storz, 2011).

Depending on target interaction and regulation, sRNAs can be divided in two classes: i) *cis*-encoded antisense sRNAs, synthesized from the strand complementary to the mRNA target and ii) *trans*-encoded antisense sRNAs, synthesized from a different genomic location respect to the mRNA target. The *cis* and *trans* acting sRNA regulate mRNAs with different mechanisms summarized in Fig. 12, as: i) blocking translation occluding the ribosome-binding site (RBS) in the 5′ untranslated region of target mRNAs, ii) recruiting the RNase E in order to degrade the mRNA after base-pairing with sRNA, iii) generate the sRNA/Hfq/RNase E complex that favors RNase E attacks; this complex then becomes a specialized RNA decay machine, iv) binding in the 5′-UTR the inhibitory sequence of RBS that becomes available, allowing initiation of translation.

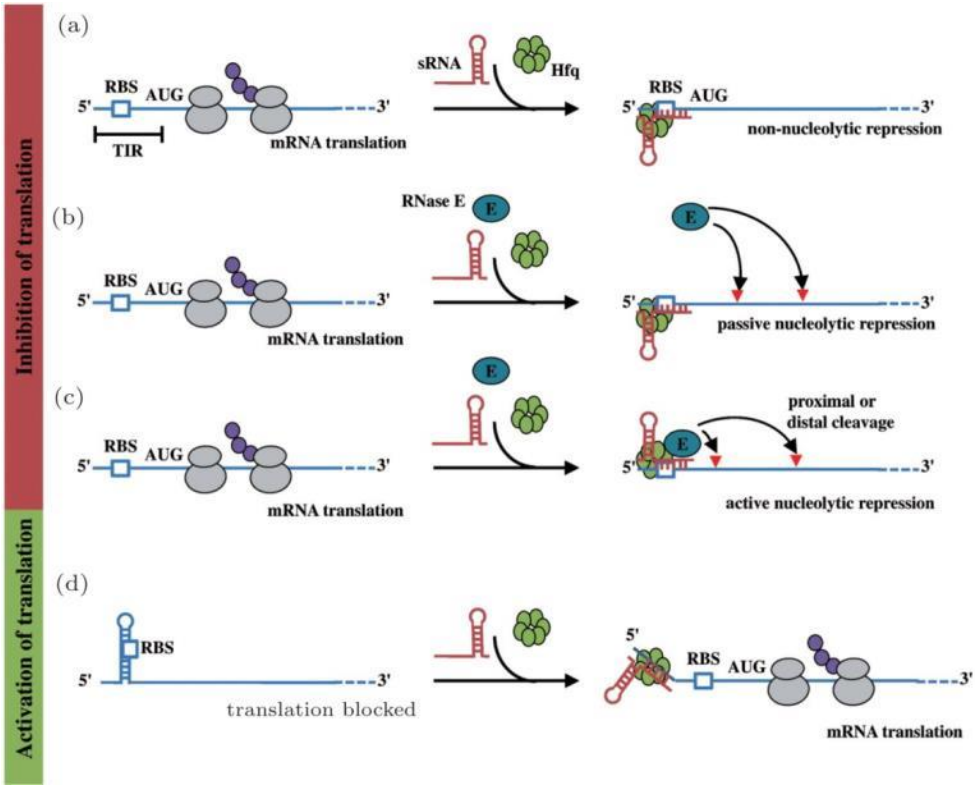

Figure 12. Small RNA-based regulatory mechanisms of mRNA expression. Inhibition of translation: (a) sRNA blocks translation of mRNA binding and occluding the ribosome binding site (RBS) of target mRNA. (b) sRNA occludes the RBS leading the exposition of the target to degradation by RNaseE. C) sRNA, Hfq and RNaseE form a complex to actively degrade the mRNA.
Activation of translation: (d) The RBS region of mRNA is embedded in a secondary structure inhibiting the translation. The sRNA binding allows the opening of the structure to be available for translation. Modified from (Lalaouna et al., 2013).

The majority of *cis*-encoded RNAs have been discovered in plasmids, phages and transposons (Brantl, 2002, 2007). They act as regulators base pairing with complete complementarity to the target mRNA and are involved in replication initiation, conjugation efficiency, suicide, transposition, mRNA degradation and translation initiation. *Cis*-RNAs encoded from plasmid and expressed constitutively, modulate the regulation of replication, conjugation or segregational stability. By contrast, chromosomally encoded antisense RNAs are expressed only under certain conditions, as in stationary phase (e.g. GadY (Opdyke et al., 2004) (Opdyke et al., 2004)) or under iron stress (e.g. IsrR (Dühring et al., 2006)).

A widespread *cis*-acting RNA element in mRNAs regulation is the metabolite-sensing riboswitch, a portion of RNA which changes the secondary structure in response to environmental stimuli. This conformational arrangement modulates the expression of the coding sequence (Breaker, 2011; Smith et al., 2010). Typically, the riboswitch feedback regulates the associated genes, which are typically involved in the uptake or metabolism of the sensed metabolite (Dambach and Winkler,

2009; Grundy and Henkin, 2006). According to sequence and structure conservation, riboswitches seem underrepresented in Gram-negative pathogens but may regulate almost 2% of the genes in Gram-positive bacteria such as *S. aureus* and *Listeria monocytogenes* (Nudler and Mironov, 2004; Waters and Storz, 2009). Another major class of *cis*-acting regulatory RNAs is represented by RNA-thermosensors. In contrast to highly specific metabolite-binding riboswitches, they respond to a fairly global physical signal, i.e. the intracellular temperature. These RNA thermometers are usually characterized by irregular stem-loop structures (ROSE, FourU motifs) that sequester the Shine-Dalgarno (SD) region of the mRNA target (Narberhaus et al., 2006). At low temperature (below 30°C), these hairpins are typically stable and, hence, the ribosome binding site (RBS) is not accessible for translation. Increasing temperature melts the stem-loop structure to promote translation (Altuvia et al., 1987, 1989, 1991)

The most studied *trans*-encoded sRNAs regulate the target with partially complementarity and usually, in Gram negative bacteria, they require a RNA-binding protein as Hfq for the mRNA interaction (Waters and Storz, 2009). The binding of Hfq can affect the structure of sRNA and target mRNAs or can modulate the local concentration of the two RNAs to facilitate the interaction. Furthermore Hfq protects many sRNAs from degradation, most likely by binding to RNase E cleavage sites within these sRNAs (Ramos et al., 2014; Soper et al., 2010).

The interaction *trans*-RNA/mRNA can occur in the surroundings of the ribosome binding site (RBS) on the target sequence, causing a competition with the ribosome that interferes with the translation. However, this mode of regulation has been reconsidered, since a number of *trans*-RNAs affect the translatability of their targets binding a region not including the RBS, or modifying the secondary structure and changing the mRNA stability (Liu and Camilli, 2010; Storz et al., 2011).

RNA-binding proteins play also a crucial role in post-transcriptional regulation. Some of these proteins and their mechanism of action are well described, as for CsrA and Hfq mentioned before. The presence of RBPs was reported in *E. coli* first, more than 20 years ago (Mu Ya Liu et al., 1995; Muffler et al., 1996). Recently, several studies increased the panel of information, in particular on the mechanism of action. Specifically, bacterial RBPs bind RNA molecules and regulate translation initiation, stability of their target, using mechanisms as: i) modifying the target RNAs in order to be more susceptible to RNases, ii) changing the accessibility of the RBS of mRNA targets and interfering with the ribosome binding, iii) acting as a chaperone for the interaction of the RNA target with other effector molecules, and iv) modulating the transcription terminator/antiterminator structure formation (Van Assche et al., 2015).

5.2 Genome-wide methodologies for investigating sRNAs regulation

Deciphering the sRNA–target interactome is a complicate but crucial step to understanding the biological role of a sRNA. Computational analyses are useful to generate a list of putative target mRNAs, basing the prediction of sRNA-mRNA interaction on several algorithms to calculate a hybridization score. However, bioinformatics tools may generate biased information, and since they mainly focused on translational starting site, sequences in the surroundings important for the interaction with the sRNA could be missed. For this reason, bioinformatics data need experimental *in vitro* and *in vivo* validations. These combined approaches provided new insights in the knowledge about sRNA physiological meaning in a number of different strains (Backofen and Hess, 2010; Pichon and Felden, 2008; Sharma and Vogel, 2009; Storz et al., 2011).

In addition, a number of high-throughput techniques have been recently introduced to expand the possibilities to identify important mechanisms of sRNAs translational regulation with a more detailed resolution. The most recent genome-wide methodologies with a short description are shown in Fig. 13.

Technology	Protocol	Applications	
Ribo-seq	Crosslink RNA to ribosomes, purify and degrade unprotected RNA. Reverse transcribe ribosome-protected RNA to cDNA and sequence.	Profiling sRNA effects on translation Deciphering the sRNA-target interactome (alongside computational analyses)	
High Throughput Point Mutagenesis	FACS sort cells containing library of target gene mutants fused to GFP. Amplify and sequence interesting targets.	Determine the effect of sRNA variants on protein expression Examination of mRNA riboswitches	
RIP-seq	Immunoprecipitate target protein crosslinked to RNA interaction partners. Degrade unbound RNA. Reverse transcribe protected RNA to cDNA and sequence.	Capturing the sRNA-protein interaction network	
RIL-seq	As RIP-seq but with additional RNA ligation and computational analysis steps.	Identifying protein-mediated sRNA-mRNA interactions	

Figure 13. Emerging genome-wide methodologies. Overview of the new technologies developed to study mechanisms of translational regulation. Modified from (Grenga et al., 2017)

However, sRNAs can also change the transcriptome profile, for example in response to particular environmental conditions or stress response, or can modulate transcript abundance regulating the degradation machinery. Therefore, the experimental approaches have included also studies of sRNA-dependent changes in gene expression (by expression microarrays, deep sequencing, or

comparative protein analyses), global searches for mRNAs that bind to a particular sRNA-regulated protein, and genetic screens for changes in reporter gene expression or altered growth phenotypes. Furthermore, sRNA-mRNA interaction is also influenced by the RNA secondary structure which could be affected by several factors (Meyer, 2017). Thus, the plasticity of sRNA regulatory networks which confers both versatility and efficiency, requires a multi-omics approach, integrating *in silico* analyses, proteomic and transcriptomic approaches in a comprehensive models for bacterial adaptation to external challenges. An example of this combined approaches has been published in 2013, and is based on the comparison of relative changes in total mRNA with translational changes (polysome fractions) and protein abundance to provide a comprehensive study of bacterial stress responses in *Rhodobacter sphaeroides* (Berghoff et al., 2013).

5.3 Small RNAs in *P. aeruginosa* virulence

Pseudomonas aeruginosa is one such example where sRNAs have been identified as critical elements of cellular adaptation and virulence. However, among the roughly 200 sRNAs discovered in *P. aeruginosa* in the past years with several high-throughput techniques, only for a few of them there are informations available about the physiological roles (Sonnleitner et al., 2012).

RsmY and RsmZ are probably the most known sRNAs involved in the switch acute-chronic infection in *P. aeruginosa*. As mentioned before, these two RNAs are part of the two component system Gac/Rsm and they are involved in the regulation of the translational repressor protein RsmA which interferes with the expression of virulence and metabolic genes (Lapouge et al., 2007; Valverde et al., 2003). For example, the Gac/Rsm system has been described to positively regulate the expression of the quorum-sensing signal *N*-butanoyl-homoserine lactone (C4-HSL) and of extracellular virulence factors, such as hydrogen cyanide (HCN), pyocyanin, and elastase (Kay et al., 2006).

Another interesting and well described sRNA is CrcZ, linked to carbon metabolism. CrcZ has five CA-motifs and it antagonizes the function of the RNA-binding protein Crc in catabolite repression. When preferred carbon source are not available, CrcZ binds to and sequesters Crc, allowing synthesis of the required catabolic enzymes for utilization of not preferred carbon source as mannitol or acetamide (Linares et al., 2010; Sonnleitner and Haas, 2011b). The expression of CrcZ is driven by CbrA/B two-component system in a σ^{54}-dependent manner. The type of carbon source determines the CrcZ level, which is low in the presence of a preferred carbon source, like succinate,

and elevated in the presence of an intermediate substrate, like glucose, and high in the presence of a non-preferred carbon source such as mannitol. In addition, the CbrAB/Crc pathway is also involved in virulence traits as pyocyanin production and in the synthesis of porins (Linares et al., 2010).

P. aeruginosa expresses the sRNA PhrS to connect QS to oxygen metabolism. PhrS is a 213 nt long sRNA, predicted by biocomputation and isolated by an RNomics approach in combination with Hfq-mediated co-immunopreciptation. PhrS regulates the expression of PQS and pyocyanin. In particular, it modulates PQS synthesis during the transition from aerobic to hypoxic conditions and this role has been suggested to stabilize PQS levels in fluctuating oxygen concentration (Sonnleitner et al., 2011). The *phrS* gene expression is dependent by ANR regulator and its promoter is activated in limited oxygen condition. Interestingly, to regulate PQS expression, PhrS binds the RBS region of a small coding sequence (*uof*) upstream *pqsR* which is co-translated with *pqsR*. Thus, PhrS regulates *pqsR* translation by modulating translation of *uof* (Sonnleitner et al., 2011; Sonnleitner and Haas, 2011b).

In *P. aeruginosa* regulation of iron homeostasis is crucial to assure adaptation avoiding toxic effect in iron-excess environment. To achieve this fine-tuned balance, *P. aeruginosa* produces siderophores, chelators and iron storage proteins such as bacterioferritin (BfrB) and enzymes that neutralize oxidative stress, e.g., catalase (KatA) and iron-containing superoxide dismutase (SodB) (Cornelis et al., 2013; Cornelissen and Sparling, 1994; Takase et al., 2000).

Furthermore, in most Gram-negative bacteria the ferric uptake regulator Fur complexed with Fe^{2+} ions prevents transcription of genes involved in iron acquisition and of iron-regulated genes involved in virulence. In *E. coli* Fur was characterized as a positive regulator of genes such as *acnA*, *fumA, ftnA, bfr*, and *sodB* encoding for iron-binding enzymes and Fe-superoxide dismutase (Chen et al., 2007; Seo et al., 2015). This Fur positive regulation is exerted through repression of a small regulatory RNA, RyhB, which causes degradation of these mRNAs when it is expressed (Massé et al., 2005b; Prévost et al., 2007).

Two ortholog of RyhB have been bioinformatically discovered in *P. aeruginosa*, PrrF1 and PrrF2. These sRNAs are encoded in tandem, with 95% of identity, and provided with a Fur binding site upstream their coding sequence. In iron-limiting condition they are both synthesized since not repressed by Fur (Oglesby et al., 2008; Sonnleitner and Haas, 2011a). As for RyhB, PrrF1 and PrrF2 block the translation of *sodB* mRNA, occluding the RBS and leading its degradation. Recently other targets and regulatory mechanism have been described for PrrF1, including

interaction with a 'sponge' derived from the 3′ end of the *katA* transcript, thus antagonizing the regulatory interaction of the 5′ end of the same mRNA (Han et al., 2016; Zhang et al., 2017).

In addition to their role in iron metabolism, PrrF represses *P. aeruginosa* genes involved aerobic and anaerobic metabolism, in anthranilate and catechol degradation (Oglesby et al., 2008). Anthranilate is usually converted in catechol, but PrrF sRNAs regulate this pathway channeling the anthranilate in the PQS biosynthetic network, affecting the expression of virulence genes (Reinhart et al., 2017). The expression in tandem of *prrF1* and *prrF2* unique in *P. aeruginosa,* allows the synthesis of a third sRNA, PrrH which is expressed in iron-deplete condition and in stationary phase of growth. This sRNA is involved in heme metabolism and homeostasis, and in heme-starvation. PrrH represses *achAB* and *sdhCDAB*, which are also known to be targets of the PrrF sRNAs, and NirL, a protein involved in biosynthesis of heme (Reinhart et al., 2017).

Finally, in the lab that hosted this thesis work, two novel sRNAs, ErsA (Ferrara et al., 2012, 2015) and ReaL (Carloni et al., 2017), have been characterized as post-transcriptional regulators in *P. aeruginosa* virulence networks as exopolysaccharides biosynthesis and quorum sensing, respectively. A third one, PesA (Ferrara et al., 2017), influences *P. aeruginosa* virulence and modulates pyocin S3 production. Both ErsA and PesA have been topics of this thesis work. For an introduction to PesA characterization, I refer you to the enclosed paper, while a brief summary of previous ErsA characterization is appended below.

5.3.1 ErsA

ErsA is a 132 bp sRNA, encoded from the same genomic context of the well-characterized *Escherichia coli* sRNA Spot 42 (Beisel and Storz, 2011), the intergenic region between the genes encoding for the highly conserved proteins PolA (DNA polymerase I) and EngB (GTPase of unknown function). Because Spot 42 and ErsA do not share obvious sequence and structural similarities, it is difficult to definitively conclude that they are orthologues. Even though their genes are located in a highly conserved genomic context and have a comparable size and the same transcription direction, ErsA is not involved in carbon source catabolism like Spot 42 (Ferrara et al., 2015). Moreover, the transcription of ErsA is strictly dependent on the envelope stress responsive sigma factor AlgU (σ^{22} /AlgT) (Ferrara et al., 2015; Potvin et al., 2008), the functional homologue of *E. coli* σ^{E} (Yu et al., 1995), that impacts *P. aeruginosa* pathogenesis. As mentioned above, AlgU is essential for the production of the exopolysaccharide alginate, whose overproduction confers a mucoid phenotype and provides *P. aeruginosa* with a selective advantage for survival in the cystic fibrosis lung (Jones et al., 2010). AlgU drive the expression of several transcriptional regulators which regulate the Pel, Psl, alginate, rhamnolipids and LPS production, the main crucial element is the phosphomannomutase AlgC. Interestingly, in (Ferrara et al., 2015) it has been shown that ErsA directly exerts a negative post-transcriptional regulation on the *algC* gene. ErsA-*algC* mRNA interaction was predicted bioinformatically using the web tool TargetRNA (Tjaden, 2008). Direct interaction was validated *in vitro* by electromobility shift assay and *in vivo* in PAO1 using the translational fusion *algC*::*sfGFP* with and without ErsA overexpression. Consistent with these results, it has been suggested that ErsA could be an additional σ^{22}-dependent regulation converging on AlgC, and the expression of AlgC is subjected to an incoherent feed-forward loop involving σ^{22} and ErsA as summarized in Fig. 14.

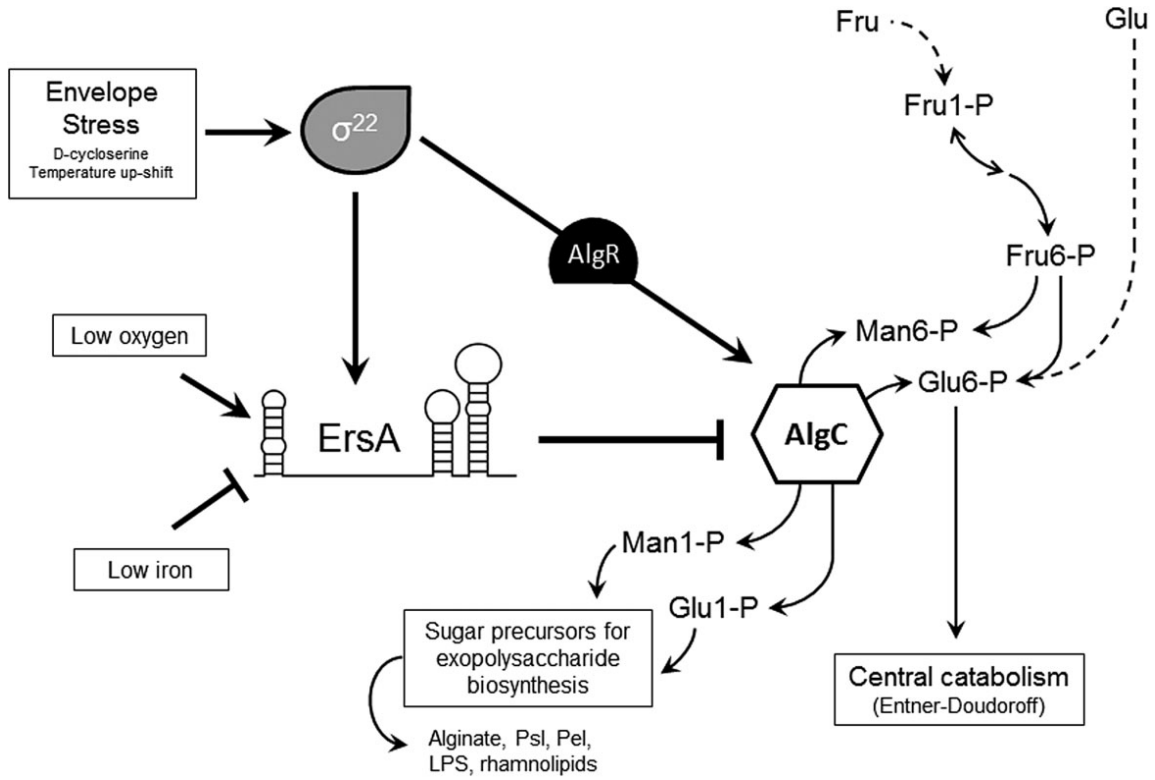

Figure 14. Model for AlgC regulation by incoherent feed forward loop. The alternative sigma factor AlgU drives the transcription of the *algC* gene and the sRNA ErsA which in turn negatively regulates *algC* mRNA at post-transcriptional level. Modified from (Ferrara et al., 2015)

In addition, ErsA is responsive to envelope stress as induced by the cell wall inhibitory antibiotic D-cycloserine and it responds to infection-relevant cues that *P. aeruginosa* can experience in mammalian host, such as oxygen availability and temperature shift (Fig. 13) (Ferrara et al., 2015). A large body of data support the notion that *P. aeruginosa* σ^{22} activation depends on a mechanism homologous to the *E.coli* RIP (regulated intramembrane proteolysis) modulated by σ^{E} and triggered by external stresses such as heat shock which lead to the accumulation of misfolded periplasmic and outer membrane proteins (Grigorova et al., 2004; Qiu et al., 2007; Walsh et al., 2003) and a role of ErsA in this response pathways could be possible.

Aim of the work

P. *aeruginosa* small RNAs are known to be crucial regulators for the pathogenicity and virulence of this human pathogen. Specifically, their contribution is pivotal to have a fast and fine-tuned response which can integrate or antagonize transcriptional changes. Furthermore, sRNAs integrate and process *stimuli* hailing from the environment and population, and as illustrated in section 5.3, they play fundamental roles in the host-pathogen interaction, marking all the stages of infection (Repoila and Darfeuille, 2009).

Interestingly, pioneering studies in *E*. *coli* and *Salmonella* revealed an important contribution of trans-encoded sRNAs also in antibiotic resistance and susceptibility, *i.e*. regulating efflux pumps (Acuña et al., 2016; Mathieu et al., 2016), and the expression of sRNAs in other species is affected in presence of antibiotics (Laureti et al., 2013; Pulido et al., 2016; Wecke and Mascher, 2011). Therefore, these evidences encourage considering sRNAs as promising target for valuable antimicrobial therapies, and raise the importance to further characterize novel sRNAs associated to pathogenic traits.

The first aim of this project was to expand the knowledge about the regulatory network of the sRNA ErsA using a combined approach to identify and validate novel target genes, *in silico, in vivo* and *in vitro*. In addition, ErsA is known to regulate *algC* mRNA (Ferrara et al., 2012, 2015). Since AlgC is crucial for the EPS production which constitutes the biofilm scaffold, we focused our attention also on the direct involvement of ErsA in biofilm development.

Compared to PAO1 strain, there are poor information about the regulatory role of sRNAs in PA14, which is known to be more virulent as usually considered the clone type of CF clinical isolate, in a wide set of hosts, including mice (Harrison et al., 2010a; Mikkelsen et al., 2011a). Thus, in parallel with the first aim of this project, we characterized the novel sRNA PesA expressed in PA14, discovered with the sRNA-sequencing approach which allowed us to identify ErsA (Ferrara et al., 2012).

Main Results

In the attached manuscript Falcone et al. (Part III), PAO1Δ*ersA* has been used to analyze the involvement of the sRNA ErsA on biofilm formation and related phenotypes, as twitching and swarming motility, compared to PAO1 wild-type strain. We used the confocal laser microscopy-flow cells technique and peg-lid microtiter biofilm assay to follow the biofilm formation at different stages. Our results show that the absence of ErsA determine a flat biofilm production compared to the wild-type which conversely develop high structures typical of 3 days-old biofilm, and a reduced biomass production when stained with crystal violet. These evidences suggest a positive contribution of ErsA on the global biofilm network, evidence that was corroborated also by motility assays. Indeed, with an opposite trend of regulation compared to biofilm, ErsA absence seems to foster the motile lifestyle, in particular the type IV pili-dependent twitching, and swarming motility. This result is in line with literature, since it is known that twitching and swarming motility are important for the early stages of biofilm formation, while the expression of flagellar genes is repressed in mature biofilm formation. The transition motility-to-biofilm involves two steps. In the short term, flagella are functionally regulated to either inhibit rotation or modulate the basal flagellar reversal frequency. Over the long term, flagellar gene transcription is inhibited and in the absence of *de novo* synthesis, flagella are likely diluted to extinction through growth. Both short term and long term control is likely important to the motility-to-biofilm transition to stabilize aggregates and optimize resource investment (Shrout et al., 2006). *P. aeruginosa* strains exhibiting more swarming phenotype developed a flat and uniform biofilm in flow cell experiments. Furthermore, twitching motility is suggested to be required for monolayer creation during the initial stages of biofilm development.

Biofilm formation and motility regulation are based on complex regulatory routes at both transcriptional and post-transcriptional level. To visualize the impact of ErsA on the transcriptome profile of *P. aeruginosa*, and to have a global approach about the regulatory networks linked to the sRNA, we performed a RNA-seq experiment.

Notably, the RNA-seq analyses revealed 168 genes differentially expressed in absence of ErsA compared to PAO1 wild-type. Among the 29 genes upregulated in the ErsA deletion mutant and so potentially repressed by ErsA, we observed genes involved in denitrification and nitrate metabolism (*narI, narJ, nirN*) and type VI and III secretion systems effectors (*tssA1, tsi4, tse6*).

The majority of genes were downregulated in absence of ErsA (139 genes); the strongest negative effect was observed for *narK1* involved in nitrate transport. The other genes comprised well described genes involved in biofilm formation and motility (*algD, esrC, ppyR, pelCDEFG, roeA*), energy and carbon compound metabolism (*prpD, prpC, coxA, coxB*), heat-shock proteins (*htpG, hslU, hslV, ibpA, dnaK, dnaJ*) and *phzS* involved in pyocyanin production.

