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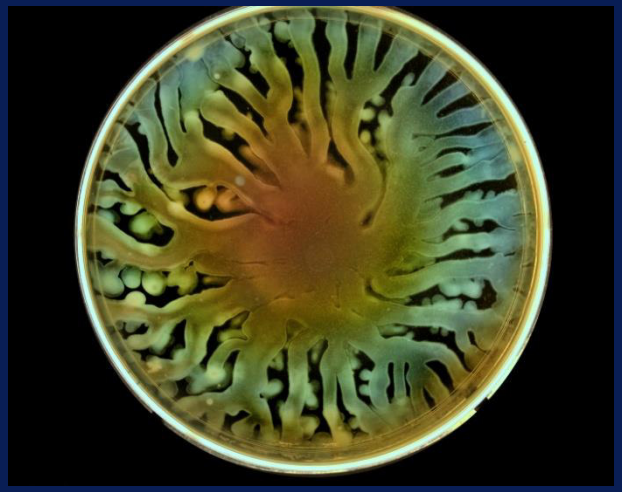
Small RNAs and regulation of virulence traits in *Pseudomonas aeruginosa*.



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Tesi di dottorato

Docente guida: Prof. Giovanni Bertoni

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Small RNAs and regulation of virulence traits in *Pseudomonas aeruginosa*

Sara Carloni

PhD Thesis

Scientific tutor: Prof. Giovanni Bertoni

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Contents

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Manuscript: Sara Carloni, Raffaella Macchi, Sara Sattin, Anna Bernardi, Silvia Ferrara, Giovanni Bertoni. The small RNA SPA0084: a novel regulatory element embedded in the *Pseudomonas aeruginosa quorum sensing* networks.

A. Abstract

This PhD project aimed at the functional characterization of novel *Pseudomonas aeruginosa* small RNAs (sRNAs) involved in virulence-associated regulatory networks. Following an initial phase of screenings for sRNA candidates involved in the regulation of virulence traits, the work focused on the sRNAs SPA0122 and SPA0084.

SPA0122, renamed ErsA, is transcribed from the same genomic context of the well-known *Escherichia coli* Spot 42. We show that, different from Spot 42, ErsA is under the transcriptional control of the envelope stress response, which is known to impact the pathogenesis of *P. aeruginosa* through the activity of the alternative sigma factor σ^{22}. The transcriptional responsiveness of ErsA RNA also spans infection-relevant cues that *P. aeruginosa* can experience in mammalian hosts, such as limited iron availability, temperature shifts from environmental to body temperature and reduced oxygen conditions. Another difference between Spot 42 and ErsA is that ErsA does not seem to be involved in the regulation of carbon source catabolism. Instead, our results suggest that ErsA is linked to anabolic functions for the synthesis of exoproducts from sugar precursors. We show that ErsA directly operates in the negative post-transcriptional regulation of the *algC* gene that encodes the virulence-associated enzyme AlgC, which provides sugar precursors for the synthesis of several *P. aeruginosa* polysaccharides. Like ErsA, the activation of *algC* expression is also dependent on σ^{22}. Altogether, our results suggest that ErsA and σ^{22} combine in an incoherent feed-forward loop to fine-tune AlgC enzyme expression.

As regards SPA0084, our results indicate that is embedded in the *P. aeruginosa quorum sensing* (QS) with the role of wiring *las* to *pqs* systems. In fact, we show that SPA0084 responds to the *las* regulator LasR and impacts positively the synthesis of the *pqs* quinolone signal PQS. Our results suggest that the stimulation of PQS synthesis is mediated by a positive post-transcriptional effect of SPA0084 on the *pqsC* gene belonging to the *pqsABCD* cluster involved in the biosynthesis of the PQS-precursor HHQ. We suggest that a fine balancing between the different already known regulatory effects of LasR on PQS synthesis and this one mediated by SPA0084 can influence timing and magnitude of expression of QS-regulated virulence factors. This view is consistent with the evidence that perturbations of SPA0084 levels affect pyocyanin synthesis, biofilm formation and swarming motility, processes that are known to be influenced by PQS synthesis. Besides being regulated by LasR, SPA0084 responds to infection relevant cues that *P. aeruginosa* can experience in mammalian hosts such as

temperature and oxygen availability. Furthermore, SPA0084 shows a growth phase-dependent pattern of expression, being up-regulated in stationary phase. Sequence analysis of the *spa0084* promoter region strongly suggests that the growth phase-dependent pattern of SPA0084 expression is due to the activity of the alternative σ factor RpoS whose significance as global factor controlling *quorum sensing* gene expression was shown previously. Together, these SPA0084 regulations are expected to contribute to the fine co-modulation of PQS synthesis.

PART I

B. State of the Art

B1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile, non-fermenting Gram-negative bacterium that has the ability to thrive in most natural environments such as soil, water, plants and animals (Figure B.1) and it can infect a broad range of different hosts [1]. *P. aeruginosa* is well known metabolically versatile bacterium and has been isolated from numerous nutrient-poor settings to the extent that is able to survive even in distilled water. Despite its optimum temperature for growth is 37°C, it is able to tolerate temperatures as high as 50°C and is capable of growing under both aerobic as well as anaerobic conditions, using preferentially nitrate as a terminal electron acceptor [2]. In humans, *Pseudomonas aeruginosa* causes a wide variety of acute (short duration, typically severe) and chronic (persisting for a long time, often refractory to treatment) infections [3] [4]. Typically it is able to infect patients with severe burn wounds, urinary tract infections, AIDS, lung cancer, chronic obstructive pulmonary disease, bronchiectasis and cystic fibrosis (CF) [5] [6]. Metabolic versatility, intrinsic and acquired antibiotic resistance, biofilm formation and production of multiple virulence (disease-causing) factors make *P. aeruginosa* a formidable pathogen difficult to eradicate.

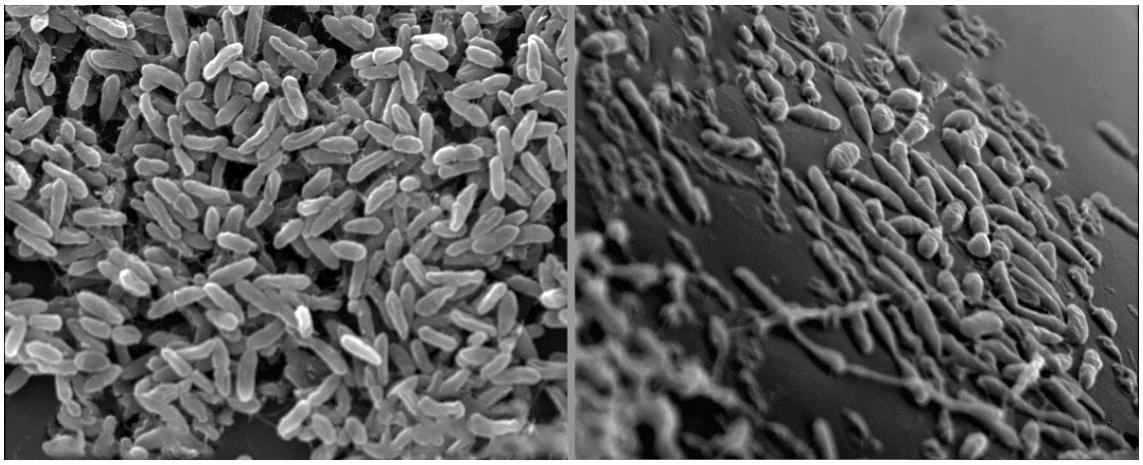

Figure B.1. Scanning electron microscopy images of *P. aeruginosa* isolates attaching to glass surfaces.

Until January 2014, 48 strains of *P. aeruginosa* from both clinical and environmental sources had been fully or partly sequenced (Pseudomonas Genome Database, PGD). Compared with most other pathogenic bacteria, *P. aeruginosa* has a relatively large genome, ranging from 6.22 to 6.91 Mb [7] arranged as a combination of conserved regions spaced by regions of genomic plasticity that contain genes unique to each strain. Despite the whole sequence of *P. aeruginosa* PAO1 genome was completed more than a decade ago [8], it was possible to assign a functional class only for 57% of its open reading frames (ORFs). Out of these, 12% have function experimentally demonstrated in *P. aeruginosa*, 8% is highly similar to a gene experimentally demonstrated in another organism and 37% are homology-based bioinformatic predictions. Thus 43% of predicted ORFs are currently genes with function not assigned or predicted.