The involvement of ErsA in biofilm architecture development emerged also from the transcriptomic analysis and intriguingly, the biofilm genes whose expression is altered in ErsA deletion mutant background are under the transcriptional control of AmrZ (Jones et al., 2014; Xu et al., 2016). AmrZ is a multifunctional transcriptional regulator of biofilm and motility, which takes place to the transition between colonizing and chronic infection and controls alginate production *via* activation of the *algD* promoter (Jones et al., 2013; Xu et al., 2016).

From literature, other transcriptional regulators are known to be modulated by sRNAs in *E. coli* (Lee and Gottesman, 2016a; Majdalani et al., 2002; Massè et al., 2005a; Ottesman et al., 1998; Prèvost et al., 2007), therefore we hypothesized that ErsA might indirectly affect the expression levels of these genes, by regulating at post-transcriptional level the *amrZ* mRNA. To investigate the interaction ErsA-*amrZ* mRNA we employed different approaches: i) bioinformatics analysis, ii) *in vitro* electrophoretic mobility shift assays, iii) *in vivo* translational fusions based on the *gfp* reporter gene. From *in silico* analyses using the IntaRNA tool, we identified a putative interaction site for ErsA on *amrZ* mRNA, close to the translational starting site. The direct interaction has been validated *in vitro* by EMSA, and *in vivo* by *amrZ*::sfGFP translational fusion that shows reduced reporter activity in PAO1Δ*ersA* compared to the reference strain, suggesting a positive regulation of ErsA on *amrZ* mRNA at the post-transcriptional level.

Overall, our results suggest that the small RNA ErsA might represent a relevant post-transcriptional regulator in biofilm development at different levels, likely interacting with different target mRNAs.

We speculate that ErsA could increase the efficiency of *amrZ* translation, by remodeling its secondary structure and this mechanism may interfere with the expression of biofilm genes which are transcriptionally controlled by AmrZ. However, we cannot exclude a direct role of ErsA on the expression of biofilm genes resulted differentially expressed in ErsA deletion mutant background.

On the whole, we propose here a mixed regulatory pattern modulated by ErsA in biofilm formation and motility regulation in which the post-transcriptional regulation exerted by ErsA on AmrZ enforce the transcriptional influence of AmrZ on biofilm genes affecting the biofilm fitness of *P. aeruginosa* PAO1 strain.

In the attached paper Ferrara et al. (Part II) (Ferrara et al., 2017) we studied the novel *P. aeruginosa* sRNA PesA, transcribed from the pathogenicity island PAPI-1 in the strain PA14.

PesA is a 260 nt non-coding RNA identified using a comparative sRNA-seq approach. It is encoded within the pathogenicity island PAPI-1 and *pesA* gene is located on the same strand and downstream *pilM2* gene and overlaps PA14_59370 gene (unknown function).

PesA is a processed product and it reaches the maximum expression in stationary phase. In addition, it is more expressed at 37°C than 20°C, and its levels are higher in anaerobic condition, with a progressive increment in the transition aerobic-anaerobic conditions. All these conditions recall the environmental stresses that *P. aeruginosa* experiences in the host, as the case of CF lungs.

Following this line, we analyzed by Northern Blot and PCR-amplification the expression of PesA in 29 clinical *P. aeruginosa* isolates from patients affected by chronic respiratory diseases (COPD) including CF, and in 5 environmental strains. Interestingly, our results show that PesA is expressed in 17 CF clinical isolates, in 2 COPD isolates and in 2 environmental isolates, and there is no difference in PesA levels at different stages of chronic infection.

We screened *in silico* the PA14 genome to identify putative PesA target genes located outside PAPI-1. One interesting prediction by *TargetRNA* tool was the *pyoS3A-I* operon coding for pyocin S3. The S3 locus comprises *pyoS3A* and *pyoS3I* and specifically, PesA is predicted to bind the region which comprises the RBS of the *pyoS3I* gene, and locates within the ORF of the *pyoS3A* gene. To evaluate the regulatory role of PesA on the operon, we generated different translational fusions. The first one, *lacZ*::*pyoS3A-I*::*sfGFP*, mimics a bi-cistron controlled by the constitutive promoter $P_{LtetO-1}$. Analyzing the GFP activity of this translational fusion, we suggested a positive role of PesA on the translability of *pyoS3A-I* operon. To test whether translation of *pyoS3A* gene was necessary to have this PesA effects on *pyoS3I*, we generated a second translational fusion, pBBR1-*pyoS3I::sfGFP* containing the same region *pyoS3A-I* present in the first translational fusion, but with only *pyoS3I* fused in frame with sfGFP. GFP analysis confirmed the positive influence of PesA on *pyoS3I* and this effect is not dependent by *pyoS3A*. Thus, to evaluate the effect of PesA on pyoS3A translation we substituted the sequence coding for the F'LacZ domain in pBBR1-*lacZ::pyoS3A-I::sfGFP* with the reporter gene *mCherry*, and measured simultaneously the activity of both mCherry and sfGFP of the translational fusion *mCherry::pyoS3A-S3I::sfGFP*. Our results confirmed the positive regulation exerted by PesA on the *pyoS3I* gene measuring the GFP activity. In addition, *mCherry* also showed an increase in activity when PesA was overexpressed, suggesting that PesA also exerts a positive regulation on the upstream gene *pyoS3A*. Thus, PesA seems to be a

positive regulator of the whole *pyoS3* operon, specifically affecting its translatability without influencing stability of the *pyoS3A-I* mRNA as confirmed by Real time PCR.

These evidences are supported also by the observation that Δ*pesA* results in strong reduction of pyocin S3 production compared to PA14 wild-type. Pyocins are bacteriocins synthesized by more than 90% of *P*. *aeruginosa* strains and are suggested to play a role in niche establishment and protection in mixed populations. The pyocin genes are usually located on the *P*. *aeruginosa* chromosome and induced by mutagenic agents that cause DNA damage such as mitomycin C and UV irradiations. In this paper, we suggest that PesA is involved in niche establishment mechanisms enhancing the production of pyocin S3. PesA was in fact also detected in environmental isolates and its expression from PAPI-1 might confer a selective advantage that favours PAPI-1 maintenance in the environment due to its regulation of pyocin S3.

We evaluated also the virulence potency of PA14Δ*pesA* compared to PA14 wildtype on the CF bronchial epithelial cell line IB3-1. Measuring the viable cell number evaluating the intact mitochondria, we observed that PA14Δ*pesA* is less virulent than wildtype strain, suggesting a role for the sRNA in *P*. *aeruginosa* acute phase of infection.

In addition, the PA14 mutant strain Δ*pesA* shows an increased sensitivity to ciprofloxacin and UV irradiation, introducing an interesting link for PesA in coordinating antibiotic resistance, DNA damage repair and DNase activity producing pyocins.

Conclusions and future Prospects

In this PhD work, we focused on the regulatory function of two sRNAs in *P. aeruginosa*, ErsA and PesA, in PAO1 and PA14 strains, respectively.

ErsA, whose expression is modulated by the alternative sigma factor AlgU, was previously suggested to work as the repressor arm of AlgU, regulating at post-transcriptional level AlgC to fine-tune the exopolysaccharides production in *P. aeruginosa* PAO1 (Ferrara et al., 2015). The results collected in this thesis expanded this framework of information assessing the positive contribution of ErsA in PAO1 biofilm development, likely by direct binding to *amrZ* mRNA, encoding for one of the most important regulators involved in this complicated process.

Interestingly, ErsA seems to retrace the role played by AmrZ in the conversion of a motile and acute-infection lifestyle in a more settled, adaptable and resistant *P. aeruginosa* strain and we propose here a mixed regulatory circuits modulated by ErsA in biofilm formation and motility regulation (Fig.1), in which the post-transcriptional regulation exerted by ErsA on AmrZ would enforce the transcriptional influence of AmrZ on biofilm genes affecting the biofilm architecture of *P. aeruginosa* PAO1.

To achieve our goals, we used a set of *in vivo* and *in vitro* assays surrounded by the high throughput RNA-seq approach. The dataset obtained by the whole transcriptome of PAO1 Δ*ersA* not only corroborated the phenotypic results revealing interesting genes involved in biofilm formation and motility regulation, but it also represents a useful starting point for future ErsA target validations and regulatory networks identification. Indeed, the transcriptomic profile of PAO1 Δ*ersA* lead us to glimpse novel regulatory routes in which ErsA could be directly or indirectly involved. For example, ErsA deletion affects the transcriptional levels of multiple heat shock response operons as *dnaK-grpE*, *dnaJ-dapB*, *htpG*-PA1597, *hslV-hslU* and this strongly suggest a contribution of ErsA, likely modulated by AlgU. Likewise stress response, type III and VI secretion systems and nitrate metabolism operons are also affected in absence of ErsA. A combinatorial approach similar to the one used to reach the aims of this PhD project has to be settled to identify ErsA direct target genes connected to these important pathways.

In addition, all the data collected by our analyses will be integrated in the future by a proteomic profile of PAO1Δ *ersA*, in order to identify target genes previously revealed by RNA-seq which are also affected at translational level. We also expected to highlight novel genes whose products are affected in absence of ErsA but not at mRNA level. To this purpose ribosome profiling or ribo-seq

technique (Ingolia, 2014) might be employed to monitor *in vivo* translation of PAO1 Δ*ersA* compared to the reference strain.

Similarly to ErsA, in this work we characterized another sRNA associate to *P. aeruginosa* virulence, named PesA. It is the first sRNA characterized to date which is encoded in a pathogenicity island in *P. aeruginosa* PA14, and our results suggest its involvement in virulence targeting genes crucial for niche establishment, virulence and stress resistance. Interestingly, our analysis revealed that PesA is expressed in several clinical isolates from patients affected by chronic respiratory diseases, including CF. PA14 Δ*pesA* is less virulent than wild-type strain as revealed by infecting the CF bronchial epithelial cell line IB3-1, suggesting also role for the sRNA in *P. aeruginosa* acute phase of infection. In addition the deletion of PesA resulted in PA14 more sensitive to ciprofloxacin treatment, one of the most common antibiotics used to treat *P. aeruginosa* infections in CF patients. On the whole, the panel of results collected by this study suggests an interesting role of both ErsA and PesA in *P. aeruginosa* virulence and pathogenesis. These observations raise the possibility to consider these two sRNAs as target for non-conventional antibacterial therapy as the case of RNA-based drugs or antibiotics targeting RNAs (Melnikova, 2007). In the last years, indeed, the increasing antibiotic resistance among the most widespread human pathogens, urged to design novel class of drugs and expand the number of their targets. Several classes of molecules have been investigated for RNA therapeutics including antisense RNA, ribozymes, RNA decoys, aptamers, small interfering RNA (siRNA) and microRNA (miRNA) in order to overcome the limited number of proteins and small molecules currently used as target for therapeutics (Dersch et al., 2017; Hong et al., 2014; Kaczmarek et al., 2017). However, target selectivity, stability, delivery and long-term safety have to be fine-tuned for RNA drugs to become a successful therapeutic category. Another possibility to target sRNAs is employing PNA (Peptide Nucleic Acid) oligomers, which can recognize complementary DNA/RNA sequences, and are characterized by a changeable peptide-like backbone (Nielsen, 2010). PNAs might be designed for example to target PesA in order to interfere with its regulatory activity and increase *P. aeruginosa* ciprofloxacin sensitivity and PNAs designed to target ErsA could affect the proper biofilm shaping and this may contribute to the anti-biofilm compound activity.

Furthermore, in the future, mouse models are required to test the involvement of ErsA and PesA in acute and chronic infection and eventually test the RNA-based therapy against the two sRNAs *in vivo*.

In conclusion, our results expanded the knowledge about sRNAs in *P*. *aeruginosa*, adding novel regulatory elements to well-known networks and we believe that detailed understanding of target genes of small RNAs may help to expand the frontiers on novel therapies against *P*. *aeruginosa* infections.

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PART II

Contents

Research article:

Silvia Ferrara, Marilena Falcone, Raffaella Macchi, Alessandra Bragonzi, Daniela Girelli, Lisa Cariani, Cristina Cigana, and Giovanni Bertoni (2017). The PAPI-1 pathogenicity island-encoded small RNA PesA influences *Pseudomonas aeruginosa* virulence and modulates pyocin S3 production. PLoS One **12**(6): e0180386.

PLOS ONE

RESEARCH ARTICLE

The PAPI-1 pathogenicity island-encoded small RNA PesA influences *Pseudomonas aeruginosa* virulence and modulates pyocin S3 production

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Abstract

Small non-coding RNAs (sRNAs) are post-transcriptional regulators of gene expression that have been recognized as key contributors to bacterial virulence and pathogenic mechanisms. In this study, we characterized the sRNA PesA of the opportunistic human pathogen *Pseudomonas aeruginosa*. We show that PesA, which is transcribed within the pathogenicity island PAPI-1 of *P. aeruginosa* strain PA14, contributes to *P. aeruginosa* PA14 virulence. In fact, *pesA* gene deletion resulted in a less pathogenic strain, showing higher survival of cystic fibrosis human bronchial epithelial cells after infection. Moreover, we show that PesA influences positively the expression of pyocin S3 whose genetic *locus* comprises two structural genes, *pyoS3A* and *pyoS3I*, encoding the killing S3A and the immunity S3I proteins, respectively. Interestingly, the deletion of *pesA* gene results in increased sensitivity to UV irradiation and to the fluoroquinolone antibiotic ciprofloxacin. The degree of UV sensitivity displayed by the PA14 strain lacking PesA is comparable to that of a strain deleted for *pyoS3A-I*. These results suggest an involvement of pyocin S3 in DNA damage repair and a regulatory role of PesA on this function.

Introduction

Bacterial small RNAs (sRNAs) have been recognized as key contributors to regulatory networks, and have been shown to play critical roles in many intra- and extracellular processes, and in pathogenesis [1–3]. Most sRNAs exert their regulatory function post-transcriptionally, acting by base pairing with the mRNA of their target genes ultimately modulating mRNA translation and/or stability. sRNAs can share extended base complementarity when they are *cis*-encoded on the opposite strand of the target mRNA, or they can interact with the target mRNA via short and imperfect base pairing, as in the case of *trans*-encoded sRNAs. The

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expression of most sRNAs is responsive to environmental stress conditions spanning from iron and oxygen limitation, to oxidative, metabolic/nutrient, pH and cell envelope stresses [4].

In the opportunistic human pathogen *Pseudomonas aeruginosa* the use of transcriptomics approaches has recently led to the identification of numerous new sRNAs, mostly in the attenuated strain PAO1 and some also in the virulent one PA14 [5–7]. The bacterium *P. aeruginosa* is known as a major cause of both acute and chronic lung infections in patients belonging to all age groups who are immunocompromised, or who have defective mucociliary clearance, previous epithelial injury or foreign body placement [8]. Lung infections caused by *P. aeruginosa* can appear as a spectrum of clinical entities ranging from a rapidly fatal pneumonia in a neutropenic patient to a multi-decade bronchitis in patients with cystic fibrosis (CF). The expression of virulence traits in *P. aeruginosa* is fine-tuned by a dynamic and intricate regulatory network [9],that leads the expression of *P. aeruginosa* pathogenic functions with a sharp timing. In this scenario, sRNAs can finely contribute to *P. aeruginosa* ability to quickly adapt to a new environment and manage to persist.

Previously, bioinformatics and approaches such as the pull-down with the RNA chaperone Hfq have been used for identifying novel *P. aeruginosa* sRNAs but only a small number of them have been characterized functionally, and even less described as being implicated in the regulation of *P. aeruginosa* virulence [10–17]. Moreover, most of the studies have been performed using the reference strain PAO1, while very little is known about the biological effects of the sRNA mediated regulation in virulent *P. aeruginosa* strains, such as PA14. Compared to PAO1, the clinical isolate PA14 is significantly more virulent in a wide range of hosts, including mice, the nematode *Caenorhabditis elegans*, the insect *Galleria mellonella*, and the plant *Arabidopsis thaliana*, and has thus become an important reference strain because of its enhanced virulence [18–20]. Generally, PAO1 and PA14 strains share highly conserved genomes, although PA14 possesses a slightly larger one, likely due to horizontal gene transfer resulting in the acquisition of pathogenicity islands (PAIs) [21].

This study focuses on a *P. aeruginosa* sRNA observed for the first time by a sRNA-sequencing approach [5], in which unique and conserved sRNAs in the *P. aeruginosa* strains PAO1 and PA14 were revealed. Here, we validated the sRNA with the operative name SPA0021, renamed PesA, as being transcribed from the pathogenicity island PAPI-1, present in PA14 strain, but not in PAO1. In addition, our results display that PesA is expressed in several *P. aeruginosa* isolates, including environmental and clinical ones isolated from CF patients. Moreover, we show that PesA operates a post-transcriptional regulation of genes involved in S-type pyocin production. Pyocins are narrow-spectrum bacteriocins synthesized by more than 90% of *P. aeruginosa* strains and presumed to play a role in niche establishment and protection in mixed populations. The pyocin genes are usually located on the *P. aeruginosa* chromosome and induced by mutagenic agents that cause DNA damage such as mitomycin C and UV irradiations. *P. aeruginosa* pyocins can be subdivided into insoluble R and F pyocins and soluble S pyocins. S-type pyocins AP41, S1, S2, and S3 are constituted of two components in which the large component carries an endonuclease C-terminal domain responsible for the killing activity that causes cell death by DNA cleavage [22–27], while pyocins S4 and S5 have tRNAse and pore-forming activities, respectively [28, 29]. More recently, also the new S-type pyocin S6, with rRNase activity, has been described functionally [30]. In our study, we show that PesA deletion leads to decreased expression of pyocin S3, associated to increased sensitivity to UV irradiation and to the fluoroquinolone antibiotic ciprofloxacin. Furthermore, PesA is induced by host-environment stimuli such as low oxygen availability and body temperature, suggesting a key role in *P. aeruginosa* adaptability to different environmental stresses. Finally, we also found that PesA is involved in the regulation of *P. aeruginosa* PA14 virulence in CF human bronchial epithelial cells. Our results suggest that a PAPI-1-encoded sRNA can contribute to

the modulation of the expression of genes outside PAPI-1, and to different aspects of *P. aeruginosa* pathogenesis during infection.

Materials and methods

Ethics Statement

Study on human *P. aeruginosa* isolates from Hannover has been approved by the Ethics Commission of Hannover Medical School, Germany [31]. The patients and parents gave oral informed consent before the sample collection. Approval for storing of biological materials was obtained by the Ethics Commission of Hannover Medical School, Germany. The study on human *P. aeruginosa* isolates from the Regional CF Center of Lombardia (S1 Table) was approved by the Ethical Committees of San Raffaele Scientific Institute and Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy, and written informed consent was obtained from patients enrolled or their parents according to the Ethical Committees rules, in accordance with the laws of the Italian Ministero della Salute (approval #1874/12 and 1084/14).

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in S2 Table. *E. coli* strains were routinely grown in Luria-Bertani broth (LB) at 37˚C. *P. aeruginosa* strains were grown at 37˚C in Brain Heart Infusion (BHI) rich medium or in LB at 120 rpm unless otherwise indicated. Carbenicillin and gentamicin were added at 300 and 20 μg/ml, respectively, unless otherwise indicated. For P_{BAD} induction in vector plasmid pGM931, arabinose was added to a final concentration of 10 mM. Anaerobic batch cultivations of *P. aeruginosa* and the shift from aerobic to anaerobic conditions were performed in a 800 ml-Biostat-Q system bioreactor (B-Braun) as described previously [17].

Plasmid constructions and mutant generations

Oligonucleotides used in this study are listed in S3 Table. To construct plasmid pGM-*pesA*, the *pesA* gene was PCR-amplified from PA14 genomic DNA with oligos 9/10, digested with *Nco*I-*Pst*I and cloned into the pHERD20T derivative vector pGM931 carrying a transcriptional terminator downstream the multicloning site [32, 33].

Plasmids pBBR1-*pyoS3I*::*sfGFP*, pBBR1-*leader-pyoS3A*::*sfGFP*, pBBR1-*lacZ*::*pyoS3A-I*::*sfGFP*, and pBBR1-*mCherry*::*pyoS3A-I*::*sfGFP* expressing *pyoS3I*::*sfGFP*, *pyoS3A*::*sfGFP*, *lacZ*::*pyos3A-I*::*sfGFP*, or *mCherry*::*pyos3A-I*::*sfGFP* translational fusions, respectively under the $P_{LtetO-1}$ constitutive promoter were constructed as follows. A DNA fragment including the last 39 codons of the open reading frame of Pyos3A and 36 first codons of PyoS3I was amplified by PCR with oligos 11/12, digested with *Nsi*I-*Nhe*I and cloned into the sfGFP reporter vectors pXG10-SF and pXG30-SF [34] giving rise to plasmid pXG10-*pyoS3I*::*sfGFP* and pXG30-*lacZ*::*pyoS3A-I*::*sfGFP*, respectively. A DNA fragment including the 278-nt UTR and the first 37 codons of the *pyos3A* open reading frame was amplified with oligos 13/14 digested with *Nsi*I-*Nhe*I and cloned into the sfGFP reporter vectors pXG10-SF giving rise to plasmid pXG10-*leader-pyoS3A*::*sfGFP*.

The DNA fragments spanning from the $P_{LtetO-1}$ promoter to the end of the GFP reporter gene were amplified by PCR respectively from pXG10-*pyo-S3I*::*sfGFP*, pXG30-*lacZ*::*pyoS3A-I*::*sfGFP* and pXG10-*leader-pyoS3A*::*sfGFP* with oligos 18/19, digested with *Cla*I-*Xba*I and cloned into the low-copy number shuttle vector pBBR1-MCS5 giving rise to constructs pBBR1-*pyoS3I*::*sfGFP*, pBBR1-*lacZ*::*pyoS3A-I*::*sfGFP* and pBBR1-*leader-pyoS3A*::*sfGFP*, respectively.

mCherry gene was amplified by PCR from the pMMR plasmid [35] using primers 15/16, (in which forward primer contained the Shine-Dalgarno sequence, and reverse primer lacked mCherry stop codon sequence), digested with *NsiI* and cloned into the pXG10-*pyoS3I*::*sfGFP* previously digested with *Nsi*I, giving rise to plasmid pXG10-*mCherry*::*pyoS3A-I*::*sfGFP*. The DNA fragment spanning from the $P_{LtetO-1}$ promoter to the end of the GFP reporter gene was amplified by PCR using oligos 18/19 digested with *Cla*I-*Xba*I and cloned in the pBBR1-MCS5 vector, giving rise to plasmid pBBR1-*mCherry*::*pyoS3A-I*::*sfGFP*. Plasmid pBBR1-*mCherry* expressing mCherry reporter gene under $P_{LtetO-1}$ was constructed as follows. The DNA fragment including the $P_{LtetO-1}$ promoter and the mCherry reporter gene was amplified from pBBR1-*mCherry*::*pyoS3A-I*::*sfGFP* using oligos 17/18 (in which reverse primer contained *mCherry* stop codon). The PCR product was digested with *Cla*I-*Xba*I and cloned in the pBBR1-MCS5 vector.

P. aeruginosa mutant in *pesA* gene was generated by an enhanced method of markeless gene replacement described previously [36] with some modifications to adapt it to *P. aeruginosa* as described previuosly [17]. PA14 mutant in *pesA* gene was obtained by allelic exchange with a deletion from—34 to + 140 positions with respect to *pesA* transcription start site as follows. TS1 region spanning left 533 bp flanking sequence of *pesA gene* was amplified by PCR with oligos 23/24. TS2 region spanning last 127 nt and right 368 bp flanking sequence of *pesA* was amplified by PCR with oligos 25/26. PCR amplifications were performed from PA14 genomic DNA. Overlap extension (SOE)-PCR with oligos 23/26 was used to join TS1 and TS2 that carried end complementary regions introduced by 25/26, respectively during their separate PCR amplification [37]. Joined TS1-TS2 DNA fragments were digested with *Eco*RI-*Pst*I and cloned in CC118 λ*pir* into the poly-linker site of pSEVA612S giving rise to pSEVApa14-*ΔpesA*.

The TS1-TS2–inserted pSEVApa14-*ΔpesA* was transferred from *E. coli* CC118 λ*pir* to PA14, with the assistance of the helper *E. coli* strain HB101(pRK600) in a conjugative triparental mating. Exconjugant *P. aeruginosa* clones were selected on M9-citrate with 60 μg ml^{-1} of gentamicin. Since pSEVA612S derivatives cannot replicate in *P. aeruginosa*, Gm^R exconjugant clones could appear only by co-integration of the construct in the genome of the recipient strain by homologous recombination between joined TS1-TS2 fragments borne by pSEVA612S and the recipient chromosome. Plasmid pSW-1 was transferred from *E. coli* DH5α to *P. aeruginosa* clones bearing genomic co-integrates of pSEVApa14-*ΔpesA* by triparental mating as above, and pSW-1-recipient *P. aeruginosa* clones were selected on M9-citrate with 300 μg ml^{-1} of carbenicillin. Cultures of resulting *P. aeruginosa* clones carrying pSW-I were grown overnight in LB with 300 μg ml^{-1} of carbenicillin and then plated on the same medium. Single colonies were screened for loss of gentamicin resistance. Gentamicin-sensitive clones carrying the deleted alleles were then screened by PCR with oligo pairs 27/28 for *pesA*.

All plasmid constructs and deletion mutant were checked by sequencing with oligos indicated in S3 Table.

RNA isolation and analysis

Total RNA was prepared as described previously [5] from 2–10 ml of bacterial cell cultures. Quality and concentration of the RNA extracted were assessed by a Biospectrometer (Eppendorf). Northern blot analyses were performed as described previously [5]. DNA oligonucleotide probes were 5'-end labeled with [γ-^{32}P]ATP (PerkinElmer, NEG502A) and T4 polynucleotide kinase (Promega, M4103) according to manufacturer's instruction. Oligo 1 and 2 were used to probe PesA and 5S RNA, respectively. Treatment with terminator-5'-phosphate-dependent exonuclease was performed in terminator reaction buffer A (Epicentre, TER51020) as described in detail previously [17].

Radioactive bands were acquired after exposure to phosphor screens using a Typhoon™ 8600 variable mode Imager scanner (GE Healthcare BioSciences) and visualized with image-Quant software (Molecular Dynamics).