B2. *Pseudomonas aeruginosa* infections

P. aeruginosa is an exceptionally diverse, opportunistic pathogen capable of many different types of interactions with the infected host. Depending on the environmental conditions and the immune status, *P. aeruginosa* can be a quiescent colonizer, enmeshed in a carbohydrate biofilm only occasionally shedding a few gene products capable of immune stimulation, or it can also be a highly virulent invader, attaching to damaged epithelial cells, injecting toxins that interfere with eukaryotic cytoskeletal integrity, and rapidly triggering apoptosis and breaches in epithelial integrity. As stated in the 2009–2010 report by the National Healthcare Safety Network (NHSN), *P. aeruginosa* is the 5th most common healthcare associated pathogen [9] and is the second most common pathogen causing respiratory infections of hospitalized patients, (70–80% mortality when confined to the lungs) [10]. *Pseudomonas aeruginosa* rarely infects the human lung without an underlying defect in immunity or mechanical barrier (e.g. mucus in CF patients), and its infections are frequently classified into ‘acute’ and ‘chronic’ infections although the distinctions between these groups are not always clear. Acute hospital infections are generally the effect of direct trauma, such as damage to the epithelium due to intubation, smoke inhalation or severe burns. On the other hand, chronic infections affect primarily patients with weak or non-effective immune response, or individuals with CF for whose chronic airway inflammation with recurrent *P. aeruginosa* infections represent the major cause of morbidity and mortality [11] (Figure B.2).

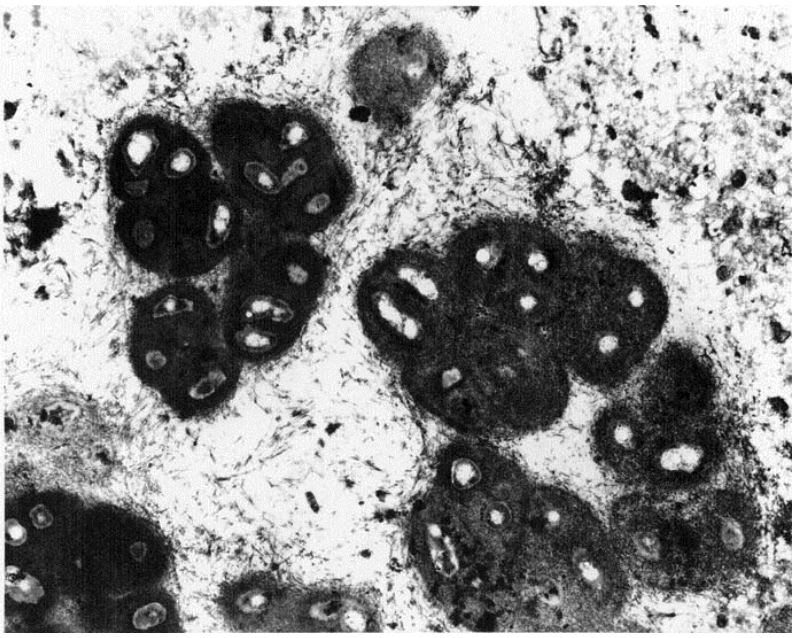

Figure B.2. Electron photomicrograph of *Pseudomonas aeruginosa* micro-colonies in CF sputum. (Pradeep Singh, University of Iowa.)

The pathogenesis of *P. aeruginosa* lung infection is complex, and its outcome depends on the virulence factors displayed by the bacteria (surface factors flagellum, pilus, LPS and secreted factors, extracellular products, type III secretion proteins, quorum sensing molecules, and alginate) as well as the host response.

Acute *P. aeruginosa* infections in specific sites (e.g. CF lung) eventually lead to chronic infections. This is due to adaptive modifications in the infecting clonal type, resulting in diverse morphotypes [12]. On establishing a chronic infection, *P. aeruginosa* overproduces extracellular polysaccharides, forms biofilms and small colony variants and upregulates the Type 6 secretion system [13], [14] [15]. Antibiotic resistance plays a major role in both types of infection, although the cells display higher levels of resistance in chronic infections [16]. The transition to a chronic infection phase is the result of numerous changes in cellular physiology in response to external stimuli [17]. The changes include down-regulation of acute virulence genes with a concomitant up-regulation of chronic infection phenotypes and antibiotic resistance, facilitating recalcitrant infections [17]. Host invasion, establishment of acute infection and the subsequent transition to the chronic phase involves tightly regulated expression of many genes associated with metabolism, virulence and antibiotic resistance. Several key players in these transition processes were identified and include transcriptional and post-transcriptional regulators [18] [19] [20]. In the particular case of CF patients, 'exacerbations' of chronic infections may clinically resemble a new acute infection. In the

absence of preceding culture data these chronic infections may be mistakenly classified as acute infections. For that reason is preferably to categorize the respiratory infections of *P. aeruginosa* into 'transient' and 'persistent' to emphasize the importance of microbiological status over one of symptoms. In figure B.3 are reported the two infections model that *P. aeruginosa* can establish upon initial infection phase [21]. The first model describes the classic 'acute' infections seen in the ICU where patients may become rapidly ill from pseudomonal pneumonia and sepsis. The second model of infection describes the acquisition of strains into an already diseased lung that may or may not result in much symptomology [21].