Quantitative RT-PCR analysis was performed on total RNA extracted from *P. aeruginosa* PA14 wild-type and Δ*pesA* at mid-, late-exponential and stationary phase (OD_{600} of 0.8, 1.6 and 2.7, respectively). cDNA synthesis was performed from 1 μg of total purified RNA using QuantiTect Reverse Transcription Kit (Qiagen). RT-PCRs were performed using QuantiTect SYBR® Green PCR Kit (Qiagen) and oligo pairs 30/31, 32/33, 34/35 for 16S, *pyoS3A* and *pyoS3I* amplification, respectively. The reaction procedure involved incubation at 95˚C for 15s and 40 cycles of amplification at 94˚C for 15 s, 60˚C for 30 s and 72˚C for 30s. 16S ribosomal RNA was used as reference.

In vitro and *vivo* assays of sRNA/mRNA interactions

Electrophoretic Mobility Shift Assay (EMSA) to analyze sRNA/mRNA interactions were performed as described previously [17].

Experiments with fluorescent reporters were carried out as described previously [17]. Fluorescence polarization $FP_{485/535}$, fluorescence intensity $FI_{590/635}$ and Abs_{595} were measured in a Tecan Infinity PRO 200 reader, using Magellan as data analysis software (Tecan). GFP and mCherry activities were expressed in Arbitrary Units (AU) as ratio $FP_{485/535}/Abs_{595}$ and $FI_{590/635}/Abs_{595}$, respectively.

RNA synthesis

RNAs for RNA/RNA interaction assays were prepared by T7 RNA polymerase transcription of gel-purified DNA fragments obtained by PCR as described previously [17]. DNA fragments for PesA RNA and *pyoS3A-I* mRNA preparations were amplified from *P. aeruginosa* PA14 genomic DNA with oligo pairs 3/4 and 5/6, respectively. The DNA fragment for RseX RNA was amplified from *E. coli* C1a genomic DNA with oligos 7/8.

Pyocin S3 spotting assay

Pyocin killing assay was performed using the spotting method as indicated previously [38] with some modifications. 10 μl of filter-sterilized supernatants from cell cultures with OD_{600} of 1 were spotted onto LB 1.5% agar plates. A lawn of the pyocin S3 sensitive *P. aeruginosa* strain ATCC 27853 containing 5×10^6 cells ml^{-1} was plated by inclusion into 0.3% soft agar over the dried spots. Plates were incubated overnight at 37˚C and checked for the formation of the clearing zone on the spotting site, which is indicative of the pyocin S3 activity.

Antibiotic disk diffusion

Susceptibilities of *P. aeruginosa* strains to antimicrobial agents were analyzed by disk diffusion measurement. Filter disks (Oxoid, CT0425B, CT0013B, CT0207B, CT0052B, CT0058, CT0010B) were placed on a lawn of 10^6 CFU/ml bacterial cells plated by inclusion into 0.3% LB agar. Plates were incubated overnight at 37˚C and the diameters of the clear zones around the disks were measured.

UV sensitivity assay

UV treatment was performed using a Stratalinker 1800 UV Crosslinker (Stratagene). Cell cultures with an OD_{600} of 1 (corresponding to 8 x 10^8 CFU/ml) were serially diluted until 10^{-7}; 3 μl of each dilution were spotted in triplicate onto LB-agar plates, dried, and exposed to

increasing amounts of UV radiation, from 0 to 100 J/m^2. Plates were incubated overnight at 37˚C.

Cytotoxicity assays in human CF respiratory cells

IB3-1 cells, an adeno-associated virus-transformed human bronchial epithelial cell line derived from a CF patient (ΔF508/W1282X) and obtained from LGC Promochem, were grown as described previously [39]. Cells were infected with *P. aeruginosa* strains at a multiplicity of infection (MOI) of 100. Cell viability was evaluated using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega, G4000), according to manufacturer's instructions.

Bacterial isolates analysis

Bacterial isolates were plated on 1.5% BHI-agar plates and grown overnight at 37˚C. Culture samples were taken and processed for genomic DNA and total RNA extraction. PAO1 and PA14 strains treated in the same conditions were used as controls. Oligos 9/10 and 37/38 were used for PCR-amplification of the genomic region containing the *pesA* and 16S (as positive PCR-control) *loci*, respectively.

Results

The sRNA PesA is encoded in the PAPI-1 and is a processed transcript

The SPA0021 sRNA was identified using a comparative sRNA-seq approach in which 52 *P. aeruginosa* novel sRNAs have been identified either in the attenuated strain PAO1 or in the virulent one PA14. SPA0021 was validated to be ≈ 260 nt in lengths, and one of the 12 sRNAs whose genetic *locus* is unique to the PA14 strain [5]. Moreover, SPA0021 was found to be encoded within the pathogenicity island PAPI-1, and because of this property, we renamed it as pathogenicity island-encoded sRNA A (PesA). The *pesA* gene locates downstream and on the same strand of *pilM2* gene (belonging to the *type IV B pilus* operon *pil2*), and overlaps the 3' of PA14_59370 (gene with unknown function) (Fig 1A and 1B). Since a rho-independent transcription terminator was predicted within the PA14_59370 sequence [40]. The cluster of the sRNA-seq reads that mapped upstream the predicted terminator was considered as the 3' of the *pesA* gene [5]. The 5'-end was also mapped by the sRNA-seq read-clustering and was in perfect agreement with the size validated by Northern blot [5].

We evaluated PesA expression along the growth-curve in the rich medium BHI (Fig 1C). Northern blot analyses showed a main band of the expected product size of ≈ 260 nt (black arrow) at each analyzed time point, and multiple bands with higher molecular weight (white arrows) especially at early exponential phase (OD_{600} = 0.2). The main band of PesA showed to accumulate in late stationary phase. The presence of high molecular weight multiple bands, and the absence of rho-independent transcription terminators downstream *pilM2*, suggested that the main band of ≈ 260 nt could derive from the processing of a longer transcript. Sensitivity to treatment with terminator 5′-phosphate-dependent exonuclease (Fig 1D), which preferentially degrades monophosphate processed transcripts confirmed that the ≈ 260 nt RNA is indeed a processed product.

PesA is widespread expressed in clinical and environmental isolates

To assess the clinical impact of PesA, we validated its presence and expression levels throughout a collection of 29 clinical *P. aeruginosa* isolates derived from respiratory samples of patients with chronic respiratory diseases, including CF and chronic obstructive pulmonary disease

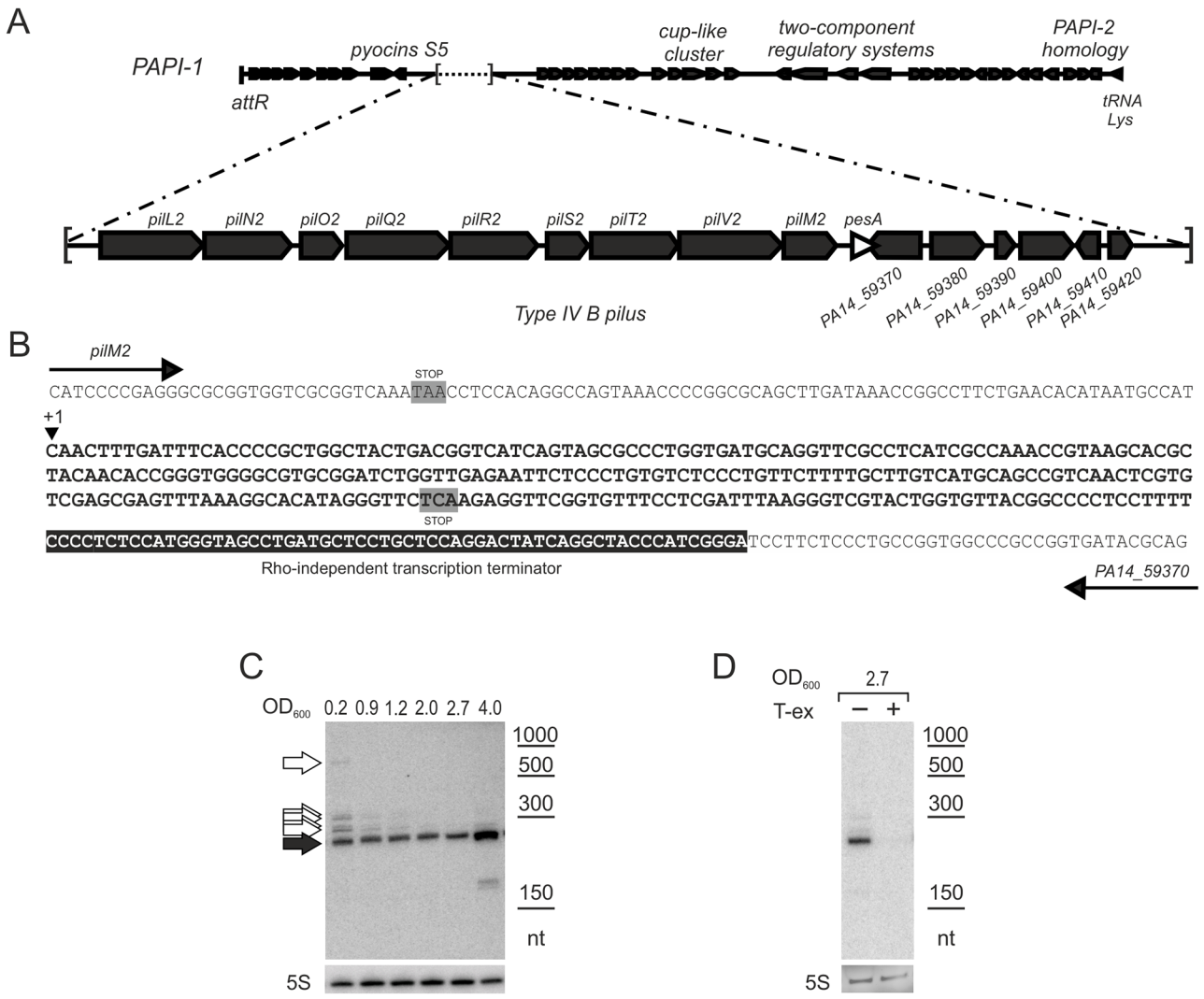

Fig 1. Genomic context and transcriptional features of *P. aeruginosa pesA* gene. A) Schematic overview of the PAPI-1 region of PA14. The *pesA* gene is indicated as white arrowhead. B) Sequence of the pilM2–PA14_59370 intergenic region of PA14. The *pesA* sequence is in bold. The 5′-end of PesA is indicated with +1. A predicted Rho-independent transcription terminator is highlighted in black. The predicted stop codons of pilM2 and PA14_59370 are highlighted in gray. C) PesA expression is induced in stationary phase. Wild-type PA14 was inoculated in BHI at an OD_{600} of 0.2 and grown for 20 h at 37˚C with agitation. At the indicated OD_{600}, culture samples were taken and processed for total RNA extraction and analysis by Northern blot. Black arrow indicates the main band of *pesA* of ≈ 260 nt; white arrows indicate bands of higher molecular weight of *pesA* present especially at early exponential phase (OD_{600} = 0.2). D) Northern blot analysis of PesA on 10 μg of total RNA extracted at the end of the exponential growth phase, treated (+) or untreated (−) with terminator 5′-phosphate-dependent exonuclease (T-ex).

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(COPD), and 5 isolates from environmental habitats. In particular, the clinical isolates were recovered both during intermittent infections and at different stages of chronic lung infection. Part of them was previously characterized both *in vitro* and *in vivo* [31, 41]. We also included in this study the Liverpool epidemic strain LESB58 [42] and PAO1 and PA14 as controls.

In Fig 2, results on *pesA*-gene amplification and Northern blot analysis show that the *pesA* gene is present in 17 out of the 27 CF clinical isolates, in the 2 COPD isolates and in 2 out of 5 environmental isolates. Notably, there was no association between the presence/expression of *pesA* gene and the *P. aeruginosa* status (chronic *vs* intermittent). In addition, no differences were observed between clonal isolates recovered from the same patients at different stages of

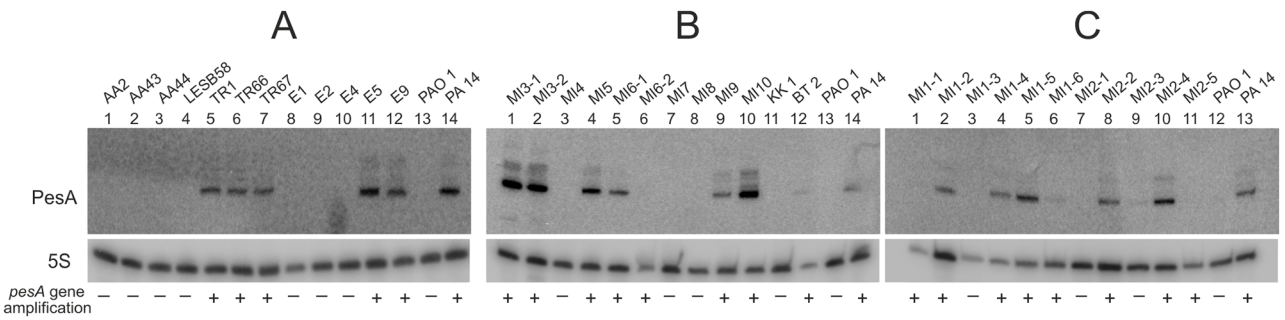

Fig 2. PesA gene dissemination and expression levels among environmental, CF and COPD clinical isolates. Assays on environmental, CF and COPD isolates are shown in three panels, A, B and C, respectively. The strain-collection was plated on BHI-agar plates. After overnight growth at 37˚C, culture samples were taken and processed for total RNA extraction and analysis by Northern blot, and for genomic DNA extraction. Positive or negative PCR-amplification outcomes are indicated as "+" or "-" in the "*pesA* gene amplification" row, below each Northern Blot. PAO1 and PA14 were used as controls of Northern Blot analysis and for negative or positive gene amplification, respectively.

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chronic infection (e.g. AA2-early, AA43-late, AA44-late or TR1-early, TR66-late, TR67-late). In the BHI-plate aerobic growth conditions of these experiments, the majority of the *pesA*-harboring isolates showed levels of expression similar to those of the PA14 strain or even higher (panel A, lanes 5–7, 11 and 12; panel B, lanes 1, 2, 4, 5, 9, 10; panel C, lanes 2–5, 8 and 10), while 5 isolates showed lower or no expression levels with respect to the PA14 (panel B, lanes 6 and 12; panel C, lanes 1, 6 and 11). In the case of PAO1 and LESB58 strains, no gene amplification was observed (panel A, lanes 4 and 13).

PesA is induced in anaerobic growth and at 37˚C

We analyzed the transcriptional responsiveness of PesA RNA to environmental or body temperature, and reduced oxygen availability. Temperature sensitivity was tested by probing PesA in early- (OD_{600} = 0.8) and mid-exponential phase (OD_{600} = 1.8) at both 20 and 37˚C and after 20 min of acclimation following a shift from 20 to 37˚C, as described in detail previously [17]. As shown in Fig 3A, the growth at 37˚C caused an up-regulation of PesA if compared to the growth at 20˚C; no increase of PesA RNA accumulation was observed during the 20 min of acclimation. PesA showed also to be responsive to oxygen availability. PesA was probed at mid- and late-exponential phase (OD_{600} of 0.8 and 2, respectively) under anaerobic conditions in BHI with nitrate to sustain anaerobic respiration, as described previously [17]. In addition, bacterial cells were grown in BHI with aeration until mid-exponential phase (OD_{600} = 0.8); then, oxygen was excluded from cultures. PesA levels were assessed immediately before oxygen exclusion and 20 (OD_{600} = 0.9) and 150 min (OD_{600} = 1.3) from the start of anaerobic conditions, as described previously [17]. PesA levels were higher in anaerobic than aerobic conditions both in mid- and late-exponential phase (Fig 3B). In addition, the shift from aerobic to anaerobic conditions caused a progressive increase of PesA levels.

PesA is involved in ciprofloxacin and UV-resistance

To explore the involvement of PesA in the regulation of cellular mechanisms, also linked to *P. aeruginosa* virulence, we constructed the knock-out mutant strain PA14 *ΔpesA* and the plasmid vector pGM-*pesA* carrying the *pesA* gene under the arabinose inducible P_{BAD} promoter with the aim to measure the effects of perturbing PesA levels on phenotypic traits of the PA14 strain. Deletion of the *pesA* gene and overexpression from the pGM-*pesA* vector in the wild-type background were ensured by Northern blot (S1 Fig).

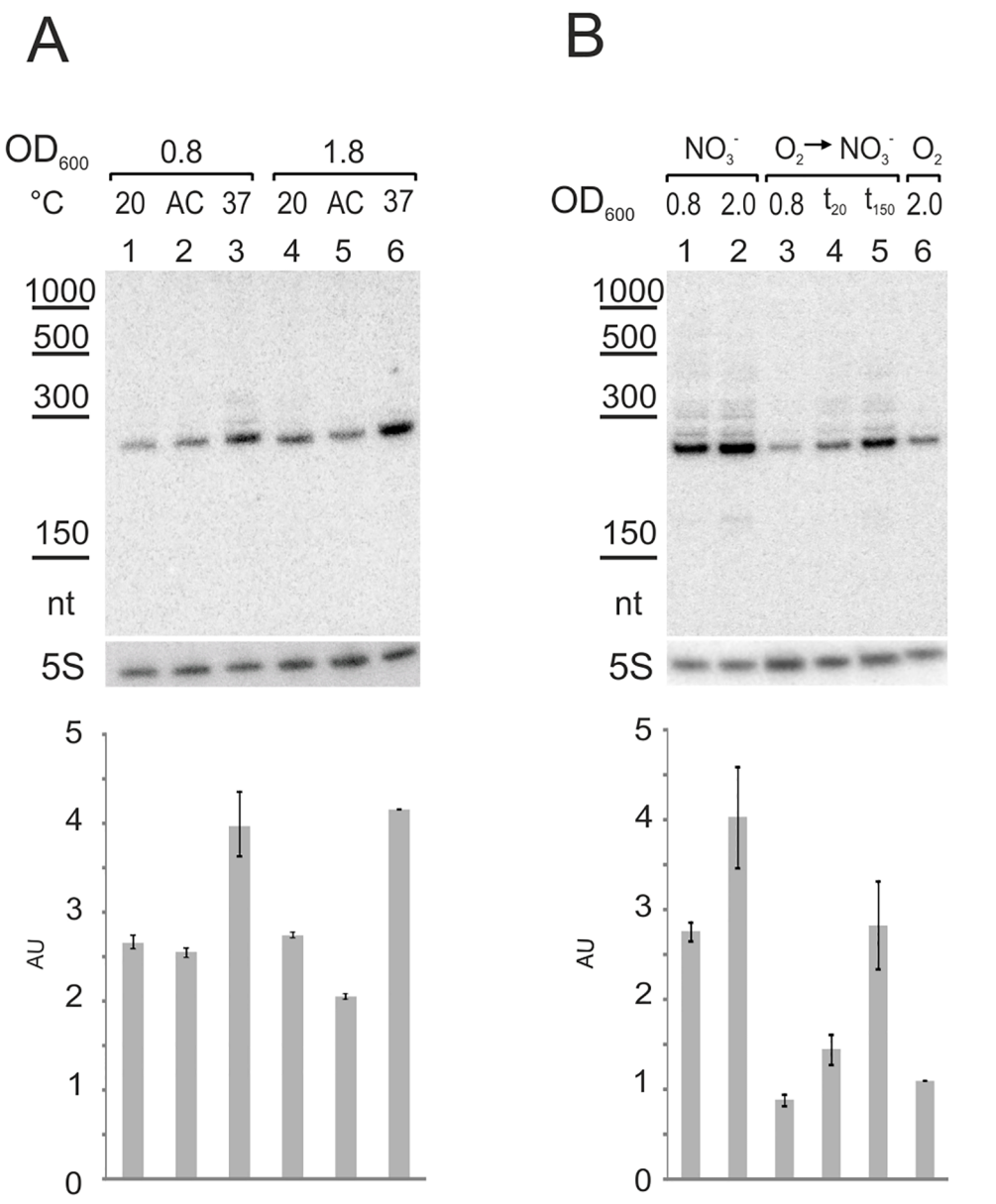

Fig 3. PesA expression is induced by temperature and low availability of oxygen. Levels of PesA RNA in: A) Wild-type PA14 grown in BHI at 20˚C (lanes 1 and 4), 37˚C (lanes 3 and 6) or following 20 min of acclimation (AC) from 20 to 37˚C (lanes 2 and 5). Culture samples were taken at middle (OD_{600} of 0.8) and late (OD_{600} of 1.8) exponential growth phase. B) Wild-type PA14 grown in BHI anaerobically (NO_3^-; lanes 1 and 2), aerobically (O_2, lane 6) and aerobically until an OD_{600} of 0.8 and then shifted to anaerobic conditions ($O_2 \rightarrow NO_3^-$; lanes 3, 4 and 5). Samples were taken 20 and 150 min after the shift to anaerobic conditions (t_{20} and t_{150}). After sampling, cell cultures were processed for total RNA extraction and analysis by Northern blot. Intensities of the bands of PesA were quantified and normalized to those of 5S RNA in the same lane. Values are expressed as arbitrary units (AU) in the histograms below each Northern blot and represent the mean ± Standard Deviation (SD) of three independent experiments.

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We performed different types of phenotype evaluations. The most evident effects of PesA deletion were on UV and ciprofloxacin susceptibility. In particular, PesA deletion resulted in an enhanced sensitivity to the antibiotic ciprofloxacin and to UV irradiation. In fact, the susceptibility of wild-type PA14 and *ΔpesA* mutant cells to antimicrobials was analyzed by

antibiotic disk diffusion on agar plates. The diameters of the inhibitory zones were measured after overnight incubation at 37˚C. As shown in Fig 4, the diameter of the clear zone around ciprofloxacin was higher for mutant *ΔpesA* (46.33 ±0.58 mm) in comparison to that of wild-type strain (40.33 ± 0.58 mm) thus suggesting a contribution of PesA in the ciprofloxacin resistance mechanism. In addition, we noticed an incremented sensitivity of the *ΔpesA* mutant strain to UV light, showing a decrease in CFUs with respect to the wild-type starting from treatment with 30 J/m^2 (Fig 5). This suggested the involvement of PesA in improving survival under conditions of genotoxic stress, such as UV irradiation treatment. Intriguingly, the deletion of *pyoS3A-I* operon, that is positively regulated by PesA (see below), gave rise to same levels of UV sensitivity as *pesA* deletion (Fig 5).

We did not observe significant differences for other phenotypes analyzed, including growth-curves analysis on rich and minimal media, susceptibility to other antimicrobial agents of different structural families, hemolytic activity, flagellum-mediated motility, pyocyanin and pyoverdine secretion.

PesA is involved in *P. aeruginosa* pathogenicity in human CF respiratory cells

We evaluated the virulence of *P. aeruginosa* PA14 strains on the CF bronchial epithelial cell line IB3-1. In particular, we assessed the killing capacity of PA14 Δ*pesA* mutant compared to the wild-type strain by the MTT assay, which provides a method of determining viable cell number measuring the conversion of 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. Our results (Fig 6) showed that cells infected with *P. aeruginosa* PA14 Δ*pesA* mutant were more viable with respect to those infected by the wild-type strain, thus indicating that *pesA* may contribute to *P. aeruginosa* PA14 acute virulence.

PesA targets *pyoS3A-I* operon

As mentioned previously, PesA is encoded *in cis* to the 3' of the gene PA14_59370 with unknown function, which makes the study of such putative target difficult to perform. Therefore, we managed to identify direct targets of PesA RNA by the use of the bioinformatics tool *TargetRNA* [43]. This tool predicted, as a high-scored output, an interaction in the region from -30 to -8 nt upstream the gene *pyoS3I* of the *pyoS3* operon (Fig 7A and S4 Table), predicted to encode pyocin S3 in the PA14 strain. The pyocin S3 genetic *locus* comprises two structural genes, *pyoS3A* (PA14_49520) and *pyoS3I* (PA14_49510), annotated to encode the killing S3A and the immunity S3I proteins, respectively. To confirm this annotation in PA14, we deleted the *pyoS3* operon and tested the pyocin S3 production by the killing assay of the sensitive ATCC 27853 *P. aeruginosa* strain [27]. As shown in Fig 8, deletion of *pyoS3A-I* completely abolished the production of pyocin S3 by PA14 strain. Remarkably, PesA deletion resulted in strong reduction of pyocin S3 production, thus suggesting a positive role of PesA on the pyocin S3 production.

The predicted region of the *pyoS3A-I* operon targeted by PesA comprises the Ribosome Binding Site (RBS) of the *pyoS3I* gene, and locates within the ORF of the *pyoS3A* gene. To assess this predicted PesA-*pyoS3* mRNA interaction, PesA RNA and the *pyoS3* mRNA region spanning –116 to +108 from *pyoS3I* translational start site were produced *in vitro*, mixed and analyzed on native polyacrylamide gels. As shown in Fig 7B, the two RNAs specifically formed a complex.

We generated distinct types of translational fusions to test the effects of PesA on the *pyoS3I* gene alone and on the *pyoS3A-I* mRNA. A first reporter plasmid, named pBBR1-*lacZ*::

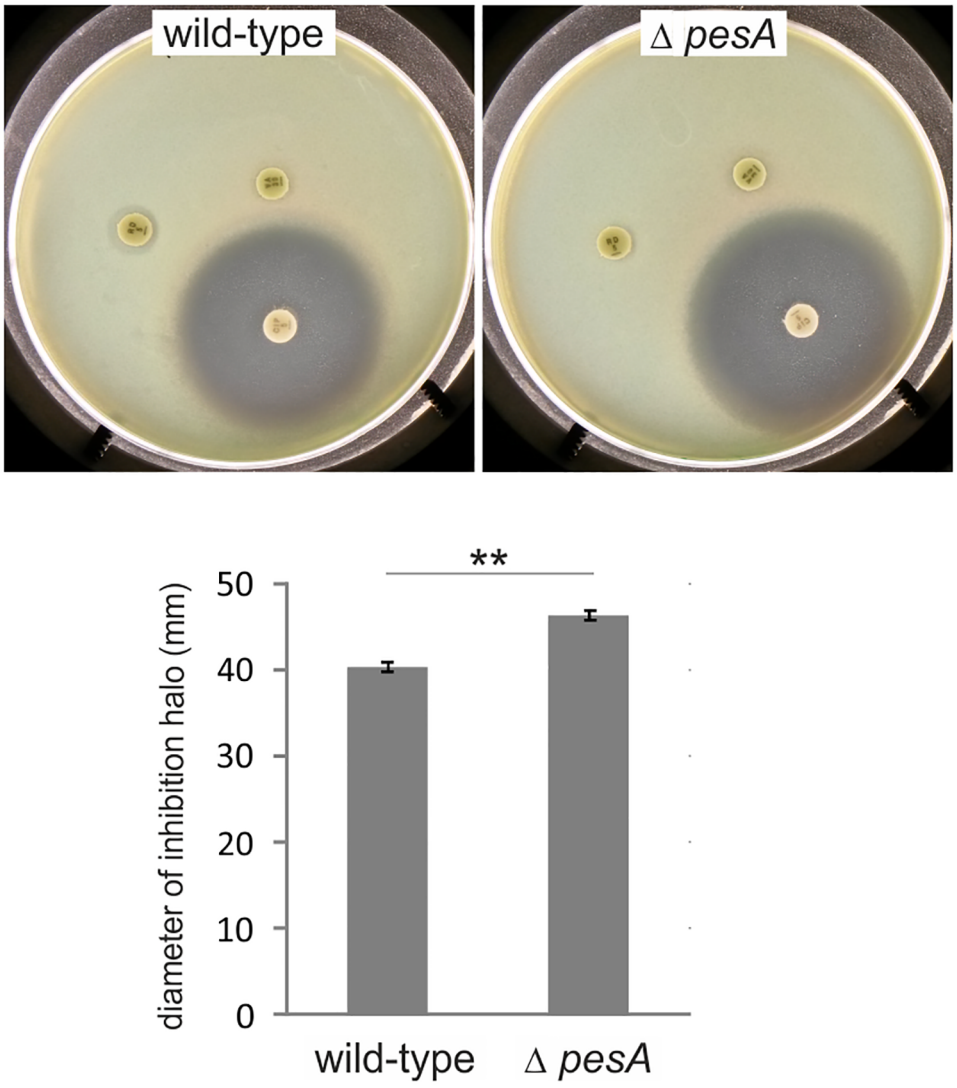

Fig 4. *PesA* deletion enhances sensitivity to ciprofloxacin. Antibiotic disk diffusion was performed on LB-agar plates spread with 10^6 CFU bacterial cells of wild-type PA14 and *ΔpesA* mutant strains. The diameters of the inhibitory zones were measured after overnight incubation at 37˚C. Data derive from three independent experiments. Values represent the mean ± SD. Statistical significance by Student's t-Test is indicated: $**p < 0.01$.

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pyoS3A-I::*sfGFP*, mimics a bi-cistron under the control of the heterologous constitutive promoter $P_{LtetO-1}$. It was obtained by cloning a region of 224 nt, comprehensive of the last 117 nt of the *pyos3A* gene and the first 108 nt of *pyoS3I* thus generating a first translational fusion of the reporter F'*lacZ* with the last 39 codons of *pyoS3A*, and a second translational fusion of the first 36 codons of *pyoS3I* gene with *sf*GFP. GFP activity was assayed in PA14 wild-type and PA14 *ΔpesA* in the absence and presence of PesA overexpression from pGM-*pesA*. As shown in Fig 9A, there was an approximately 25% reduction in GFP activity in the PA14 *ΔpesA* background. In the presence of pGM-*pesA* overexpressing PesA, in wild-type background, GFP activity increased approximately 30%. These results suggested that PesA positively regulates *pyoS3I* expression. To test whether translation of *pyos3A* gene was necessary to have this PesA effects on *pyos3I*, we generated a second mono-cistronic reporter derivative, pBBR1-*pyoS3I*::

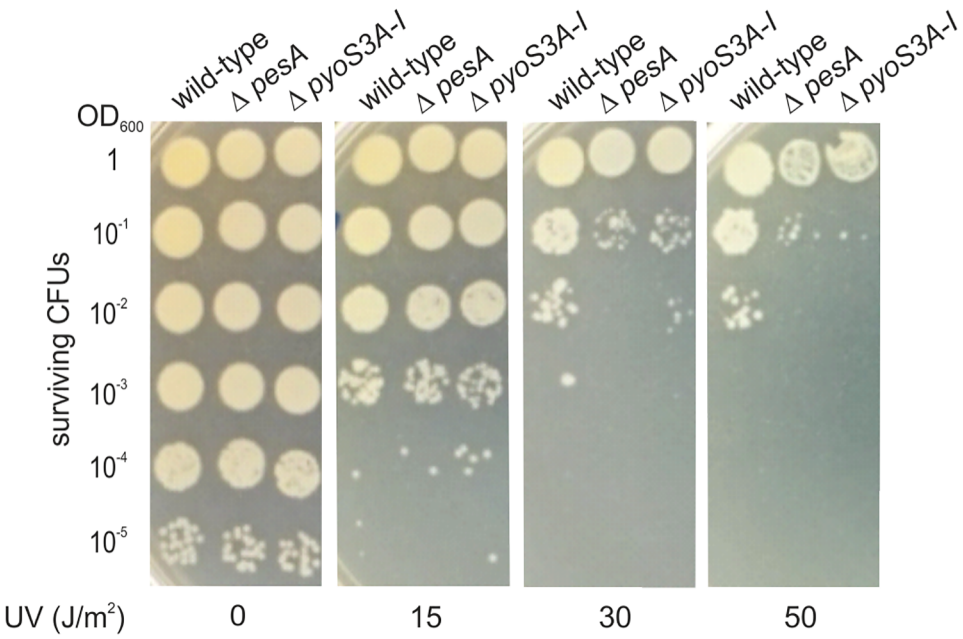

Fig 5. PesA deletion enhances UV sensitivity similarly to *pyoS3* operon deletion. 3 μl of cultures of PA14 *wt*, Δ*pesA and* Δ*pyoS3*, serially diluted 10-fold, were spotted onto LB-agar plates, and treated with UV light at the indicated doses. Surviving CFUs were observed after overnight incubation at 37˚C.

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sfGFP, carrying the same *pyoS3A-I* region as pBBR1-*lacZ*::*pyoS3A-I*::*sfGFP* in which only the *pyoS3I* gene was translationally fused with sfGFP. GFP analyses confirmed that PesA influence in a positive manner the regulation of *pyoS3I* in the mono-cistronic construct, with a ~20% increment in GFP activity in the presence of PesA overexpression in the wild-type background, and a ~40% decrease in the PA14 *ΔpesA* background (Fig 9B). This suggested that the translation of the two genes is not merely and solely coupled and that the effect of PesA on *pyoS3I* do not require the translation of *pyoS3A*.

To assess the influence of PesA also on the *pyoS3A* gene, we substituted the sequence coding for the F'LacZ domain in pBBR1-*lacZ*::*pyoS3A-I*::*sfGFP* with the one of the reporter gene *mCherry*, and monitored simultaneously the activity of both mCherry and sfGFP of the translational fusion *mCherry*::*pyoS3A-S3I*::*sfGFP*. As shown in Fig 9C, the ~50% increase in sfGFP activity followed by PesA overexpression, and the ~25% decrease in the PA14 *ΔpesA* background reconfirmed the positive regulation exerted by PesA on the *pyoS3I* gene. mCherry also showed an increase in activity when PesA was overexpressed, suggesting that PesA also exerts a positive regulation on the upstream gene *pyoS3A*. Thus, PesA seems to be a positive regulator of the whole *pyoS3* operon.

Quantitative RT-PCR on total mRNA of wild-type and Δ*pesA* strains was also performed to check whether PesA influenced *pyoS3* mRNA levels. Samples were taken at mid-, late-exponential and stationary phase (OD_{600} of 0.8, 1.6 and 2.7, respectively) and both genes, *pyoS3A* and *pyoS3I*, were analyzed for their expression levels. No significant differences were observed either for *pyoS3A* or *pyoS3I* in wild-type and Δ*pesA* backgrounds at any time-point. Notably, the expression levels of the two genes were comparable at every time-point and seemed not to be influenced by the growth phase, being constant along the growth curve (S5 Table). We conclude that PesA exerts positive regulation on *pyoS3A-I* mRNA by modulating mRNA translatability and without influencing its stability.

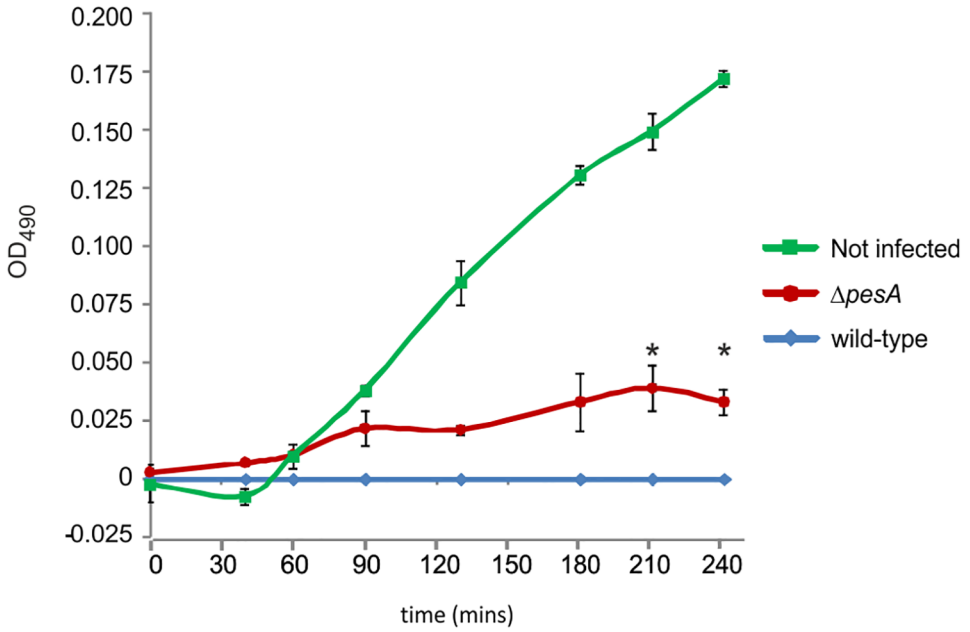

Fig 6. Time course of cell viability of IB3-1 cells following bacterial infection with *P. aeruginosa* PA14 wild-type and Δ*pesA*. Cell viability, assessed as a reduction of MTT salt, was quantified by the optical density (OD) at 490 nm. IB3-1 cells were seeded at a density of 5×10^4 cells/well into 96-well microplates, and infected with 5×10^6 bacterial cells (MOI 1:100). At every time point, data are shown as the difference in OD_{490} between the PA14 wild-type strain and the sRNA-deleted mutant Δ*pesA*. Uninfected cells were used as positive control of cell viability. Data derive from three independent experiments. Results are shown as the difference in the OD_{490} reached at the different time points by IB3-1 cells infected by the mutant strain or non-infected, subtracted of the OD_{490} reached by IB3-cells infected with the wild-type strain. Values represent the mean ± standard error of the mean (SEM). Statistical significance between wild-type and Δ*pesA* strains by Student's t-Test is indicated: **p*< 0.05.

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By the use of the *IntaRNA* web tool [44, 45], we also detected a putative interacting region between PesA and the leader sequence of the *pyoS3* operon, from -76 to -42 from the TTG start codon of the *pyoS3A* gene. To evaluate whether PesA was also able to influence the S3-operon translation by acting on its leader sequence, we generated the translational fusion *leader-pyoS3A*::*sfGFP*, by cloning the whole leader region of the *pyoS3* operon and the sequence encoding the first 37 aminoacids of *pyos3A* gene, in frame with the *sfGFP* reporter. The comparison of the fluorescence activity of the *leader-pyoS3A*::*sfGFP* translational fusion between the wild-type and Δ*pesA* genetic background did not show any significant difference, not even in presence of PesA overexpression from pGM-*pesA* in wild-type (S2 Fig). Spurious outside interactions of PesA with sfGFP and mCherry open reading frame were ruled out using alternative reporter plasmids carrying the *sfGFP* and *mCherry* genes alone (S2 Fig).

Discussion

We studied the novel *P. aeruginosa* sRNA PesA, which was originally identified as being transcribed from the horizontally acquired pathogenicity island PAPI-1 in the strain PA14. Our analysis revealed that PesA is widespread in clinical isolates from patients affected by chronic respiratory diseases, such as CF, being expressed in 55% of the cases tested. Moreover, PesA expression is responsive to low oxygen conditions, a hallmark of CF and COPD, and impacts *P. aeruginosa* pathogenicity in CF bronchial cells. These results suggest that PesA could be relevant during *P. aeruginosa* infection in chronic respiratory diseases.

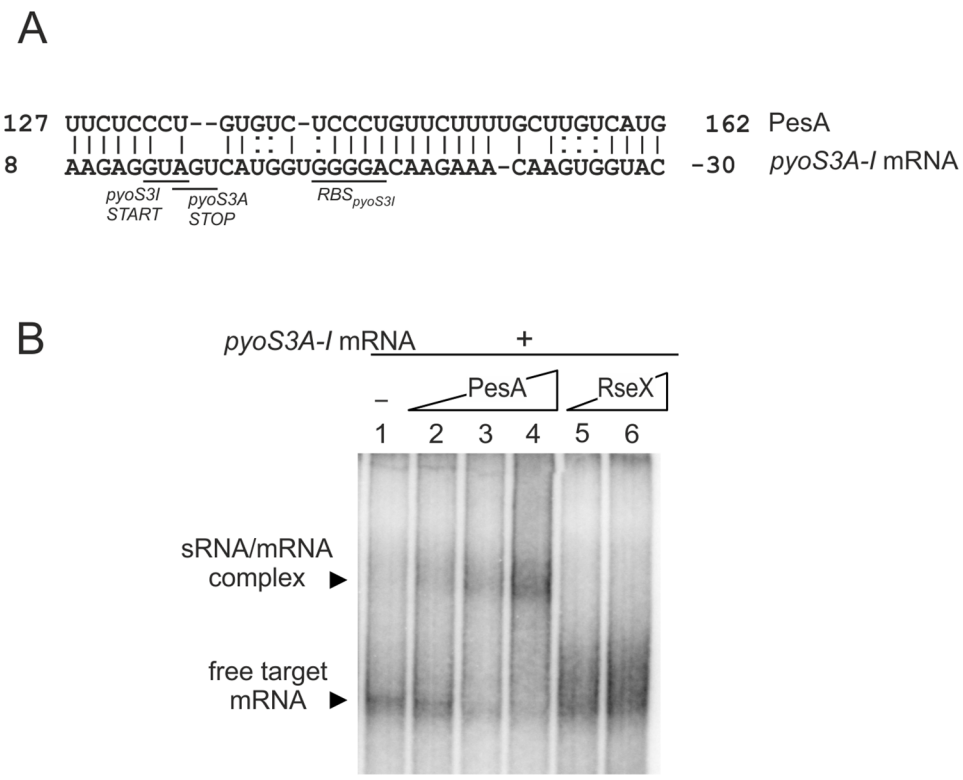

Fig 7. Interaction of PesA with *pyoS3A-I* mRNA. A) Prediction by *TargetRNA* software of the base-pairing interactions between PesA and *pyoS3A-I* mRNA. B) *In vitro* interaction between PesA RNA and *pyoS3A-I* mRNA by an electrophoretic mobility shift assay. Increasing amounts of PesA RNA (0, 0.08, 0.15, and 0.25 pmol; lanes 1–4) or, as a negative control, *E. coli* RseX RNA (0.25 and 2.5 pmol; lanes 5 and 6) were incubated at 37˚C for 20 min with 0.15 pmol radiolabeled *pyoS3A-I* mRNA and loaded onto a native 6% polyacrylamide gel.

https://doi.org/10.1371/journal.pone.0180386.g007

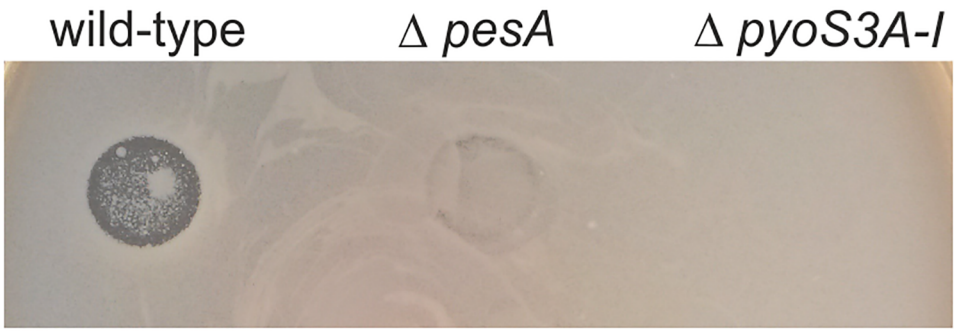

Fig 8. PesA deletion impairs the production of pyocin S3. Plate showing the effects of pyocin S3 present in the filtered supernatants of PA14 wild-type, Δ*pesA* and Δ*pyoS3A-I* on the killing of the indicator strain *P. aeruginosa* ATCC 27853. Drops of 5 μl of filtered supernatants from PA14 wild-type, Δ*pesA* and Δ*pyoS3A-I* cultures at OD_{600} = 1 were deposited on Luria-Bertani agar plates. A layer of the indicator strain *P. aeruginosa* ATCC 27853 was plated over the dried drops by inclusion in 0.7% agar. Plates were incubated overnight at 37˚C. Clearer haloes represent inhibition (killing) of the indicator strain by pyocin S3 present in the sedimented supernatants.

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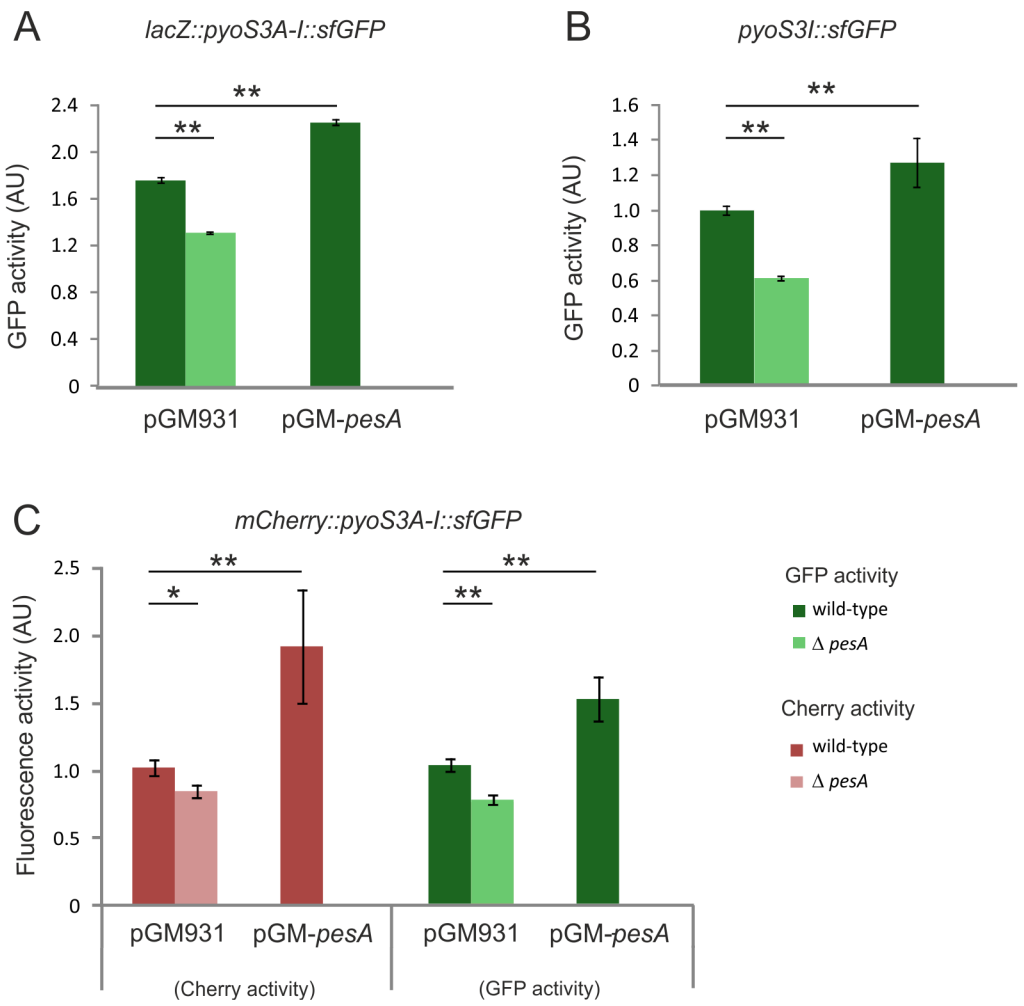

Fig 9. PesA positively regulates the expression of both the *pyoS3A* and *pyoS3I* translational fusions in PA14. Comparison of the sfGFP and mCherry activities expressed in arbitrary units (AU) resulting from the translational fusion of (A) *lacZ::pyos3A-I::sfGFP*, (B) *pyoS3I::sfGFP* and (C) *Cherry::pyoS3A-I::sfGFP* combined with the control vector (pGM931) or the plasmid overexpressing PesA (pGM-*pesA*), in PA14 wild-type and PA14 Δ*pesA*. The strains were grown to an OD_{600} of 1.8 in LB medium supplemented with gentamicin and carbenicillin, to maintain pBBR1- and pGM- plasmids, respectively, and arabinose, to induce PesA overexpression. Cells were harvested and treated for sfGFP and mCherry activity determination by measuring fluorescence polarization $FP_{485/535}$ and fluorescence intensity $FI_{590/635}$, respectively. sfGFP and mCherry activities are expressed as ratio $FP_{485/535}/Abs_{595}$ and $FI_{590/635}/Abs_{595}$, respectively. Data derive from three independent experiments. Values represent the mean ± SD. Statistical significance by Student's t-Test is indicated: *p<0.05; **p< 0.01.

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We speculated that PesA had the potential to act as a *trans*-encoded base-pairing sRNA involved also in the post-transcriptional regulation of genes located outside PAPI-1 and thus performed genome-wide bioinformatics screenings for target genes. One predicted target was the *pyoS3A-I* operon coding for pyocin S3. We then confirmed that both *pyoS3A* and *pyoS3I* genes are positively influenced by PesA. The interaction of PesA with the bicistronic *pyoS3A-I* mRNA is suggested to simultaneously stimulate translation initiation and termination of *pyoS3I* and *pyoS3A*, respectively, without influencing stability of the *pyoS3A-I* mRNA. Interestingly, PesA could impact a putative mechanism of translation coupling between *pyoS3I* and

pyoS3A [22] that remains to be elucidated. However, in this paper we demonstrate that stimulation of *pyoS3I* by PesA does not require translation of the upstream *pyoS3A* thus suggesting that the two genes are not strictly translationally coupled. This scenario, as a whole, is compatible with the role of PesA in assuring balanced expression of toxin S3A and antitoxin S3I to prevent deleterious effects on the producing host. Overall, these data obtained with the reporter genes are consistent with the observation that PesA deletion results in strong reduction of pyocin S3 production (Fig 8).

It is conceivable that PesA can be involved in mechanisms of niche establishment via pyocin S3. PesA was in fact also detected in environmental isolates and its expression from PAPI-1 might confer a selective advantage that favours PAPI-1 maintenance in the environment due to its regulation of pyocin S3.

It is likely that PesA has a broad set of target genes whose functions go beyond the niche establishment. The observation that PesA deletion induces less killing in infected CF bronchial epithelial cells suggests that PesA could modulate, directly or indirectly, virulence factors of *P. aeruginosa*. It was previously shown that pyocin S2 is endowed with cytotoxic activity on human cell lines [46]. It can be argued that pyocin S3 has similar effects and can act as virulence factor whose regulation is under the control of PesA.

Furthermore, PesA could regulate the expression of genes involved in DNA damage repair as suggested by the increased sensitivity of *ΔpesA* mutant to fluoroquinolone antibiotic ciprofloxacin and to UV irradiation. Intriguingly, the degree of UV sensitivity displayed by the *ΔpesA* mutant is comparable to that of a strain deleted for *pyoS3A-I*. These results imply a potential involvement of the DNase activity of pyocin S3 in DNA damage repair, and introduce an intriguing network among sRNAs, pyocins and DNA damage repair that will require additional experiments to be elucidated. To our knowledge this is the first work characterizing a sRNA encoded in a pathogenicity island in *P. aeruginosa*. In addition, our results indicate that PesA is able to modulate key genes located outside the PAPI-1. In summary, the horizontal acquisition of PAPI-1 could provide the new host with a regulatory function that can switch the expression of genes involved in niche establishment, virulence and stress resistance.

Supporting information

S1 Fig. Validation of PesA deletion and overexpression. A) PA14 wild-type and PA14 Δ*pesA* were grown in BHI medium until an OD_{600} of 2.7. Culture samples were taken and processed for total RNA extraction and analysis by Northern blot. B) PA14 strains harbouring pGM-*pesA* or the control empty vector pGM931 were grown in BHI medium with carbenicillin until an OD_{600} of 0.8. Cells were split into two flasks, and 10 mM arabinose (ara) was added to one. Culture samples were taken at the indicated OD_{600} and processed for total RNA extraction and analysis by Northern blot probing PesA RNA. Intensities of the bands of PesA were quantified and normalized to those of 5S RNA in the same lane. Values are expressed as arbitrary units (AU) in the histograms below the Northern blot.
(TIF)

S2 Fig. Fluorescence activity to check PesA regulation on the leader of *pyoS3A* gene, and spurious outside interactions of PesA with *sfGFP* and *mCherry* open reading frames. A) Comparison of the sfGFP activity resulting from the translational fusion *leader-pyoS3A::sfGFP* in PA14 wild-type and PA14 Δ*pesA* (-), and combined with the control vector (pGM931) or the plasmid overexpressing PesA (pGM-*pesA*) in PA14 wild-type. B) Comparison of the fluorescence activity of the reporter gene *sfGFP* combined with the control vector (pGM931) or the plasmid overexpressing PesA (pGM-*pesA*), in PA14 wild-type and PA14 Δ*pesA*. C)

Comparison of fluorescence activity of the reporter gene *mCherry* combined with the control vector (pGM931) or the plasmid overexpressing PesA (pGM-*pesA*), PA14 wild-type and PA14 Δ*pesA*.
(TIF)

S1 Table. Clinical isolates.
(PDF)

S2 Table. Strains and plasmids.
(PDF)

S3 Table. Oligonucleotides.
(PDF)

S4 Table. List of mRNA targets of PesA predicted by bioinformatics analysis conducted with the *TargetRNA* web-tool.
(PDF)

S5 Table. Quantitative RT-PCR analyses of *pyoS3A* and *pyoS3I* mRNA levels in wild-type and Δ*pesA* backgrounds.
(PDF)

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Writing – review & editing: SF GB.

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PART III

Contents

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Marilena Falcone, Silvia Ferrara, Elio Rossi, Helle Krogh Johansen, Søren Molin and Giovanni Bertoni. The small RNA ErsA of *Pseudomonas aeruginosa* contributes to biofilm development and motility through post-transcriptional modulation of AmrZ.