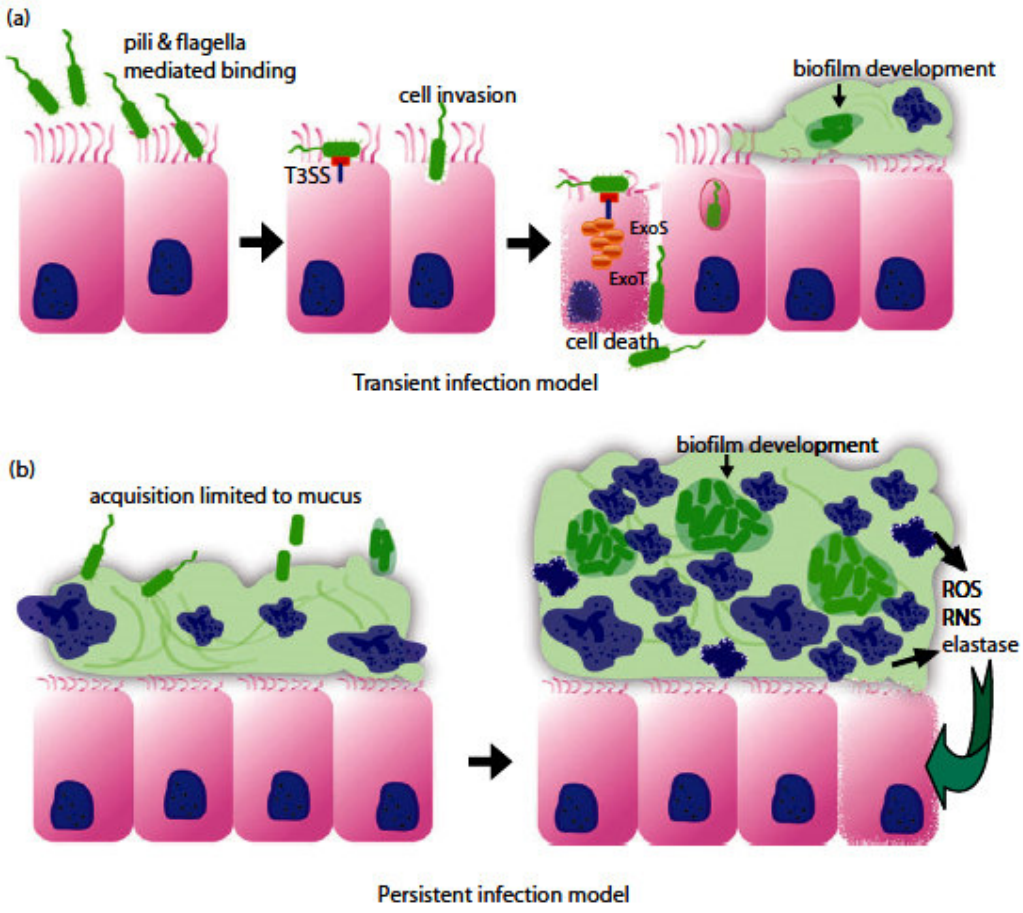

Figure B.3. Models of *Pseudomonas aeruginosa* infection establishment. In (a) *P. aeruginosa* is equipped with a full arsenal of virulence traits including pili, flagella, type 3 secretion systems (T3SS) and secreted virulence factors. Epithelial cell binding occurs via flagella and pili to various structures including asialoGM1. Toxin elaboration injures the surrounding host tissue. The significance of cytosolic invasion during human infections is not clear, but breach of the epithelial surface occur after cell death from toxin injury. Epithelial injury also results in loss of mechanical clearance mechanisms and establishment of the pseudomonal biofilm leading to a persistent infection. In (b) *P. aeruginosa* infects an already inflamed surface with a defective muco ciliary elevator. The infecting organism mayor may not be piliated, and may already exist in a 'biofilm' state if acquired from another patient. The infection occurs strictly in the mucous layer where 'nests' of pseudomonads bind to cell debris and extracellular DNA rather than the epithelial surface. The pseudomonal micro-colonies are a strong inflammatory stimulus, but are resistant to the actions of neutrophils, which may injure surrounding tissue in their efforts to remove the stimulus. Neutrophil death may provide more substrate for pseudomonal growth. (Image source Williams, J. Dehnbostel, Sblackwell *Pseudomonas aeruginosa*: hoste defence in lung desease, 2010, Respirology).

B3. *Pseudomonas aeruginosa*-host interaction

Pseudomonas aeruginosa may be the most versatile bacterial pathogen studied to date. It is a natural pathogen to plants and protozoa, and an opportunistic pathogen of mammals. To achieve a broad host range it must survive extremes of environmental exposure such as temperature and pH fluctuations, nutrient limitations and antimicrobial assaults from soil microorganisms or patient prescriptions. *P. aeruginosa* must harbour an extraordinary complement of genes to encode for virulence traits, broad ranging nutrient acquisition pathways and an arsenal of defence mechanisms. *Pseudomonas aeruginosa* expresses a wide array of virulence traits that are powerful manipulators and destroyers of host cells; however, it is clear that *P. aeruginosa* does not express all of these at all times. Probably the most important aspect of *P. aeruginosa* versatility is its ability to quickly adapt to a new environment, and then accumulate traits/mutations that allow it to persist [22].

Virulence factors include pili and flagella, which play a critical role in motility and adhesion to the epithelium as well as elastase, alkaline protease, the endotoxin lipopolysaccharide (LPS) and four effector proteins already identified: exotoxin A, exoenzyme S (ExoS), exoenzyme U (ExoU), exoenzyme T (ExoT), exoenzyme Y (ExoY). All these factors together are crucial for maximum virulence of *P. aeruginosa*; however, based on several studies in diverse animal models, the relative contribution of any given factor may vary with the type of infection [23].

B3.1 Biofilm

A key feature to *P. aeruginosa* environmental sustainability is its ability to form biofilm. While this mode of growth may have evolved to attach pseudomonads to environmental substrates, such as rocks in a stream, it is clear that it affords *P. aeruginosa* a distinct survival advantage in the diseased or damaged lung.

Biofilms are complex organized structures of bacteria attached to one another and to a surface [24], and their formation is intricately linked to quorum sensing (QS) [25]. These highly organized structures are characterized by extracellular polymeric substances (EPS), composed by polysaccharides, nucleic acids, lipids, and proteins [26]. By forming such a multicellular aggregate, cells are deeply protected from penetration of toxic chemicals (e.g., antibiotics, host defence molecules) and can also escape, or at least slow down significantly the process of phagocytosis by mammalian host [27]. Furthermore, it is known that bacteria within the biofilm differ substantially respect their planktonic form, mainly regarding their transcriptional profile: indeed more than 25% of the PAO1 genome was differentially expressed in biofilms condition [28]. The transition of *P. aeruginosa* from the motile to aggregate state in biofilms is a complex multi-step process (Figure B.4) that involves a multitude of physiological changes [26]. The first phase, an initial reversible adhesion to the surface, is mostly controlled by physiochemical properties such as Van der Waals interactions, electrical charge and hydrophobicity [29]. In the presence of opportune conditions, adhesion, mediated by type IV pili, flagella, and Cup fimbria [30], become irreversible and cells start to duplicate forming a monolayer called micro-colony. Finally, micro-colonies multiply and, producing the EPS matrix, a mature biofilm could develop. Three different polysaccharides (alginate, Pel and Psl) are synthesized by *P. aeruginosa* EPS, with various relevance and relative percentages according to different strains, environmental conditions and age of the biofilm [31].