The small RNA ErsA of *Pseudomonas aeruginosa* contributes to biofilm development and motility through post-transcriptional modulation of AmrZ

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Abstract

The small RNA ErsA of *Pseudomonas aeruginosa* was previously suggested to be involved in biofilm formation via negative post-transcriptional regulation of the *algC* gene that encodes the virulence-associated enzyme AlgC, which provides sugar precursors for the synthesis of several polysaccharides. In this study, we show that a knock-out *ersA* mutant strain form a flat and uniform biofilm, not characterized by mushroom-multicellular structures typical of mature biofilm. Conversely, the knock-out mutant strain showed enhanced swarming and twitching motilities. To assess the influence of ErsA on the *P. aeruginosa* transcriptome, we performed RNA-seq experiments comparing the knock-out mutant with the wild-type. More than 160 genes were found differentially expressed in the knock-out mutant. Parts of these genes, important for biofilm formation and motility regulation, are known to belong also to the AmrZ transcriptional regulator regulon. Here, we show that ErsA binds *in vitro* and positively regulates *amrZ* mRNA at post-transcriptional level *in vivo* suggesting an interesting contribution of the ErsA-*amrZ* mRNA interaction in biofilm development at several regulatory levels.

Introduction

Biofilm formation is considered to be an adaptive strategy of the human pathogen *Pseudomonas aeruginosa,* and the switch from the motile to a sessile mode of growth represents an important step in the virulence of this pathogen (Costerton et al., 1999).
Biofilms are microbial communities assembled in a self-produced matrix of exopolysaccharides, proteins and DNA (Ma et al., 2006), generating conditions that confer resistance and protection against antimicrobial agents and the immune system. The biofilm lifestyle cycle of *P. aeruginosa* PAO1 develops through coordinated stages. Adhesion to a surface is the first step in the colonization of *P. aeruginosa* and is followed by cell-to-cell agglutination. Attachment is an irreversible condition characterized by formation of microcolonies that confluence in more structured and three-dimensional clusters. During these two stages, the bacterial cells display three types of motility, swimming movement in liquid or low-viscosity conditions, swarming on semisolid surface and twitching on a solid surface. Swarming motility is based on flagella and type IV pili as well as on biosurfactants, swimming is flagella-dependent, and twitching relies on extension and retraction of type IV pili (Kohler et al., 2000; O'Toole and Kolter, 1998; Wang et al., 2014). The final stage of biofilm development is bacterial dispersion, in which the bacteria re-enter the planktonic state of spreading and colonizing other surfaces (Flemming et al., 2007; Wang et al., 2014).
As summarized in Fig. 1, intertwined regulatory pathways and numerous regulators control transcriptionally and post-transcriptionally biofilm development. Most of these regulators are coordinated by the alternative sigma factor AlgT/U (σ^{22}) (Potvin et al., 2008), a mediator of stress response and a functional homologue of *E. coli* σ^{E} (Yu et al., 1995). AlgU regulates the alginate production driving the expression of *algD* operon, and activating two transcriptional regulators, AlgR and AmrZ, both required for alginate production in multiple mucoid strains (Mohr et al., 1991, 1992; Yu et al., 1995). AmrZ, besides the interaction with *algD*, also directly affects the *P. aeruginosa* exopolysaccharides profile. In fact, as shown in Fig.1, AmrZ triggers the expression of the exopolysaccharide Pel interacting with a member of the *pel* operon (*pelB*), represses the expression of the exopolysaccharide Psl binding to the *pslA* promoter. In addition, AmrZ affects the intracellular levels of the signaling molecule bis (3'-5')-cyclic diguanylic monophosphate (c-di-GMP) (Jones et al., 2014; Petrova et al., 2014; Xu et al., 2016). Pel and Psl exopolysaccharides are the major contributors to *P. aeruginosa* biofilm structure and development. Psl supports the cell-to-cell interactions during the initial attachment and adhesion phase, forming a fiber web to constitute a scaffold for the biofilm shaping, and Pel provides structural stability to the global configuration (Jennings et al., 2015; Ma et al., 2006, 2007; Yang et al., 2011).
Biosynthesis of Pel, Psl and LPS uses common sugar precursors supplied by the AlgU-induced AlgC enzyme, which coordinates the levels of exopolysaccharides in the cell, catalyzing the conversion of Man-6-P and glucose-6-P (Glc-6-P) to Man-1-P and Glc-1-P, respectively (Coyne et al., 1994; Ma et al., 2012). AlgC is positively regulated by AlgR at the transcriptional level, and negatively regulated by the small RNA (sRNA) ErsA at the post-transcriptional level (Ferrara et al., 2015; Wozniak and Ohman, 1994; Zielinski et al, 1991). ErsA is a novel sRNA recently characterized in *P. aeruginosa* whose expression responds to several infection cues such as limited iron availability, temperature shifts from environmental to body temperature and reduced oxygen conditions. The incoherent feed-forward loop settled by ErsA and AlgU to fine-regulate AlgC was supposed to be an additional regulatory route in the complex process of biofilm shaping, in particular balancing the sugar precursors production in the exopolysaccharides biosynthesis (Ferrara et al., 2015).
In a recent study (Zhang et al., 2017), ErsA has been described to bind and regulate at the post-transcriptional level *oprD* mRNA, coding for a porin which highly contributes to carbapenems

sensitivity. The overexpression of ErsA negatively affects translation of *oprD* mRNA and consequently the OprD protein level, reducing susceptibility to meropenem treatment. These findings contribute to enforce the role of ErsA in *P. aeruginosa* pathogenesis by regulating different virulence traits.

sRNAs can regulate multiple targets, allowing the cells to have a faster response to stress conditions and adapt in a short time frame to environmental changes (Beisel and Storz, 2010).

ErsA provides a relevant regulatory contribution balancing metabolism and virulence routes by regulating the checkpoint enzyme AlgC and it was conceivable to hypothesize novel ErsA targets in the large landscape of regulatory routes connected to exopolysaccharides production and biofilm formation.

In this study, we scrutinized for the first time the regulatory pattern of ErsA in *P. aeruginosa* biofilm formation revealing a positive contribution of the sRNA in biofilm maturation and shaping. An RNA-seq approach allowed us to identify several genes involved in this process, whose expression was deregulated in an ErsA deletion mutant. Most of these genes belong to AmrZ regulon, which was shown to be a novel direct target for ErsA (Fig. 1).

MATERIALS AND METHODS

Bacterial strains and media. Bacteria and plasmids used in this study are listed in Table S1. *Escherichia coli* strains were grown 37°C in Lysogeny Broth (LB). *P. aeruginosa* strains were grown at 37°C in LB or in Brain Heart Infusion Broth (BHI) or Artificial Sputum Medium (ASM) in flasks at 200 r.p.m.. When required, for *E.coli* strains the media were supplemented with 10 ug/ml gentamycin, 100 ug/ml ampicillin, 25 ug/ml kanamycin, and for *P. aeruginosa* strains with 50 ug/ml gentamycin and 300 ug/ml carbenicillin. For monitoring biofilm development in flow-chambers conditions, PAO1 wild-type and PAO1 Δ*ersA* (Ferrara et al., 2015) were chromosomally tagged with green fluorescent protein (GFP) and grown in modified FAB medium (Heydorn et al., 2000) supplemented with 0.3 mM glucose.
ErsA overexpression was obtained from pGM-*ersA* plasmid (Ferrara et al., 2015) using arabinose 0.2% when required.

Plasmid construction and mutant generation. Oligonucleotides used in this study are listed in Table S2. Translational fusions pBBR1 *amrZ::sfGFP*, *amrZ*CIS1::*sfGFP*, *amrZ*ΔIS2::*sfGFP and amrZ*CIS1ΔIS2::*sfGFP* under the $P_{LtetO-1}$ constitutive promoter were generated as follows. A DNA fragment of 161 bp including 56 nt of UTR-region and 35 codons of the open reading frame (ORF) of *amrZ* was amplified by PCR with oligos 1/2 (Table S2), digested with *Nsi*I-*Nhe*I and cloned into the *sfGFP* reporter vectors pXG10-SF resulting in the plasmid pXG10-*amrZ::sfGFP*. Likewise for *amrZ::sfGFP*, 161 bp including 56 nt of UTR-region and 35 codons of the open reading frame (ORF) of *amrZ* were amplified by PCR with oligos 1/2 (Table S2) from pUCIDT *amrZ*CIS plasmid, carrying the synthetic and modified sequence of *amrZ*, digested with *Nsi*I-*Nhe*I and cloned into pXG10-SF to generate the translational fusion pXG10-*amrZ*CIS1::*sfGFP*. The translational fusion pXG10-*amrZ*ΔIS2::*sfGFP* was generated amplifying a fragment of 119 bp including 56 nt of UTR-region and 21 codons of the *amrZ* ORF with oligos 1/3 (Table S2) digested with *Nsi*I-*Nhe*I and cloned into pXG10-SF. *AmrZ*CIS1ΔIS2::*sfGFP* was constructed amplifying a fragment of 119 bp including 56 nt of UTR-region and 21 codons of the *amrZ* ORF with oligos 1/3 (Table S2) from pUCIDT *amrZ*CIS plasmid. All the fragments from the $P_{LtetO-1}$ promoter to the end of the GFP reporter gene, including the different versions of *amrZ,* were amplified from pXG10-*amrZ::sfGF, amrZ*CIS1::*sfGFP, amrZ*ΔIS2::*sfGFP* and *amrZ*CIS1ΔIS2::*sfGFP,* using oligos 9/10, digested with *Cla*I-*Xba*I and cloned into the low-copy number shuttle vector pBBR1-MCS5 generating the pBBR1-*amrZ::sfGFP, amrZ*CIS1::*sfGFP, amrZ*ΔIS2::*sfGFP and amrZ*CIS1ΔIS2::*sfGFP*, respectively. All the plasmids were then transformed into *P. aeruginosa* strains as reported previously (Ferrara et al., 2015).

Mini-Tn7- *gfp* strain construction. A PrrB1-*gfp*-a transposon cassette was inserted into the chromosome of PAO1 wild-type and Δ*ersA* by conjugation using pBK-miniTn7-ΩGm as a delivery plasmid carrying the cassette inserted into *Not*I site as reported previously (Lambertsen et al., 2004).

Biofilm adhesion in 96-wells peg-lid microtiter. A quantity of 200 ul of overnight bacterial cultures grown in BHI or ASM and diluted to OD_{600}= 0.01, with the addition of carbenicillin 300 ug/ml and arabinose 0.2% when required, was aliquoted into 96-well peg-lid microtiter plates (Nunclon Delta Surface Cat. No.167008, Nunc TSP Cat. No.445497, Thermo Scientific) as reported previously (Harrison et al., 2010). The plates were incubated at 37°C in aerobic conditions with 100 r.p.m. stirring. After 20 hrs of incubation, growth was monitored by measuring the OD_{600}, and the ability of the *P. aeruginosa* strains to adhere to the polystyrene peg-lid was tested by crystal violet staining. Briefly, the peg-lid was washed twice with saline

solution and then stained with 0.1% crystal violet for 20 min (O´Toole, 2011). Excess of stain was rinsed off by placing the peg-lid in saline solution before to solubilize the dye in absolute ethanol per well. The optical density of each well was measured at 590 nm. Biofilm formation was expressed in adhesion units as the result of the OD_{590}/OD_{600} ratio and statistical analysis were performed using T-Test.

Biofilm development in flow-cells system. Biofilms were grown at 30°C in flow chambers composed by three individual channels as described previously (Møller et al., 1998). PAO1 wild-type and Δ*ersA* overnight cultures diluted to OD_{600} = 0.01 were inoculated into each flow channel with a small syringe. After 1 hr without flow, each channel was supplied with a flow of 3 ml/h of FAB medium with glucose 0.3 mM, using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow cells was 0.2 mm/s.

Confocal laser scanning microscopy and image processing. The microscopic analyses were performed using a Zeiss LSM510 confocal laser scanning microscope (CLSM; Carl Zeiss, Jena, Germany) equipped with an Ar/Kr laser and filter sets for GFP detection (excitation, 488 nm; emission, 517 nm). Images were obtained using a 40×/1.3 Plan-Neofluar oil objective. Simulated shadow projection images and cross sections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland).
The experiment was performed in triplicate for each strain acquiring 7 random images for each channel every day for 3 days. Thus, 21 images for each time point were employed for the statistical analyses using COMSTAT 2.1 software (Heydorn et al., 2000; Vorregaard, 2008, www.comstat.dk)

Co-twitching and co-swarming motility assays. Swarming assays were performed using Nutrient Broth (Nutrient Broth n°2 Oxoid) medium plates supplemented with 0.5% glucose and 0.5% Bacto-agar (Difco). Overnight cultures normalized at the same OD_{600} of PAO1 wildtype and Δ*ersA* were spotted on the same plate suitably spaced each other and placed at both 28°C and 37°C for 24 hrs.
Twitching was performed on LB plates supplemented with 1% Bacto-agar (Difco). The inoculation was performed with a sterile toothpick dipped in the overnight cultures and followed at 37°C for 24 hrs. Statistical analysis was performed on 3 independent replicates with GraphPad Prism software.

RNA sequencing and data analysis. For RNA-Seq, cultures of wild-type PAO1 and Δ*ersA* strains were grown to early stationary phase (OD_{600} = 2.7) in BHI medium. For each strain, total RNA was extracted from at least two independent biological replicates using Trizol reagent (Thermo Fisher Scientific Inc.) followed by RNA clean & concentrator kit (Zymo Research, Irvin, USA) accordingly to vendors' protocols. RNA quality was checked using RNA Nano kit on an Agilent Bioanalyzer 2100 machine. Samples with an RNA integrity number (RIN) greater than 9 were used in downstream analysis. Strand-specific sequencing libraries were prepared using 50 ng of mRNA-enriched samples as input for TruSeq stranded mRNA library preparation kit (Illumina) following vendor's recommendations. Sequencing was performed on an Illumina NextSeq 500 to a depth of 15-20 million reads per sample. After quality filtering, raw reads were aligned using BWA aligner against *P. aeruginosa* PAO1 genome (NC_002516.2). Read count for gene relative abundance was obtained using HTSeq-count tool from HTSeq package (Anders et al., 2015), while differential expression analysis and statistical analysis were performed as previously described (Peano et al., 2014). RNA-seq data have been deposited in the

ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6247.

RNA isolation and synthesis. Total RNA was extracted as reported previously (Ferrara et al., 2012). RNA for RNA/RNA interaction assays was prepared by T7 RNA polymerase transcription of gel-purified DNA fragments. DNA fragments for ErsA RNA and *amrZ* mRNAs (*amrZ*, *amrZ*CIS1, *amrZ*ΔIS2, *amrZ*CIS1ΔIS2) preparations were amplified from *P. aeruginosa* PAO1 genomic DNA with oligo pairs 4/5 or 4/6 and 7/8, respectively. The transcription reactions were performed using the Riboprobe® System-T7 (Promega) with 300 ng of DNA template. DNA probe was 5′-end–labelled with (γ-^{32}P) ATP and T4 polynucleotide kinase (Promega) according to manufacturer's instruction. Synthesized RNA was precipitated and resuspended in diethylpyrocarbonate-treated water. Purified RNA was checked by denaturing polyacrylamide gel electrophoresis and quantified using a Qubit Fluorometer.

***In vitro* and *in vivo* assays of sRNA/mRNA interactions.** To assess the ErsA/*amrZ* mRNA interactions *in vitro*, the binding reactions were set up as described previously (Ferrara et al., 2015). After the electrophoresis, the membrane was UV-crosslinked and hybridized with a [^{32}P]-labelled oligo and the radioactive bands were acquired using a Typhoon™ 8600 variable mode imager scanner (GE Healthcare BioSciences) and visualized with ImageQuant software (Molecular Dynamics).
Non-radioactive EMSA were performed using Mini-Protean® Electrophoresis System (BioRad) at 4°C and 150 V for 45 min. The gel was stained in SYBR™ Gold Nucleic Acid Gel Stain diluted in 0.5 × TBE. Images were acquired by Gel Doc™ XR+ (BioRad) imaging system.
Fluoresence measurements of *P. aeruginosa* strains carrying the reporters pBBR1-*amrZ::gfp* were carried out as previously reported (Ferrara et al., 2015). Abs_{595} and fluorescence polarization $FP_{485/535}$ were measured in a Tecan Infinity PRO 200 reader, using Magellan as data analysis software (Tecan). GFP activities were expressed in Arbitrary Units (AU) as ratio $FP_{485/535}/Abs_{595}$. Statistical analysis performed on three individual clones per strain using T-test.

Results

ErsA is required for biofilm adhesion and development

We investigated the effects of deleting the *ersA* gene on biofilm formation using a semi-quantitative microtiter "peg-lid" assay in Brain Heart Infusion medium (BHI). As shown in Fig. 2A, the ErsA deletion resulted in decreased biofilm formation in BHI compared to PAO1 wild-type strain, and the complemented strain carrying the plasmid pGM-*ersA* produces more biofilm than the *ersA* deletion mutant strain carrying the pGM931 empty vector (Fig. 2B).
To examine the role of ErsA in *P. aeruginosa* biofilm architecture development, we cultivated the PAO1 wild-type and the Δ*ersA* GFP-tagged strain, in flow-chambers continuously supplied with modified FAB medium supplemented with glucose. Biofilm development stages were followed and visualized daily for 3 days by Confocal Laser Microscopy (CLSM). In agreement with biofilm formation in the microtiter "peg-lid" assays in BHI medium, the PAO1 Δ*ersA* strain developed less biofilm biomass than the wild-type, which showed the mushroom-like structures typical of 3-days old *P. aeruginosa* biofilms in flow-cells system (Fig. 2C). The statistically significant differences in biomass and spatial structure between PAO1 wild-type and Δ*ersA* biofilms were determined by the COMSTAT 2.1 software (Heydorn et al., 2000; Vorregaard, 2008) as represented in Fig. 2C. We further noticed the positive influence of ErsA on adhesion and biofilm formation when

overexpressed in PAO1 wild-type and Δ*ersA* strains, grown in artificial sputum medium (ASM) (Fig. S1), which is defined to reflect the chemical environment of CF lungs (Haley et al., 2012; Sriramulu and Shre, 1999).

ErsA negatively regulates swarming and twitching motility

Motility is crucial in cell-to-cell adherence and attachment in early biofilm stages and it has been suggested an inverse regulation of motility and biofilm during biofilm development (Caiazza et al., 2007; Wang et al., 2014). Several transcriptional and post-transcriptional regulators are involved in these pathways and some of them coordinate both sessile and motile lifestyles (Gloag et al., 2013; O'Toole and Kolter, 1998; Ramsey and Whiteley, 2004; Shrout et al., 2006). To further investigate the involvement of ErsA on these biofilm-related phenotypes, we performed co-swarming, swimming and co-twitching experiments comparing PAO1 wild-type with Δ*ersA* strain. Our results reveal a negative influence of ErsA on both swarming and twitching motility (Fig. 3) and the temperature conditions do not affect ErsA regulation on swarming motility (Fig. 3A). No differences between PAO1 wild-type and Δ*ersA* mutant strain were observed for swimming motility (Fig. S2).

ErsA deletion affects the transcriptional levels of 168 genes in *P. aeruginosa* PAO1

Small RNAs are usually involved in post-transcriptional regulation, and the role of ErsA in biofilm development and motility shown in this study, suggested interference with the translation of transcriptional regulators. Thus, to expand the panel of ErsA targets in *P. aeruginosa* PAO1, and to have a better view of the effect of ErsA activity on the genome-wide gene expression, we performed an RNA-seq experiment comparing PAO1 wild-type to ErsA deletion mutant strains, grown to late exponential phase (OD_{600} of 2.7) in BHI medium. We observed 168 genes (Table S3 and the most representative genes listed in Table 1) differentially expressed in the *ersA* deletion mutant when compared to the wild-type strain. Among the 29 genes upregulated in the *ersA* deletion mutant we identified genes involved in denitrification and nitrate metabolism (*narI, narJ, nirN*) as well as type VI and III secretion systems effectors (*tssA1, tsi4, tse6*).
The majority of genes were downregulated in absence of ErsA (139 genes); the strongest negative effect was observed for *narK1* involved in nitrate transport. The other hits with a change of $Log_2(FC) \leq -1.5$, comprise well described genes involved in biofilm formation and motility (*algD, esrC, ppyR, pelCDEFG, roeA*), energy metabolism and carbon metabolism (*prpD, prpC, coxA, coxB*), heat-shock proteins (*htpG, hslU, hslV, ibpA, dnaK, dnaJ*) and *phzS* involved in pyocyanin production.

ErsA binds *in vitro* and positively regulates *in vivo amrZ* mRNA at the post-transcriptional level

In order to investigate the possibility that ErsA regulates expression of AmrZ at the post-transcriptional level through direct binding of ErsA to the *amrZ* mRNA, we used a plasmid based GFP-reporter system and an electromobility-shift assay for the *in vivo* and *in vitro* validation, respectively. Before this, however, we used the full-length ErsA RNA sequence and the *amrZ* mRNA (including the 5′ untranslated region, 5'-UTR), as inputs in the web tool *IntaRNA* (Wright et al., 2014) to predict ErsA-*amrZ* mRNA interactions. This tool identified two putative interaction sites for ErsA on the *amrZ* mRNA. The interaction site 1 (IS1) involves part of the ErsA U-rich unstructured region, from nt 41 to 52 and is predicted to bind to *amrZ* mRNA in the region

spanning +5 to +14 from the translational starting site AUG (Fig. 4A). The ErsA interaction site 2 (IS2) on *amrZ* mRNA is predicted at positions +65 to +89 and covers a longer region on ErsA unstructured structure, from nt 26 to 56 (Fig. 4A).
To test the ErsA post-transcriptional regulation on *amrZ* mRNA, we generated a translational fusion between the 5'-UTR along with the first 35 codons of *amrZ* mRNA and the superfolder variant gene of the green fluorescent protein (sfGFP) under the control of the heterologous constitutive promoter $P_{LtetO-1}$. This GFP reporter fusion was transformed into *P. aeruginosa* PAO1 wild-type and Δ*ersA* strains, respectively. As shown in Fig. 4B, the ErsA deletion caused a reduction in GFP activity of the *amrZ::sfGFP* translational fusion compared to the wild-type and it was possible to increase the *amrZ::sfGFP* translational levels in Δ*ersA* mutant strain by inducing with arabinose the expression of *ersA* from the pGM-*ersA* plasmid (Fig.S3), suggesting a direct effect of ErsA on *amrZ* translation efficiency. The lack of a full genetic complementation could be explained by the fact that we observed by Northern blot that the ErsA levels expressed by pGM-*ersA* in a Δ*ersA* strain are lower than those expressed by the chromosomal copy of *ersA* gene (data not shown). This scenario is different from that observed for the expression of ErsA from pGM-*ersA* in a *wt* background where the ErsA levels resulted to be 5-6 fold higher than those expressed by the chromosomal copy of *ersA* gene (Ferrara et al., 2015). This would suggest a higher ErsA degradation in a Δ*ersA* background.
Interactions of ErsA with the GFP open reading frame were previously controlled using a plasmid carrying exclusively the *gfp* gene (Ferrara et al., 2015). These results strongly suggested positive regulation by ErsA on translation of the *amrZ* gene. This regulation does not depend on Hfq (data not shown). Furthermore, to document the predicted ErsA–*amrZ* mRNA interaction also *in vitro*, ErsA RNA and *amrZ* mRNA were synthesized, mixed and analyzed by electrophoresis on native polyacrylamide gels. As shown in Fig. 4C, ErsA specifically formed a complex with the *amrZ* mRNA.
To further document the specific ErsA-*amrZ* mRNA interactions, we generated three *amrZ* mRNA fragments, i) *amrZ*CIS1, in which the interaction site 1 has been substituted with its complementary sequence, ii) *amrZ*ΔIS2 characterized by the deletion of the interaction site 2 and iii) *amrZ* CIS1ΔIS2 containing both the modifications present in *amrZ*CIS1 and in *amrZ*ΔIS2. The *in vitro* analysis showed that ErsA forms a complex with both *amrZ*CIS1 and *amrZ*ΔIS2 (Fig. 5A and B), and it does not bind to *amrZ* CIS1ΔIS2 mRNA (Fig. 5C). This suggested that both interaction sequences are involved in ErsA-*amrZ* binding (Fig. S4). *In vitro* results were corroborated by *in vivo* experiments, measuring the translational levels of *amrZCIS1::sfGFP*, *amrZΔIS2::sfGFP* and *amrZCIS1ΔIS2::sfGFP* in PAO1 wild-type and Δ*ersA* strains. The absence of the interaction sites for ErsA causes a reduction of translational fusions activity in both genetic backgrounds (Fig.5D), associated also to a transcriptional instability (data not shown).

Discussion

ErsA is a 132 nt long sRNA expressed in *P. aeruginosa* in concert with other stress-induced genes. We have previously reported that ErsA regulates exopolysaccharide production, negatively affecting at the post-transcriptional level *algC* mRNA translation in an incoherent feed-forward loop driven by the alternative sigma factor σ^{22} (Ferrara et al., 2015). Several sRNAs can regulate broad spectra of mRNA targets, usually governing similar or correlated cellular processes (Storz et al., 2011). In this work, we expanded the target spectrum of ErsA, validating its direct interactions with the transcriptional regulator AmrZ, which is involved in biofilm and motility, in particular promoting multicellular colony formation and repressing swarming and twitching motility.

P. aeruginosa strains exhibiting increased swarming phenotype generally develop flat and uniform biofilm in flow cell experiments (Shrout et al., 2006). Likewise, twitching motility is suggested to be required for monolayer creation during the initial stages of biofilm development (Guttenplan and Kearns, 2013; Shrout et al., 2006). In addition, in gram-negative bacteria, biofilm formation and cellular motility are inversely regulated (O'Toole and Kolter, 1998; Wang et al., 2014). According to these observations, inactivation of *ersA* gene results in increased twitching and swarming motility leading to a less structured biofilm matrix resulting in development of homogeneous monolayers with high surface to volume ratios to the wild-type strain.
These phenotypes were supported by genome-wide expression analysis, showing that inactivation of ErsA affects expression of several genes involved in biofilm development and motility regulation, such as *pelCDEFG*, *algD*, *ppyR* and *roeA*. All these genes are known to be directly or indirectly regulated by the transcriptional regulator AmrZ (Jones et al., 2014; Xu et al., 2016).
Small RNAs can positively or negatively affect translation of transcriptional regulators. For example, three sRNAs, DsrA, MicF and GcvB, inhibit translation of the *lrp* gene, coding for a transcriptional regulator involved in amino acid transport and use (Majdalani et al., 2002; Massè et al., 2005a; Ottesman et al., 1998; Prèvost et al., 2007). The results of this work strongly suggest that ErsA positively affects *amrZ* translation through direct binding to *amrZ* mRNA at two different segments located on the mRNA, IS1 and IS2, with the former positioned close to the translational starting site. ErsA binds to these two regions with the same segment as involved in the *algC* interaction (Ferrara et al., 2015). Likewise ErsA, other sRNAs are known to regulate target expression via multiple interactions. SgrS, a regulator of the *manXYZ* operon binds two different sites, both involved in RNaseE-dependent degradation of the mRNA (Rice et al., 2012); the aforementioned GcvB sRNA, interacts with two independent regions on the *lrp* mRNA (Lee and Gottesman, 2016); and RyhB is suggested to repress expression of *msrB*, a methionine oxidase gene, interacting with two sites on the same mRNA (Bos et al., 2013).
It is possible that concomitant binding of two ErsA RNAs to the *amrZ* mRNA, is required to remodel *amrZ* mRNA secondary structure in order to release the AUG from the interaction with the anti-AUG sequence present in *amrZ* mRNA in its unbound form (Fig. S4). These interactions would expose the translational starting site and improve the efficiency of translation of *amrZ* transcript, thus explaining the positive contribution of ErsA at the post-transcriptional level.
Even though we identified biofilm genes being part of the AmrZ regulon and therefore differentially expressed in the absence of ErsA, the transcriptomics data does not reflect in all cases the known regulation exerted by AmrZ. For example, the *roeA* and *ppyR* genes, suggested to be positively regulated by ErsA, are known to be repressed by AmrZ (Jones et al., 2014; Merritt et al., 2010; Sternberg et al., 2008). We cannot exclude that ErsA may also stabilize directly these transcripts, for instance protecting them from degradation, or that these effects depend on the activity of other regulators affecting *roeA* and *ppyR* expression. Therefore, ErsA seems to overlap with the AmrZ regulon in guiding the switch from a motile life-style into the biofilm mode, extending our previous findings of its involvement in extracellular matrix production (Ferrara et al., 2015). ErsA thus stimulates indirectly exopolysaccharide production through its control of AmrZ translation; acting on AlgC, it may redirect the sugar precursor fluxes providing more building blocks for extracellular polysaccharides biosynthesis (Fig. 1). ErsA, in this sense, may be part of a mixed-regulatory circuit, like that involved in high osmolarity response in *E.coli* (Guillier et al., 2006).
A *P. aeruginosa* mixed-regulatory circuit could be used to take advantage of ErsA to have a more rapid and enhanced response compared to transcriptional regulators, in particular in stress conditions (Shimoni et al., 2007) or for niche-competition in case of mixed-species biofilms. Indeed, ErsA has recently been described to be overexpressed in *P. aeruginosa* biofilm grown with

Staphylococcus aureus. However, the role of ErsA in neutralizing *S*. *aereus* agents has to be investigated (Miller et al., 2017).
Thus, ErsA may be employed as a "fast switcher" in the regulation of biofilm development at multiple stages and regulatory levels, fine-tuning the main routes controlled by the alternative sigma factor σ^{22} in the transition between acute and chronic infection of *P*. *aeruginosa*.

Author Contribution Statement

Conceived and designed the study: GB, SF, SM; Conceived the experiments: MF, SF, GB, SM; Designed and performed the experiments: MF, SF, ER; Analyzed the data: MF, GB, SF, SM, ER, HJ; Contributed reagents/materials/analysis tools: GB, SM, HJ; Wrote the paper: MF, GB, SM.

Conflict of Interest Statement

No conflict of interests to declare.

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Figure 1. Schematic representation of different levels of AlgU-dependent regulatory routes in *Pseudomonas aeruginosa*. AlgU drives the expression of the alginate biosynthetic operon by activating the expression of *algD* promoter and it modulates exopolysaccharides (Pel and Psl) production by inducing the expression of transcriptional regulators, as AmrZ and AlgR, and the small RNA ErsA, which regulates *algC* at the post-transcriptional level. Green arrows represent positive regulations, red arrows negative regulations.

Figure 2. Biofilm formation of PAO1 wild-type, Δ*ersA*, wild-type/pGM931, Δ*ersA*/pGM931 and Δ*ersA*/pGM-*ersA* strains. **A.** PAO1 *ersA* mutant strain produces less biofilm in BHI medium when compared to the wild-type strain. **B.** The phenotype is rescued when the *ersA* mutation is complemented by the pGM-*ersA* plasmid (4 replicates for each strain, 24 hrs at 37°C. Adhesion units are expressed as the ratio of biofilm formation optical density OD_{590} normalized for the bacterial growth OD_{600}). T.Test p.value < 0.001 ***, < 0.01 **, < 0.1 *. **C**. Spatial distribution of 3 days-old flow-chamber-grown biofilms of PAO1 wild-type and Δ*ersA* GFP-tagged strains. The larger central plots are simulated fluorescence projections, in which long shadows indicate large, high micro-colonies. The scale bars shown are also valid for the right and lower frames. Surface to volume ratio, thickness distribution and biomass of PAO1 wild-type and Δ*ersA* values are means of data from 21 image stacks (seven image stacks from three channels). The statistical analysis was performed using GraphPad Prism software (p.value < 0.01 **; < 0.001 ****).

Figure 3. PAO1 and PAO1 Δ*ersA* motility. ErsA deletion results in more swarming motility compared to the PAO1 wild-type on 0.5% Nutrient Broth agar plates supplemented with 0.5% glucose at 37°C and 28°C (**A**), and twitching motility at the plastic-1.0% LB agar interface stained with 0.1% crystal violet (**B**). Statistical analysis was performed on 3 independent replicates with GraphPad Prism software (p.value < 0.05 *; < 0.01 **). The best representative pictures are displayed.

Figure 4. Interaction of ErsA with *amrZ* mRNA. **A.** Prediction by IntaRNA software of the two base-pairing interactions between ErsA and *amrZ* mRNA. ErsA is predicted to bind to *amrZ* mRNA at two different sites in the ORF; the interaction sequence 1 is close to the ATG (highlighted in gray). **B.** Comparison of the fluorescence polarization expressed in arbitrary units (AU) resulting from the translational fusion *amrZ*::*sfGFP* in PAO1 and PAO1 *ΔersA*. The absence of ErsA results in a reduction of the reporter activity compared to the reference strain (statistical analysis performed on three individual clones per strain using T-test, p.value < 0,01 **). **C.** *In vitro* interaction between ErsA RNA and *amrZ* mRNA by electrophoretic mobility shift assay. Increasing amounts of ErsA RNA (0.15, 0.3, 0.6, 1.2 pmol; lanes 3–6) or, as a negative control, yeast tRNA (0.89 and 8.9 pmol; lanes 7 and 8) were incubated with 0.3 pmol of *amrZ* mRNA at 37°C for 20 min and loaded onto a native 6% polyacrylamide gel. Nucleic acids were transferred onto Hybond N+ nylon membranes. After blots, the ErsA-mRNA interactions were tested using oligonucleotide probes for the mRNA target. Free target mRNA is indicated with open arrowheads, sRNA/mRNA complex with filled arrowheads.

Figure 5. *In vitro* (non-radioactive EMSA) and *in vivo* analysis of ErsA interactions with *amrZ* and *amrZ* modified transcripts. **A.** Interactions between ErsA-*amrZ* mRNA and ErsA- *amrZ*CIS1 mRNA generated by substitution of IS1 with its complement sequence. **B.** Interactions between ErsA-*amrZ* mRNA and ErsA- *amrZ*ΔIS2 mRNA carrying the deletion of IS2. **C.** Interactions between ErsA-*amrZ* mRNA and ErsA- *amrZ*CIS1ΔIS2 mRNA characterized by both the modifications present in *amrZ*CIS1 and *amrZ*ΔIS2. ErsA specifically binds *amrZ*, *amrZ*CIS1 and

*amrZ*ΔIS2 mRNAs (black arrows) but no complex is formed when combined to *amrZ*CIS1ΔIS2 mRNA. Binding reactions were performed mixing the *amrZ* mRNAs forms (5 pmol) with increasing amount of ErsA RNA (ratio 1:0.5, 1:1, 1:2). ErsA RNA free form 10 pmol (A lane 9, B lane 9, C lane 5), *amrZ* mRNA free form 5 pmol (A lane 1, B lane 1, C lane 6). **D.** Comparison of the fluorescence intensity expressed in arbitrary units (AU) deriving from *amrZ::sfGFP*, *amrZ*CIS1*::sfGFP*, *amrZ*ΔIS2*::sfGFP* and *amrZ*CIS1ΔIS2*::sfGFP* in PAO1 wild-type and Δ*ersA* strains. Modification in ErsA interaction site IS1 and/or IS2 causes a reduction in the translational levels of *amrZ* mRNA in PAO1 wild-type, comparable to those measured in *ersA* deleted strain. T-test p.value < 0.001 ***.

Table 1 Selection of the most representative genes differentially expressed in PAO1 ErsA deletion mutant with Log_2 (FC) ≤ -1 or Log_2 (FC) ≥ 1.
† Log_2 (FC) = log_2 of fold change calculated as ratio between gene expression of PAO1 wildtype versus PAO1 Δ*ersA*.
‡ Genes involved in biofilm formation and motility regulation.

Locus Tag	Name and description	Log2(FC)†	Fold Change
Down-regulated in PAO1ΔersA strain			
PA0105	*coxB*	-1,70889	-3,269092
PA0106	*coxA*	-1,82114	-3,5336031
PA0792	*prpD*, propionate catabolism	-3,035	-8,1964546
PA0795	*prpC*, citrate synthase 2	-2,68584	-6,4345978
PA1107‡	*roeA*, RoeA	-1,57126	-2,9716413
PA1596	*htpG*, heat shock protein HtpG	-1,69763	-3,2436766
PA2663‡	*ppyR*, Psl and pyoverdine operon regulator, PpyR	-2,18138	-4,5358722
PA3058‡	*pelG*, PelG	-1,4459	-2,7243272
PA3059‡	*pelF*, PelF	-2,06261	-4,1774136
PA3060‡	*pelE*, PelE	-1,89954	-3,7309422
PA3061‡	*pelD*, PelD	-1,54687	-2,9218255
PA3062‡	*pelC*, PelC	-1,75174	-3,3676448
PA3126	*ibpA*, heat-shock protein IbpA	-2,64174	-6,240839
PA3540‡	*algD*, GDP-mannose 6-dehydrogenase AlgD	-1,5095	-2,8471135
PA3877	*narK1*,nitrite extrusion protein 1	-3,06065	-8,3434844
PA3879	*narL*, two-component response regulator NarL	-1,67047	-3,1831828
PA4217	*phzS*, flavin-containing monooxygenase	-1,60294	-3,037617
PA4596	*esrC*, EsrC	-2,53876	-5,8108934
PA4760	*dnaJ*, DnaJ protein	-1,23304	-2,3506178
PA4761	*dnaK*, DnaK protein	-1,72393	-3,3033504
PA5053	*hslV*, heat shock protein HslV	-1,52351	-2,87491619
PA5054	*hslU*, heat shock protein HslU	-2,14174	-4,4129396
Up-regulated in PAO1ΔersA strain			
PA0082	*tssA1*, TssA1	1,424308	2,6838574
PA0093	*tse6*, Tse6	1,500855	2,8301039
PA0509	*nirN*, NirN	2,22837	4,6860424
PA2775	*tsi4*, Tsi4	1,314079	2,4864355
PA3872	*narI*, respiratory nitrate reductase gamma chain	3,080462	8,4588527
PA3873	*narJ*, respiratory nitrate reductase delta chain	2,190598	4,5649467
PA3874	*narH*, respiratory nitrate reductase beta chain	1,653484	3,1459244

Figure 1.

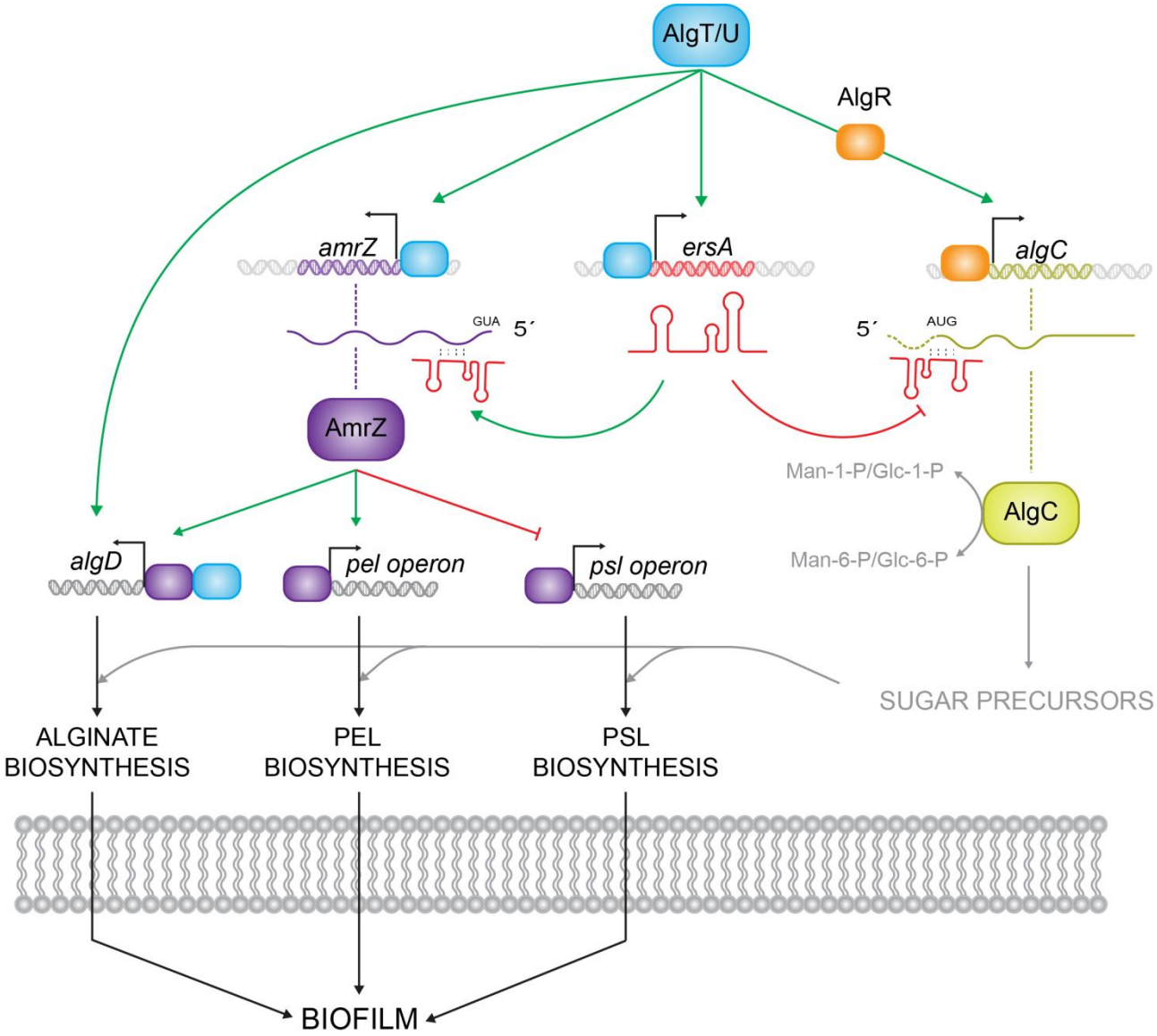

AlgT/U
AlgR
amrZ
ersA
algC
GUA
5´
5´
AUG
AmrZ
Man-1-P/Glc-1-P
AlgC
Man-6-P/Glc-6-P
algD
pel operon
psl operon
SUGAR PRECURSORS
ALGINATE BIOSYNTHESIS
PEL BIOSYNTHESIS
PSL BIOSYNTHESIS
BIOFILM

Figure 2.

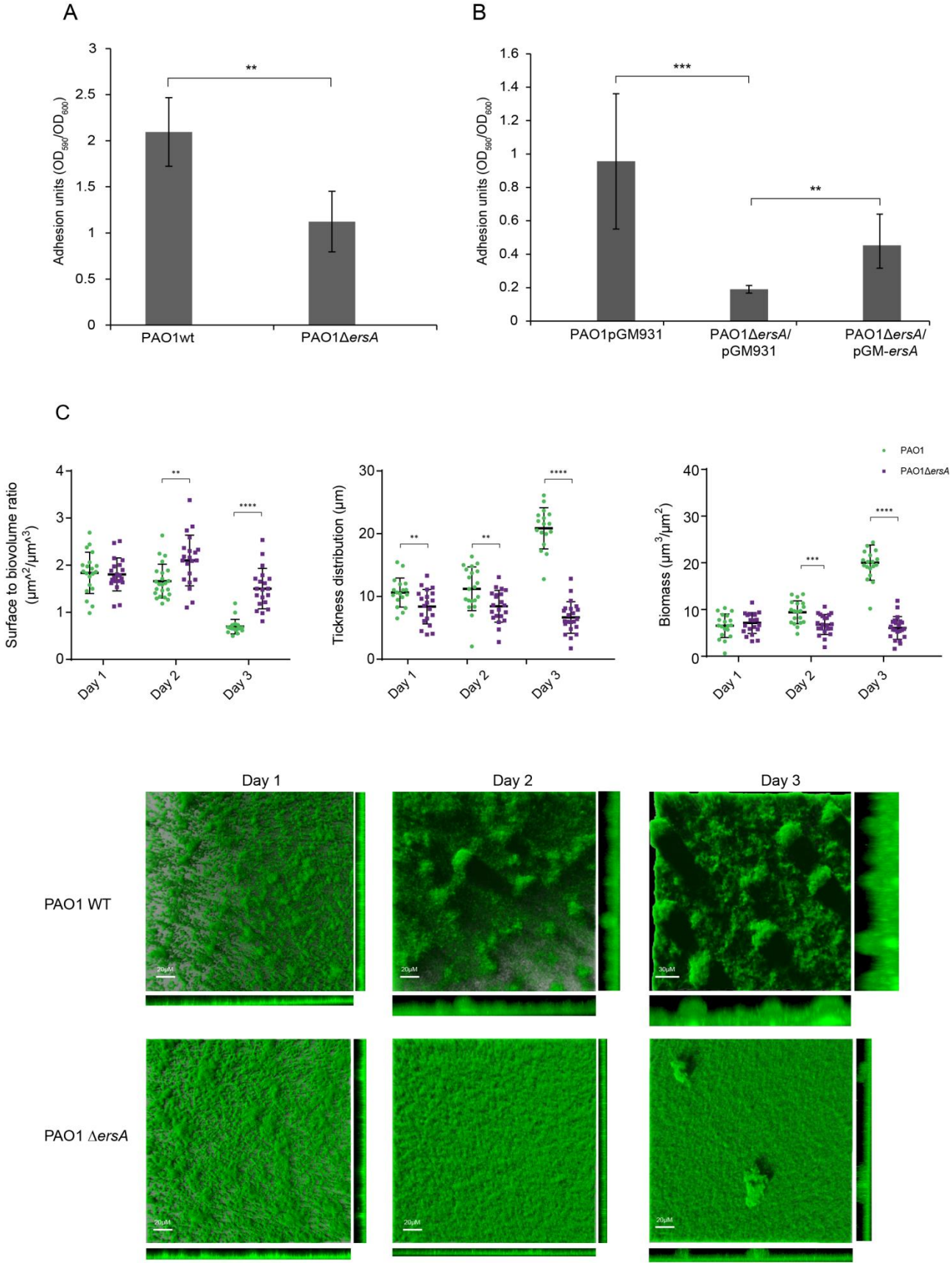

A
Adhesion units (OD_{590}/OD_{600})
PAO1wt
PAO1Δ*ersA*
B
Adhesion units (OD_{590}/OD_{600})
PAO1pGM931
PAO1Δ*ersA*/ pGM931
PAO1Δ*ersA*/ pGM-*ersA*
C
Surface to biovolume ratio ($\mu m^2/\mu m^3$)
Tickness distribution (μm)
Biomass ($\mu m^3/\mu m^2$)
PAO1
PAO1Δ*ersA*
Day 1
Day 2
Day 3
PAO1 WT
PAO1 Δ*ersA*

Figure 3.

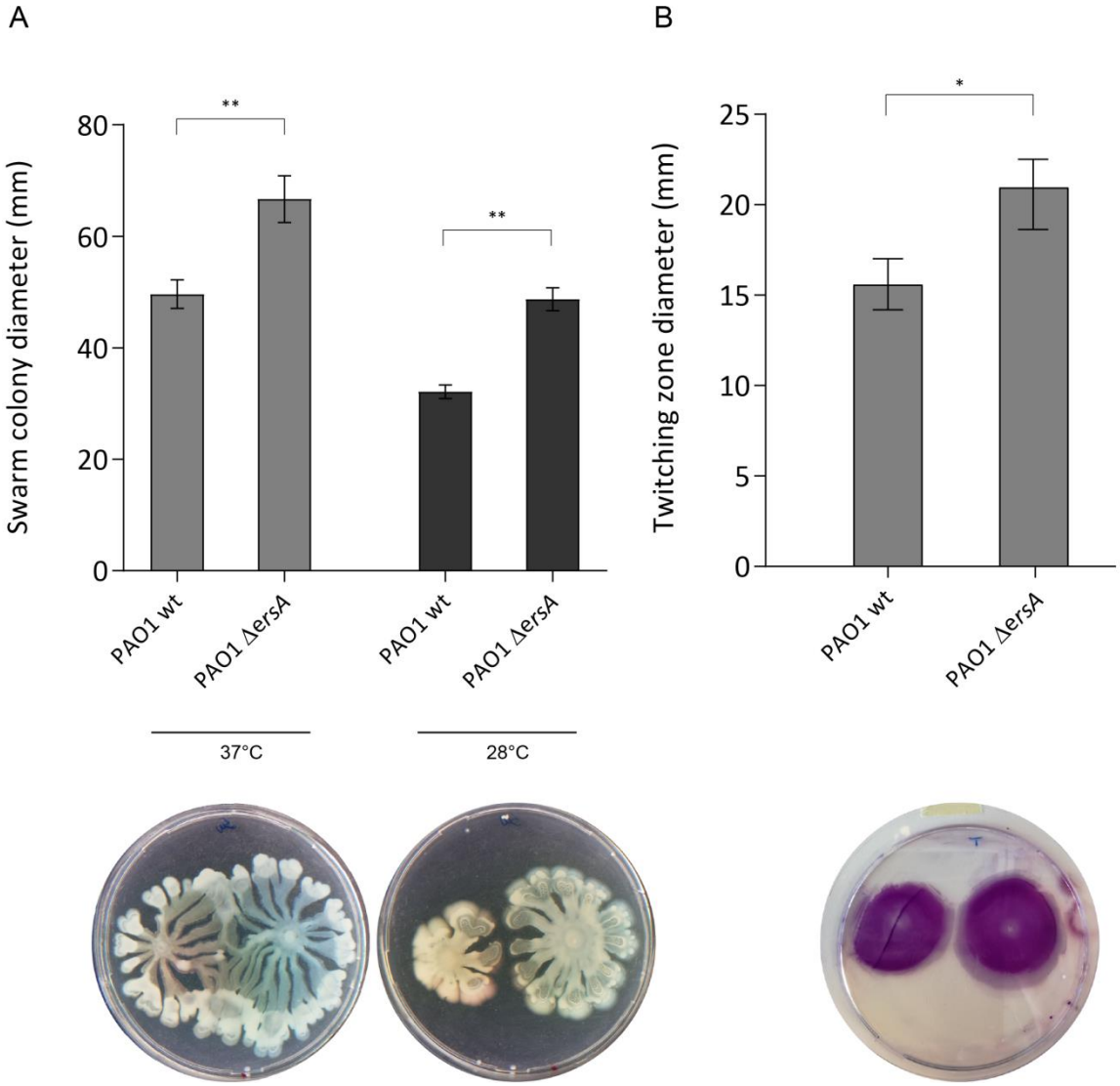

A
B
Swarm colony diameter (mm)
Twitching zone diameter (mm)
**
**
*
80
60
40
20
0
25
20
15
10
5
0
PAO1 wt
PAO1 ΔersA
PAO1 wt
PAO1 ΔersA
PAO1 wt
PAO1 ΔersA
37°C
28°C

Figure 4.

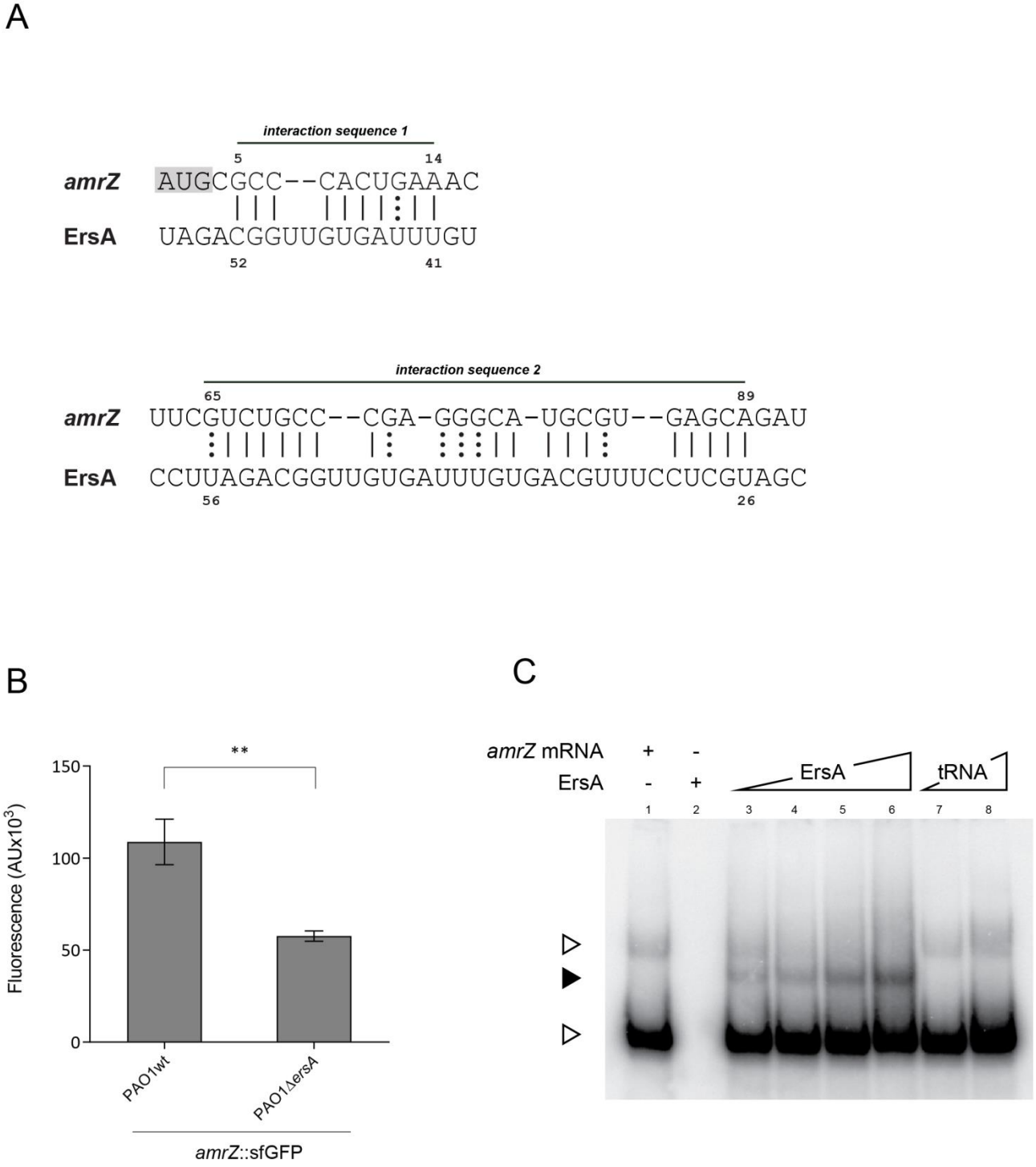
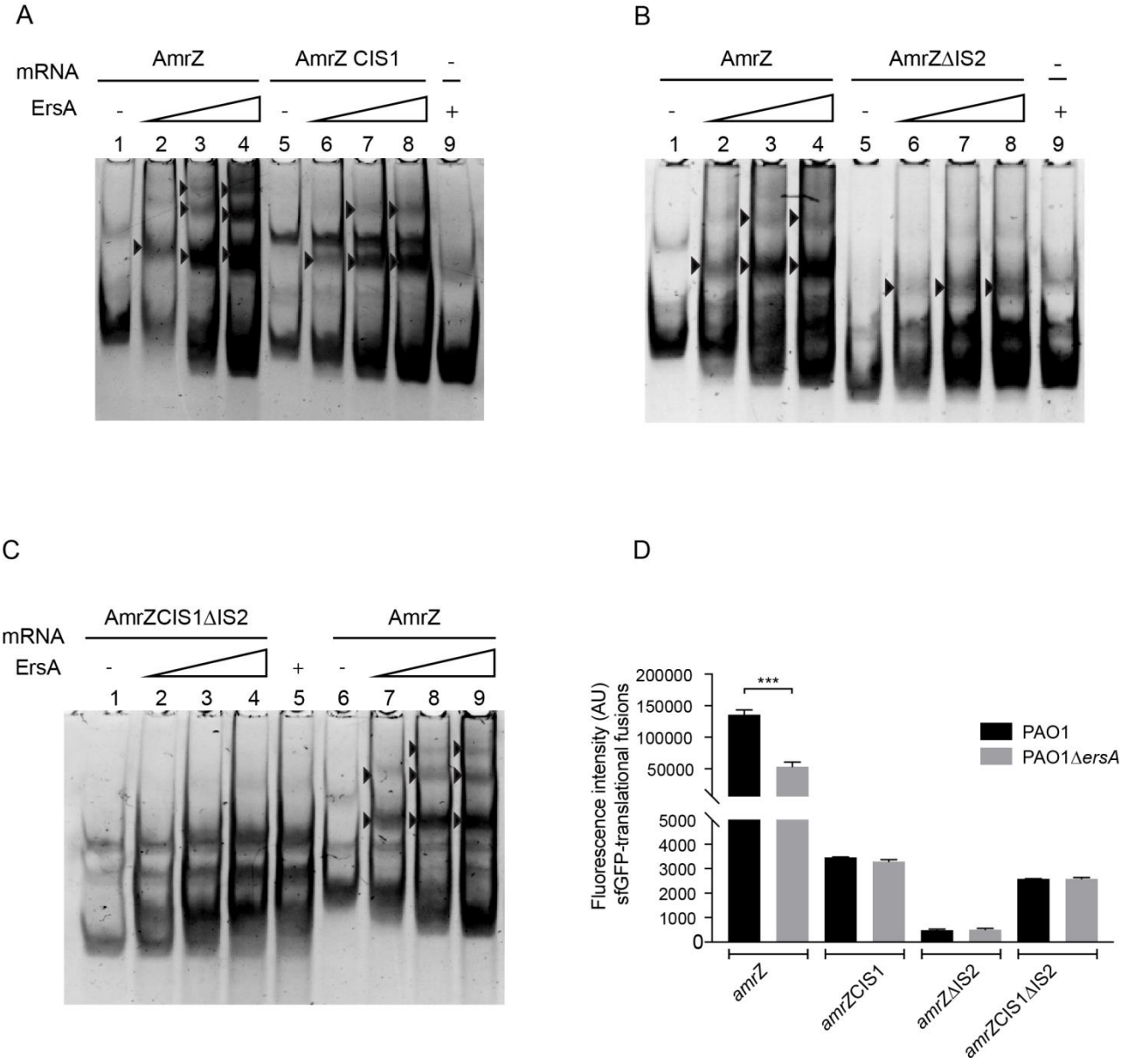

A
interaction sequence 1
5
14
amrZ
AUGCGCC--CACUGAAAC
ErsA
UAGACGGUUGUGAUUUGU
52
41
interaction sequence 2
65
89
amrZ
UUCGUCUGCC--CGA-GGGCA-UGCGU--GAGCAGAU
ErsA
CCUUAGACGGUUGUGAUUUGUGACGUUUCCUCGUAGC
56
26
B
**
Fluorescence (AUx10^3)
150
100
50
0
PAO1wt
PAO1ΔersA
amrZ::sfGFP
C
amrZ mRNA + -
ErsA - +
ErsA
tRNA
1 2 3 4 5 6 7 8

Figure 5.


A
mRNA
AmrZ
AmrZ CIS1
-
ErsA
-
-
+
1 2 3 4 5 6 7 8 9
B
AmrZ
AmrZΔIS2
-
-
-
+
1 2 3 4 5 6 7 8 9
C
mRNA
AmrZCIS1ΔIS2
AmrZ
ErsA
-
+
-
1 2 3 4 5 6 7 8 9
D
Fluorescence intensity (AU)
sfGFP-translational fusions
200000
150000
100000
50000
5000
4000
3000
2000
1000
0
***
PAO1
PAO1Δ*ersA*
amrZ
amrZCIS1
amrZΔIS2
amrZCIS1ΔIS2

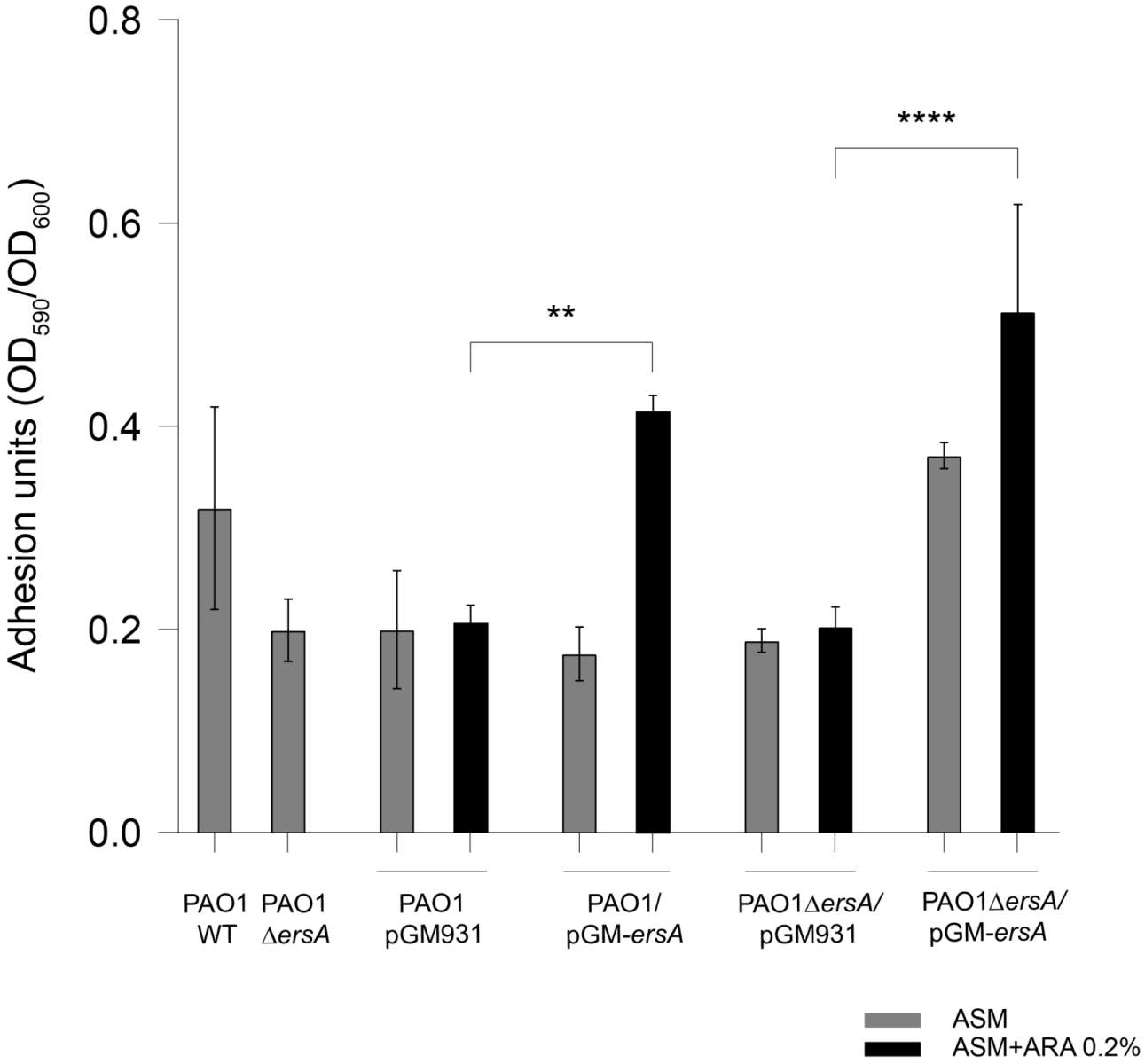


Figure S1. Biofilm assay in peg-lid microtiter. PAO1wild-type (WT) and Δ*ersA* background strains were grown in ASM at 37°C and 100 rpm shaking for 20h in presence of arabinose 0.2% to induce the *ersA* overexpression. The overexpression of ErsA induces an increment of biomass attached to the abiotic surface as quantified by crystal violet staining. The statistical analysis was performed using GraphPad Prism software (p.value < 0,01 **; < 0,001 ****).

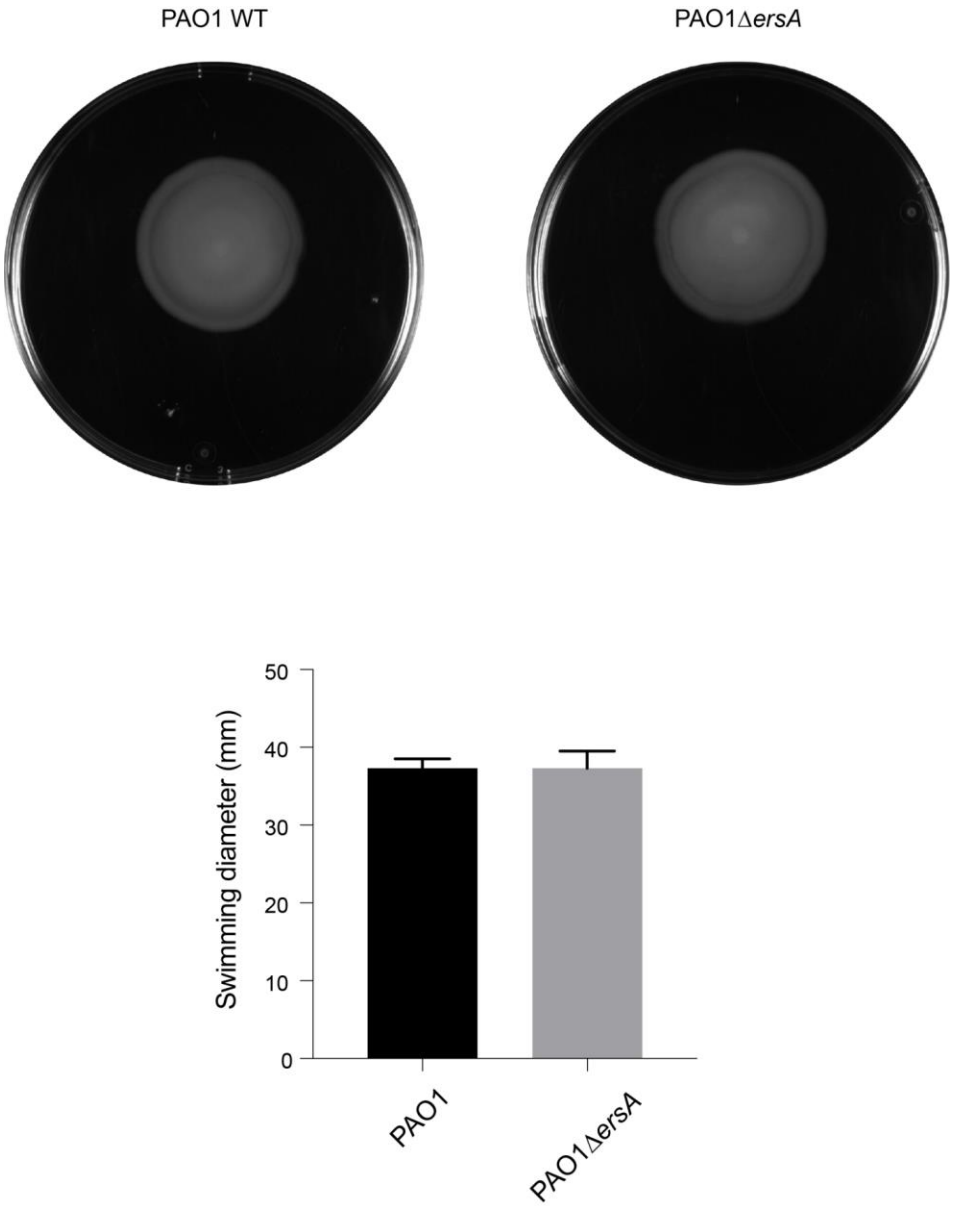


Figure S2. Swimming motility of PAO1 wild-type and Δ*ersA* strains performed in LB medium supplemented with agarose 0.3%, at 37°C for 18 hrs.

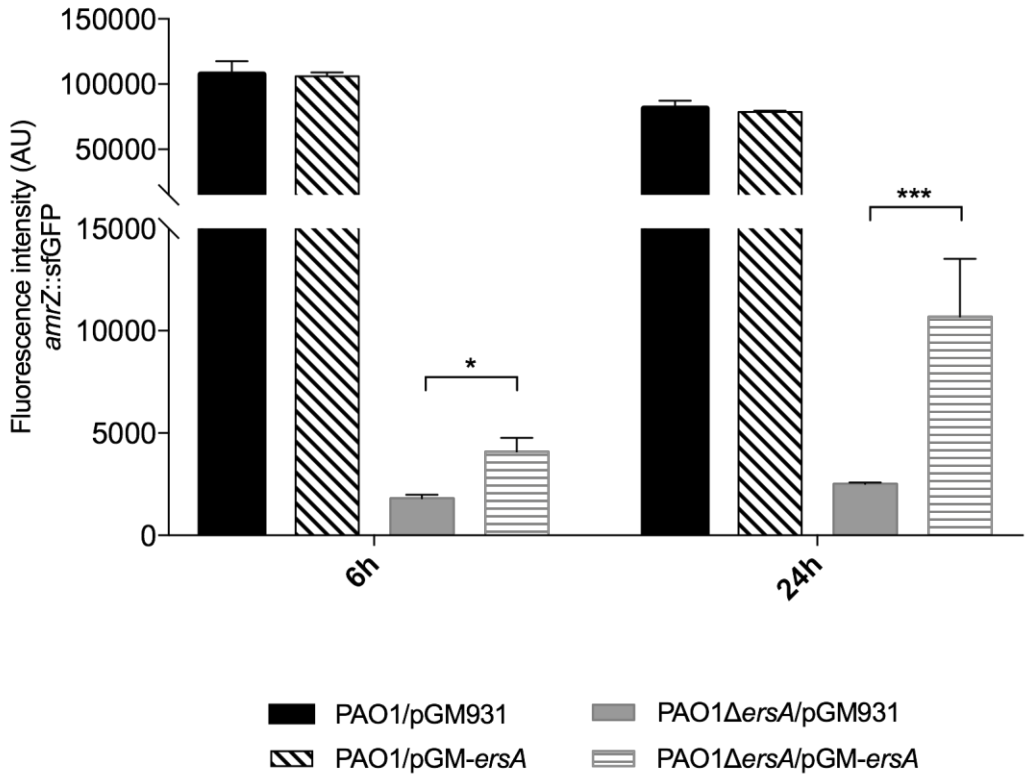


Figure S3. Comparison of fluorescence intensity of *amrZ::sfGFP* translational fusion combined with the control vector pGM931 and pGM-*ersA* in PAO1 wild-type and Δ*ersA* strains, in presence of arabinose 0.2% to induce *ersA* expression. Fluorescence intensity was measured after 6 and 24 hrs from induction with arabinose.

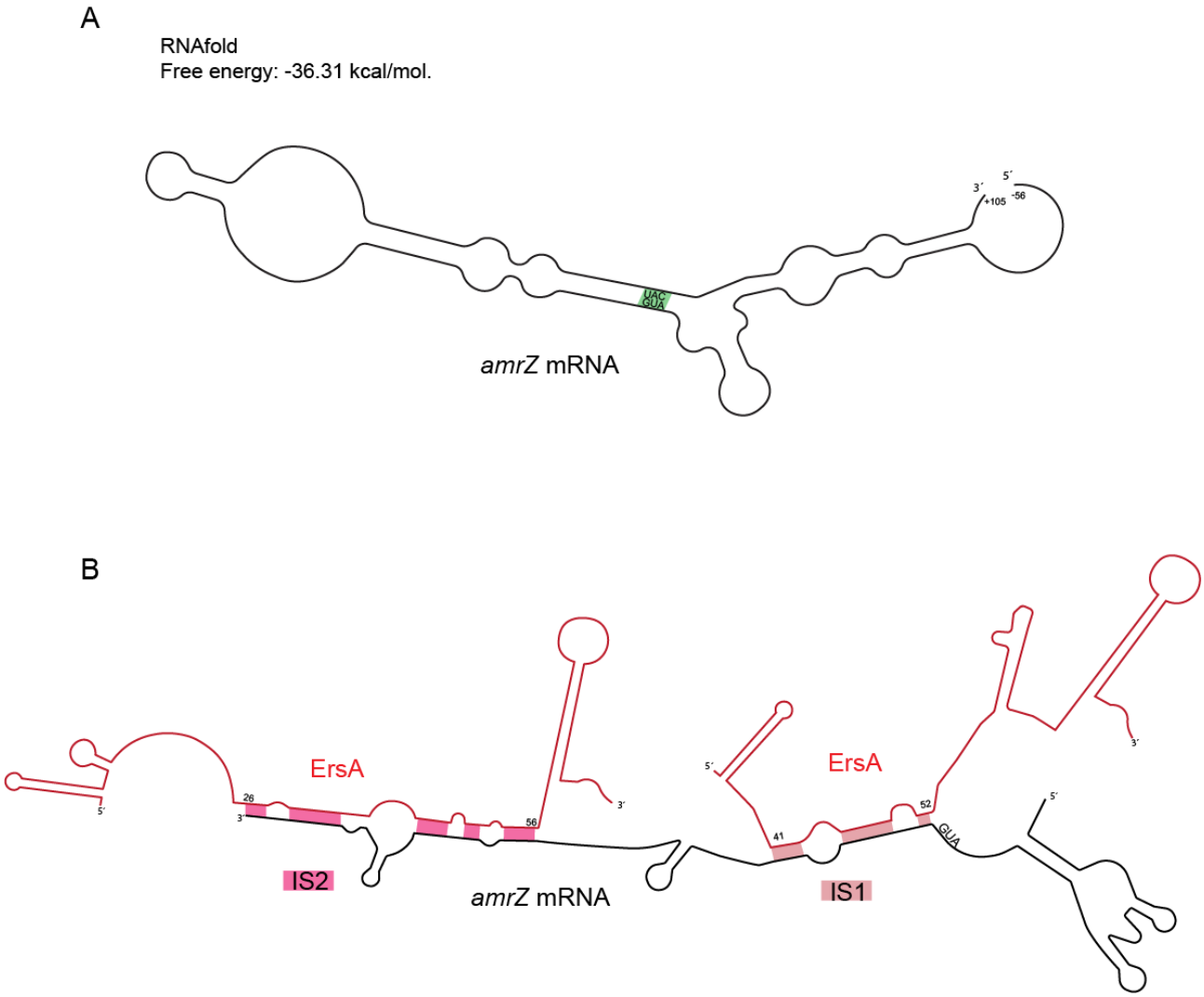


Figure S4. A. *amrZ* (from -56 to +105) secondary structure predicted by RNAfold software. **B.** Proposed model for ErsA-*amrZ* interaction. Two molecules of ErsA are suggested to bind *amrZ* mRNA in IS1 and IS2, remodeling the *amrZ* secondary structure and exposing the AUG.

Table S1. Bacterial strains and plasmids used in this study

Strains	**Description**	**Construction**	**Reference**
Pseudomonas aeruginosa			
PAO1	Wild-type		(Olson et al., 2000)
PAO1Δ*ersA*	Markerless Δ*ersA*		(Ferrara et al., 2015)
PAO1 miniTn7(Gm)P$_{rrnB1}$-*gfp*-a	Gm^r, Cm^r	PAO1 wild-type containing the chromosomal insertion miniTn7(Gm)P$_{rrnB1}$-*gfp* located just downstream of the coding region of *glmS* gene.	This study
PAO1Δ*ersA* miniTn7(Gm)P$_{rrnB1}$-*gfp* a	Gm^r, Cm^r	PAO1 Δ*ersA* containing the chromosomal insertion miniTn7(Gm)P$_{rrnB1}$-*gfp* located just downstream of the coding region of *glmS* gene.	This study
Escherichia coli			
TOP10	*mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZDM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG*		Invitrogen
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*		Invitrogen
Plasmids	**Description**	**Construction**	**Reference**
pXG10-SF	sfGFP reporter plasmid; *lacZ::gfp* under P$_{LtetO-1}$, Cm^r		(Corcoran et al., 2012)
pXG10-*amrZ::sfGFP*	pXG10-SF derivative; P$_{LtetO-1}$ -*amrZ::sfGFP*, Cm^r	contains PCR product using oligos #1 and #2	This study
pXG10-*amrZ*CIS1*::sfGFP*	pXG10-SF derivative; P$_{LtetO-1}$ -*amrZ* CIS1*::sfGFP*, Cm^r	contains PCR product from pUCIDT-*amrZ* CIS1 using oligos	This study

		#1 and #2	
pXG10-*amrZ*ΔIS2::*sfGFP*	pXG10-SF derivative; $P_{LtetO-1}$ -*amrZ*ΔIS2*::sfGFP*, Cm^r	contains PCR product using oligos #1 and #3	This study
pXG10-*amrZ*CIS1ΔIS2::*sfGFP*	pXG10-SF derivative; $P_{LtetO-1}$ - *amrZ*CIS1ΔIS2*::sfGFP*, Cm^r	contains PCR product from pUCIDT-*amrZ* CIS1 using oligos #1 and #3	This study
pBBR1-MCS5	$lacZ_\alpha$, Gm^r		(Kovach et al., 1995)
pBBR1-*amrZ::sfGFP*	pBBR1-MCS5 derivative; $P_{LtetO-1}$-*amrZ::sfGFP*, Gm^r	contains PCR product using oligos #9 and #10	This study
pBBR1-*amrZ*CIS1::*sfGFP*	pBBR1-MCS5 derivative; $P_{LtetO-1}$-*amrZ*CIS1*::sfGFP*, Gm^r	contains PCR product using oligos #9 and #10	This study
pBBR1-*amrZ*ΔIS2::*sfGFP*	pBBR1-MCS5 derivative; $P_{LtetO-1}$-*amrZ*ΔIS2*::sfGFP*, Gm^r	contains PCR product using oligos #9 and #10	This study
pBBR1-*amrZ*CIS1ΔIS2::*sfGFP*	pBBR1-MCS5 derivative; $P_{LtetO-1}$-*amrZ* CIS1ΔIS2*::sfGFP*, Gm^r	contains PCR product using oligos #9 and #10	This study
pUCIDT-*amrZ* CIS1	Plasmid cloning vector containing the synthetic gene *amrZ* CIS1. Amp^r	Generated by IDT (integrated DNA technologies)	This study
pBK-miniTn7(Gm)P_{rrnB1}-*gfp*-a	P_{rrnB1}, Amp^r, Gm^r, Cm^r		(Lambertsen et al., 2004)
pRK600	Helper plasmid, RP4/RK2 conjugation system, ColE1, Cm^r		(Christensen et al., 1998)
pUX-BF13	R6K, RP4 *mob*, Amp^r		(Bao et al., 1991)
pGM931	pHERD20T derivative, araC/*PBAD* - tΩ, Amp^r		(Ferrara et al., 2015)
pGM-*ersA*	pGM931 derivative, *ersA* under *PBAD*, Amp^r		(Ferrara et al., 2015)

Bao, Y., Lies, D. P., Fu, H., and Roberts, G. P. (1991). An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. *Gene* 109, 167–168. doi:10.1016/0378-1119(91)90604-A.

Christensen, B. B., Sternberg, C., Andersen, J. B., Eberl, L., Møller, S., Givskov, M., et al. (1998). Establishment of new genetic traits in a microbial biofilm community. *Appl*. *Environ*. *Microbiol*. 64, 2247–2255. doi:10.1128/IAI.68.6.3140-3146.2000.

Corcoran, C. P., Podkaminski, D., Papenfort, K., Urban, J. H., Hinton, J. C. D., and Vogel, J. (2012). Superfolder GFP reporters validate diverse new mRNA targets of the classic porin regulator, MicF RNA. *Mol*. *Microbiol*. 84, 428–445. doi:10.1111/j.1365-2958.2012.08031.x.

Ferrara, S., Carloni, S., Fulco, R., Falcone, M., Macchi, R., and Bertoni, G. (2015). Post-transcriptional regulation of the virulence-associated enzyme AlgC by the σ(22) -dependent small RNA ErsA of *Pseudomonas aeruginosa*. *Environ*. *Microbiol*. 17, 199–214. doi:10.1111/1462-2920.12590.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., et al. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166, 175–6.

Lambertsen, L., Sternberg, C., and Molin, S. (2004). Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ*. *Microbiol*. 6, 726–732. doi:10.1111/j.1462-2920.2004.00605.x.

Olson, M. V., Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., et al. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964. doi:10.1038/35023079.

Table S2. Oligonucleotides used in this study. Lowercase: sites for restriction enzymes. Bold: T7 promoter sequence.

Oligo #	Oligo name	Sequence (5´→ 3´)	Application
1	AmrZ_NsiI_Fw	GTTTTatgcatGAGAACAATGAACGCTTC	Amplification of part of the 5' UTR and 35 codons of *amrZ* ORF with *Nsi*I/*Nhe*I ends for cloning in frame with *sfGFP* in pXG10-SF vector
2	AmrZ_NheI_Rv	GTTTTgctagcAGCGACTTCTGCGATCTG	
3	AmrZ ΔIS2_NheI_Rv	GTTTTgctagcAACGACGAATTTGTCAGCGGT	Amplification of part of the 5' UTR and 21 codons of *amrZ* ORF to generate *amrZ* ΔIS2 with *Nsi*I/*Nhe*I ends for cloning in frame with *sfGFP* in pXG10-SF vector
4	T7_AmrZ_Fw	**CTAATCGACTCACTATAGGG**GAGAACAATGAACG	Amplification of *amrZ* in fusion with RNA polymerase T7 promoter for *in vitro* transcription
5	AmrZ_Rv_EMSA	AGCGACTTCTGCGATCTG	
6	AmrZ ΔIS2_Rv_EMSA	AACGACGAATTTGTCAGCGGT	Combined to primer number 3 for amplification of *amrZ* ΔIS2 in fusion with RNA polymerase T7 promoter for *in vitro* transcription

7	T7_ErsA_Fw	**CTAATACGACTCACTATAGGG**CGAATGGCTTCTTGAGCC	Amplification of ErsA in fusion with RNA polymerase T7 promoter for *in vitro* transcription
8	ErsA_Rv_EMSA	AAAAAAAACCCCGAGCTTCGTATGGGGAG	
9	Ptet-O1_ClaI_Fw	GTTTTatgcatTCCCTATCAGTGATAGAG	Amplification of the fragment spanning from the $P_{LtetO-1}$ promoter to the stop codon of *gfp* of pXG10-*amrZ::*sf*gfp* with *Cla*I/*Xba*I ends for cloning in pBBR1-MCS5
10	sfGFP_TAA_XbaI_Rv	TGATGCCtctagaTTATTTGTAGAGCTC	
11	sfGFP_+96_r	TTGTGCCCATTAACATCACCATC	Reverse primer on *gfp* for verification of constructs

Table S3. RNA-seq**.** List of all genes identified by RNA-seq analysis, whose expression is significantly different comparing the Δ*ersA* mutant to PAO1wildtype. Negative fold change indicates that the expression was lower in the ErsA mutant strain than the wildtype, while positive fold change indicates that the expression was higher in the Δ*ersA* mutant strain than the PAO1 wildtype.

locus	logFC	logCPM	PValue	FDR	description	classification
PA0052	-1,486381603	4,357914112	0,000895017	0,035588872	hypothetical protein	hypothetical protein
PA0082	1,424307629	8,410532522	8,84076E-05	0,007637065	tssA1 ,TssA1	Protein secretion/export apparatus
PA0093	1,500855258	7,294120187	3,54053E-05	0,00457211	tse6 ,Tse6	Secreted Factors (toxins, enzymes, alginate)
PA0105	-1,70889424	7,453265041	0,001263491	0,043775333	coxB ,cytochrome c oxidase, subunit II	Energy metabolism
PA0106	-1,821143918	5,281470126	0,001052621	0,03960923	coxA ,cytochrome c oxidase, subunit I	Energy metabolism
PA0108	-2,163994052	5,850188873	0,000599543	0,028847335	coIII ,cytochrome c oxidase, subunit III	Energy metabolism
PA0109	-1,232082437	6,119032597	0,001104463	0,040487462	hypothetical protein	hypothetical protein
PA0111	-2,319686143	3,946662672	0,000658207	0,030655204	hypothetical protein	hypothetical protein
PA0112	-2,675957811	2,881962254	0,00040649	0,021430804	hypothetical protein	hypothetical protein
PA0129	-1,643722443	5,381296242	0,000241353	0,014745907	bauD ,Amino acid permease	Carbon compound catabolism; transport of small molecules
PA0130	-1,635845382	7,245832331	0,000964077	0,037264517	bauC ,3-Oxopropanoate dehydrogenase	Carbon compound catabolism; putative enzyme
PA0131	-1,377707391	5,484677047	0,000930447	0,036460696	bauB ,BauB	Carbon compound catabolism
PA0132	-1,884969077	7,413086579	0,000348735	0,019052995	bauA ,Beta-alanine:pyruvate transaminase	Amino acid biosynthesis and metabolism; Carbon compound catabolism
PA0200	-1,269747745	8,820474677	0,001040419	0,039411083	hypothetical protein	hypothetical protein
PA0447	-1,470881988	8,497664545	0,001160437	0,041562948	gcdH ,glutaryl-CoA dehydrogenase	Carbon co Fatty acid and phospholipid metabolism; Amino acid biosynthesis and metabolism
PA0506	-1,958005426	9,24301037	6,18862E-05	0,006169073	probable acyl-CoA dehydrogenase	Putative enzymes
PA0509	2,228370122	7,673960252	2,90491E-06	0,000784267	nirN ,NirN	Energy metabolism
PA0526	-1,968860338	4,306679935	0,00068377	0,031586846	hypothetical protein	hypothetical protein

PA0563	1,508365422	8,883332826	6,14524E-05	0,006169073	conserved hypothetical protein	hypothetical protein
PA0579	1,416799202	7,669928774	0,001194967	0,041912354	rpsU ,30S ribosomal protein S21	Translation, post-translational modification, degradation
PA0730	1,593220206	4,511261043	0,000771933	0,033058707	probable transferase	Putative enzymes
PA0743	-1,906720148	6,254725832	0,000426168	0,022215499	probable 3-hydroxyisobutyrate dehydrogenase	Carbon compound catabolism
PA0779	-2,06862047	10,05140707	2,08525E-07	0,000131649	asrA ,AsrA	Adaptation, Protection; Putative enzymes
PA0788	-1,8013369	5,908231027	0,000207298	0,013234471	hypothetical protein	hypothetical protein
PA0792	-3,034996275	7,842282409	2,80284E-07	0,000137802	prpD ,propionate catabolic protein PrpD	Carbon compound catabolism
PA0793	-2,823705858	8,006381343	8,3997E-06	0,001524363	hypothetical protein	hypothetical protein
PA0794	-2,548853599	9,091375842	3,10632E-05	0,004202402	probable aconitate hydratase	Energy metabolism
PA0795	-2,685849977	9,138011074	8,58494E-06	0,001524363	prpC ,citrate synthase 2	Carbon compound catabolism; Central intermediary metabolism
PA0836	-1,573102769	8,23015672	0,000192502	0,012429488	ackA ,acetate kinase	Putative enzymes
PA0929	1,605939897	5,86187194	0,000751212	0,033058707	two-component response regulator	Transport of small molecules; two component system
PA0989	1,609367518	5,645432615	0,000227701	0,014217557	hypothetical protein	hypothetical protein
PA1034	1,408768468	6,394545063	0,000598692	0,028847335	hypothetical protein	hypothetical protein
PA1107	-1,571257791	3,45203355	0,001189683	0,041912354	roeA ,RoeA	Motility & Attachment
PA1132	1,246261645	6,41327154	0,001171203	0,041592357	hypothetical protein	hypothetical protein
PA1190	-2,475525761	6,584036638	1,47418E-05	0,002326741	conserved hypothetical protein	hypothetical protein
PA1196	-2,536798332	7,355571976	1,85559E-06	0,000585749	probable transcriptional regulator	Transcriptional regulators
PA1218	-2,002678408	4,603968093	0,000842421	0,034685763	hypothetical protein	hypothetical protein
PA1337	-1,281080279	7,694788092	0,001315195	0,04494938	ansB ,glutaminase-asparaginase	Amino acid biosynthesis and metabolism
PA1396	1,359104784	6,957766978	0,000295226	0,016944165	probable two-component sensor	Two-component regulatory systems
PA1410	-1,437880762	5,422576241	0,000261585	0,01581198	probable periplasmic	Transport of small molecules

					spermidine/putrescine-binding protein	
PA1429	-2,276852207	9,984959974	2,35923E-06	0,000670257	probable cation-transporting P-type ATPase	Membrane proteins; Transport of small molecules
PA1517	-1,557673361	4,581205473	0,001329021	0,04494938	conserved hypothetical protein	hypothetical protein
PA1524	-1,583283239	5,54387962	1,1913E-05	0,001933991	xdhA ,xanthine dehydrogenase	Nucleotide biosynthesis and metabolism
PA1542	-1,867406734	2,827746636	0,000318388	0,01773608	hypothetical protein	hypothetical protein
PA1546	-1,586357763	9,248310884	8,87093E-05	0,007637065	hemN ,oxygen-independent coproporphyrinogen III oxidase	Biosynthesis of cofactors, prosthetic groups and carriers
PA1596	-1,697633266	11,33063176	3,94579E-06	0,000896798	htpG ,heat shock protein HtpG	Chaperones & heat shock proteins
PA1597	-1,528478523	7,224544169	4,86115E-05	0,005524207	hypothetical protein	hypothetical protein
PA1604	-2,034411866	7,22651663	6,57465E-07	0,000262305	hypothetical protein	hypothetical protein
PA1639	1,78322982	7,161013567	1,1849E-06	0,000396035	hypothetical protein	hypothetical protein
PA1673	-1,293844473	9,768384051	0,00124955	0,04355793	hypothetical protein	hypothetical protein
PA1674	1,274420975	6,114888654	0,000284204	0,016647931	folE2 ,GTP cyclohydrolase I precursor	Biosynthesis of cofactors, prosthetic groups and carriers
PA1761	-1,793264705	7,139156335	0,000185003	0,012223131	hypothetical protein	hypothetical protein
PA1762	-1,295277265	5,175482022	0,001090676	0,040487462	hypothetical protein	hypothetical protein
PA1789	-1,571557853	9,281083172	4,57314E-05	0,005302974	hypothetical protein	hypothetical protein
PA1826	-1,422217931	4,941576164	0,000231243	0,014281775	probable transcriptional regulator	Transcriptional regulators
PA1869	1,405843393	7,047178114	0,000301792	0,017147805	probable acyl carrier protein	Fatty acid and phospholipid metabolism
PA1920	-2,061167812	4,507906456	1,84012E-05	0,002751464	nrdD ,class III (anaerobic) ribonucleoside-triphosphate reductase subunit, NrdD	Nucleotide biosynthesis and metabolism
PA2024	-2,167043927	3,74018552	2,93376E-05	0,004065765	probable ring-cleaving	Putative enzymes

					dioxygenase	
PA2026	-1,512413894	4,478636893	0,0008218	0,034456689	conserved hypothetical protein	hypothetical protein
PA2119	-1,705093158	8,463537883	7,2353E-05	0,006739501	alcohol dehydrogenase (Zn-dependent)	Putative enzymes
PA2127	-1,579251119	7,570129351	0,000373507	0,020021369	cgrA ,cupA gene regulator A, CgrA	Transcriptional regulators
PA2250	-1,711186752	10,20272628	0,001323012	0,04494938	lpdV ,lipoamide dehydrogenase-Val	energy metabolism
PA2324	-2,007460134	2,261862324	0,000874666	0,035247167	hypothetical protein	hypothetical protein
PA2343	-2,516893618	1,839472778	9,62401E-05	0,008041711	mtlY ,xylulose kinase	Carbon compound catabolism
PA2464	1,216377127	7,454333432	0,000871262	0,035247167	hypothetical protein	hypothetical protein
PA2474	-2,067393307	1,977689662	0,000550344	0,027539148	hypothetical protein	hypothetical protein
PA2479	-1,429845391	4,600638873	0,001428272	0,048020376	probable two-component response regulator	Transcriptional regulators; two component system
PA2618	-1,368197903	5,562230821	0,000332934	0,018366336	hypothetical protein	hypothetical protein
PA2619	1,433370671	7,354660538	0,001103763	0,040487462	infA ,initiation factor	Translation, post-translational modification, degradation
PA2662	-2,035487396	5,002624166	0,000191391	0,012429488	conserved hypothetical protein	hypothetical protein
PA2663	-2,181380673	3,567509999	9,32256E-05	0,007906085	ppyR, psl and pyoverdine operon regulator, PpyR	Membrane proteins; Cell wall / LPS / capsule
PA2665	-1,301177259	5,627821823	0,000616217	0,029177854	fhpR,Transcriptional activator of P. aeruginosa flavohemoglobin, FhpR	Transcriptional regulators; Adaptation, Protection
PA2753	-1,843609985	7,817436746	4,15874E-05	0,005129215	hypothetical protein	hypothetical protein
PA2754	-1,905910651	7,968972939	1,96844E-05	0,002867867	conserved hypothetical protein	hypothetical protein
PA2762	-1,627145074	5,564951181	0,000314882	0,01771443	hypothetical protein	hypothetical protein
PA2773	1,236179165	5,776039239	0,000729442	0,032894349	hypothetical protein	hypothetical protein
PA2775	1,314078872	6,400031615	0,000556999	0,027539148	tsi4 ,Tsi4	Membrane proteins; adaptation, protection
PA2814	-1,668768495	6,396720658	6,90168E-05	0,006646672	hypothetical protein	hypothetical protein
PA2889	-1,926403174	6,419970826	0,000163599	0,011336196	atuD ,citronellyl-CoA	Putative enzymes

					dehydrogenase, AtuD	
PA2891	-1,626360101	6,608258073	0,000128271	0,009849148	atuF ,geranyl-CoA carboxylase, alpha-subunit (biotin-containing)	Putative enzymes
PA2892	-1,550284953	5,243081867	0,000550877	0,027539148	atuG ,GCase, alpha-subunit (biotin-containing)	Putative enzymes
PA2937	-1,939994686	4,516782811	0,000152509	0,010969065	hypothetical protein	hypothetical protein
PA2983	1,172053103	6,04142966	0,000955386	0,037181544	probable tolQ-type transport protein	Transport of small molecules
PA3006	-1,359413079	9,353388135	0,000552265	0,027539148	psrA ,transcriptional regulator PsrA	Transcriptional regulators
PA3058	-1,445901886	5,236528455	0,000604159	0,028847335	pelG ,PelG	Cell wall / LPS / capsule
PA3059	-2,06260642	5,662797153	4,5007E-08	4,87587E-05	pelF ,PelF	Cell wall / LPS / capsule
PA3060	-1,899537785	4,592844727	5,56601E-05	0,005919674	pelE ,PelE	Cell wall / LPS / capsule
PA3061	-1,5468726	6,109840959	0,000268508	0,016059594	pelD ,PelD	Cell wall / LPS / capsule
PA3062	-1,751739614	4,282513647	0,000281873	0,016647931	pelC ,PelC	Cell wall / LPS / capsule
PA3126	-2,641737435	10,14035387	2,27419E-10	6,46097E-07	ibpA ,heat-shock protein IbpA	Chaperones & heat shock proteins
PA3272	-1,333438674	8,853855569	0,000717383	0,032640734	probable ATP-dependent DNA helicase	DNA replication, recombination, modification and repair
PA3330	-1,238040797	7,667042755	0,001089632	0,040487462	probable short chain dehydrogenase	Putative enzymes
PA3331	-1,256920345	8,361497488	0,000718073	0,032640734	cytochrome P450	Adaptation, Protection; carbon compound metabolism
PA3337	-2,025641091	10,04127883	3,59378E-06	0,000850827	rfaD ,ADP-L-glycero-D-mannoheptose 6-epimerase	Cell wall / LPS / capsule
PA3418	-1,791209415	7,042476679	0,000115773	0,009265095	ldh ,leucine dehydrogenase	Amino acid biosynthesis and metabolism
PA3458	-1,977009973	7,903780872	3,19222E-05	0,004218188	probable transcriptional regulator	transcriptional regulators
PA3465	-1,578735427	8,136997465	0,00086984	0,035247167	conserved hypothetical	hypothetical protein

					protein	
PA3504	-1,33979329	4,740780822	0,000746254	0,033058707	probable aldehyde dehydrogenase	Putative enzymes
PA3505	-1,552158405	3,088781818	0,000784377	0,03325991	hypothetical protein	Hypothetical, unclassified, unknown
PA3506	-1,76030667	4,372125097	5,88205E-05	0,006076689	probable decarboxylase	Putative enzymes
PA3540	-1,509501357	3,502495829	0,00116306	0,041562948	algD ,GDP-mannose 6-dehydrogenase AlgD	Cell wall / LPS / capsule; Secreted Factors (toxins, enzymes, alginate); Adaptation, Protection
PA3572	-1,935065078	7,423468374	8,48073E-07	0,000301172	hypothetical protein	hypothetical protein
PA3613	-1,775915796	10,13693129	0,000144578	0,010668729	hypothetical protein	hypothetical protein
PA3614	-1,567657759	9,311377957	7,68531E-05	0,007007459	hypothetical protein	hypothetical protein
PA3661	1,995960333	3,427480855	2,42968E-05	0,003451354	hypothetical protein	hypothetical protein
PA3839	-1,895189451	7,930640809	5,62588E-05	0,005919674	probable sodium:sulfate symporter	Transport of small molecules; Membrane proteins
PA3870	2,370292019	4,734620681	7,25933E-06	0,001374916	moaA1 ,molybdopterin biosynthetic protein A1	Biosynthesis of cofactors, prosthetic groups and carriers
PA3871	2,409157425	5,57885567	1,06185E-05	0,001774546	probable peptidyl-prolyl cis-trans isomerase, PpiC-type	Translation, post-translational modification, degradation; Chaperones & heat shock proteins
PA3872	3,080461986	4,375501772	2,91029E-07	0,000137802	narI ,respiratory nitrate reductase gamma chain	Energy metabolism
PA3873	2,190597545	3,075665833	0,000773066	0,033058707	narJ ,respiratory nitrate reductase delta chain	Energy metabolism
PA3874	1,653484185	5,31260064	0,000182836	0,012222077	narH ,respiratory nitrate reductase beta chain	Energy metabolism
PA3877	-3,060653511	6,508278182	5,14875E-08	4,87587E-05	narK1 ,nitrite extrusion protein 1	Transport of small molecules; Membrane proteins
PA3879	-1,670466911	6,765918086	0,000102382	0,008413013	narL ,two-component response regulator NarL	Two-component regulatory systems; Energy metabolism
PA3915	-1,710625615	5,296846594	0,000442618	0,022863237	moaB1 ,molybdopterin biosynthetic protein B1	Biosynthesis of cofactors, prosthetic groups and carriers
PA3952	-1,583421507	8,150864353	4,33302E-05	0,005129215	hypothetical protein	hypothetical protein
PA3973	-1,641791297	6,217274353	0,000103645	0,008413013	probable transcriptional regulator	transcriptional regulators

PA4042	1,218951781	5,430195849	0,000773813	0,033058707	xseB ,exodeoxyribonuclease VII small subunit	DNA replication, recombination, modification and repair
PA4217	-1,602942607	8,822863447	0,000830169	0,034456689	phzS ,flavin-containing monooxygenase	Putative enzymes
PA4318	1,415699596	5,245761436	0,000140983	0,010668729	hypothetical protein	hypothetical protein
PA4319	1,424817203	6,285627442	7,76962E-05	0,007007459	conserved hypothetical protein	hypothetical protein
PA4328	-1,442092131	6,782442644	0,000118183	0,009326582	hypothetical protein	hypothetical protein
PA4352	-1,420121748	10,609043	0,000625601	0,029377375	conserved hypothetical protein	hypothetical protein
PA4385	-1,372534781	11,62521786	0,000155577	0,011049843	groEL ,GroEL protein	Chaperones & heat shock proteins
PA4387	-1,397518722	7,355606697	0,000219982	0,013888173	conserved hypothetical protein	hypothetical protein
PA4503	-1,509523883	7,528460931	0,001006439	0,038379769	probable permease of ABC transporter	Membrane proteins; transport of small molecules
PA4504	-2,178887794	5,576845941	2,08879E-06	0,000624659	probable permease of ABC transporter	Membrane proteins; transport of small molecules
PA4505	-2,715212868	7,446295376	6,28516E-07	0,000262305	probable ATP-binding component of ABC transporter	Transport of small molecules
PA4506	-2,41955681	7,34667466	3,6698E-08	4,87587E-05	probable ATP-binding component of ABC dipeptide transporter	Transport of small molecules
PA4517	1,910381753	4,181540614	1,78138E-05	0,002735629	conserved hypothetical protein	hypothetical protein
PA4542	-2,167136015	12,299015	3,06922E-08	4,87587E-05	clpB ,ClpB protein	Translation, post-translational modification, degradation
PA4577	-1,592929829	7,68331787	0,000146601	0,010679318	hypothetical protein	hypothetical protein
PA4596	-2,538759396	6,795390063	6,92464E-07	0,000262305	esrC ,EsrC	Transcriptional regulators
PA4610	-1,860033595	6,014083064	3,51545E-06	0,000850827	hypothetical protein	hypothetical protein
PA4624	-1,328690003	7,949064339	0,000895672	0,035588872	cdrB ,cyclic diguanylate-regulated TPS partner B, CdrB	Cell wall / LPS / capsule

PA4702	-1,529968116	5,240196126	0,000909648	0,035893191	hypothetical protein	hypothetical protein
PA4759	-1,21872377	9,181139967	0,000762104	0,033058707	dapB ,dihydrodipicolinate reductase	Amino acid biosynthesis and metabolism
PA4760	-1,233038234	9,179295041	0,000566301	0,027738964	dnaJ ,DnaJ protein	Chaperones & heat shock proteins; DNA replication, recombination, modification and repair; Adaptation, Protection
PA4761	-1,723932624	11,24781421	4,2419E-06	0,000927018	dnaK ,DnaK protein	Chaperones & heat shock proteins; DNA replication, recombination, modification and repair; Adaptation, Protection
PA4762	-1,296616559	8,583085127	0,000830793	0,034456689	grpE ,heat shock protein GrpE	Chaperones & heat shock proteins; DNA replication, recombination, modification and repair
PA4910	-1,606528486	4,897611095	0,001124244	0,040948443	branched chain amino acid ABC transporter ATP binding protein	Transport of small molecules
PA4921	-1,250310599	6,629011551	0,00131446	0,04494938	choE ,cholinesterase, ChoE	Hypothetical, unclassified, unknown
PA5026	-1,572912027	4,608735021	0,000287333	0,016659436	hypothetical protein	hypothetical protein
PA5027	-2,000510116	7,940537305	5,47629E-06	0,001111296	hypothetical protein	hypothetical protein
PA5053	-1,523519901	7,398840175	0,000119827	0,0093268	hslV ,heat shock protein HslV	Chaperones & heat shock proteins
PA5054	-2,141737621	9,255398557	7,26367E-08	5,89603E-05	hslU ,heat shock protein HslU	Chaperones & heat shock proteins
PA5055	-1,414280433	4,999426464	0,00017431	0,011790859	hypothetical protein	hypothetical protein
PA5082	-2,337011035	7,398403488	5,29188E-05	0,005782398	dguC ,DguC	Transport of small molecules
PA5083	-1,989818096	4,366979275	0,001155089	0,041562948	dguB ,DguB	Hypothetical, unclassified, unknown
PA5169	-1,564616373	7,124069203	0,000157869	0,011074202	dctM ,DctM	Transport of small molecules; Membrane proteins
PA5170	-2,093798779	11,21908069	4,55087E-06	0,000957705	arcD ,arginine/ornithine antiporter	Transport of small molecules; Membrane proteins; Aminoacid biosynthesis and metabolism
PA5207	-1,642733837	6,147653	0,000738484	0,033039897	probable phosphate transporter	Transport of small molecules; Membrane proteins
PA5232	-1,294873803	9,564994926	0,000998342	0,038328236	conserved hypothetical	hypothetical protein

					protein	
PA5379	-1,338922687	5,236283763	0,000557375	0,027539148	sdaB ,L-serine dehydratase	Amino acid biosynthesis and metabolism
PA5381	-1,579000349	5,090219755	0,000407344	0,021430804	hypothetical protein	hypothetical protein
PA5396	-1,611177205	7,66033075	5,1258E-05	0,005710748	hypothetical protein	hypothetical protein
PA5397	-2,191218608	4,483709091	3,68647E-05	0,004654781	hypothetical protein	hypothetical protein
PA5398	-1,946188108	6,811016424	9,93465E-06	0,001710566	dgcA ,DgcA, Dimethylglycine catabolism	Amino acid biosynthesis and metabolism
PA5399	-1,839906241	6,177880726	6,35469E-05	0,006225407	dgcB ,DgcB, Dimethylglycine catabolism	Amino acid biosynthesis and metabolism
PA5410	-1,60320599	7,481687455	8,41342E-05	0,007469537	gbcA ,GbcA	Amino acid biosynthesis and metabolism
PA5411	-1,567845279	6,319258669	0,000173641	0,011790859	gbcB ,GbcB	Amino acid biosynthesis and metabolism
PA5415	-1,780184969	6,034628028	4,3152E-05	0,005129215	glyA1 ,serine hydroxymethyltransferase	Amino acid biosynthesis and metabolism
PA5416	-2,175820976	6,913652076	2,33123E-07	0,000132461	soxB ,sarcosine oxidase beta subunit	Amino acid biosynthesis and metabolism
PA5417	-2,423597986	2,752971173	6,07912E-06	0,001191088	soxD ,sarcosine oxidase delta subunit	Amino acid biosynthesis and metabolism
PA5418	-2,058628457	7,181932599	3,03659E-06	0,000784267	soxA ,sarcosine oxidase alpha subunit	Carbon compound metabolism
PA5420	-1,794993097	6,502862793	0,000371249	0,020021369	purU2 ,formyltetrahydrofolate deformylase	Nucleotide biosynthesis and metabolism
PA5446	-2,177166881	5,345420413	1,67286E-07	0,000118815	hypothetical protein	hypothetical protein
PA5460	-1,841110506	3,509458329	0,000143566	0,010668729	hypothetical protein	hypothetical protein
PA5475	-1,657216406	9,742869348	7,07723E-05	0,00670214	hypothetical protein	hypothetical protein
PA5492.1	-3,957996038	4,93094617	3,64939E-18	2,07358E-14	ersA ,ErsA	