Alginate is an anionic acetylated polysaccharide composed of monomers of β-1,4-linked L-guluronic and D-mannuronic acids [32]. Its suggested role is to protect the bacterial community from the host response [33]. The glucose-rich Pel polysaccharide is encoded by the pel cluster, and together with the mannose-rich Psl polysaccharide, mainly mediates cell-to-surface and cell-to-cell interactions, which are essential for *P. aeruginosa* biofilm formation and maintenance [34] [35].

As the micro-colony develops, the EPS forms and encloses the biofilm in a mushroom-shaped structure that is crossed with specifically designed channels allowing the exchange of waste products and nutrients to the deep rooted cells [36] [37].

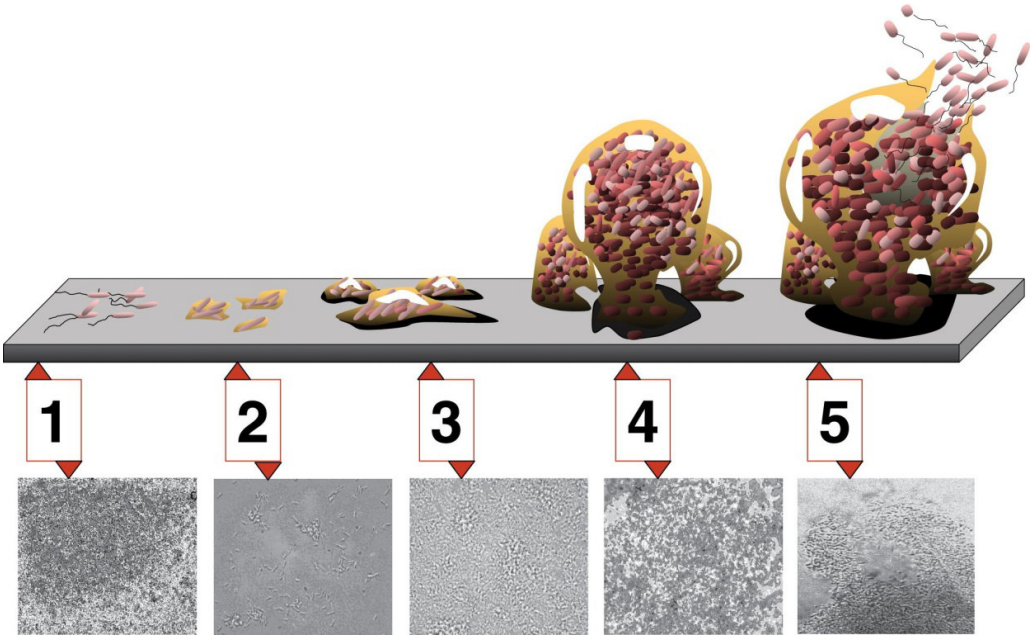

Figure B.4. Schematic representation of biofilm maturation model. Stage 1, initial attachment; stage 2, irreversible attachment; stage 3, maturation I; stage 4, maturation II; stage 5, dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. (Image source from Monroe, 2007).

In the last step, biofilm undergo to a structural fragmentation with the release of cells through the environment, where they are able to adhere to another surface, renewing this cycle. Although this process can be influenced by mechanical events, biofilm detachment and desegregation is a cell-driven process carried out in response to different environmental signals, such as nutrient reduction [38] or accumulation of oxygen and nitrogen reactive species [39] [40].

B3.2 Lipopolysaccharides

Lipopolysaccharide is a complex glycolipid that constitutes the main component of the outer membrane of Gram negative bacteria and plays a crucial role in antigenicity, inflammatory response, exclusion of external molecules, and in mediating interactions with antibiotics [41]. The *P. aeruginosa* LPS consist of three different regions: the membrane-anchored lipid A, the polysaccharide core region, and a highly variable O-specific polysaccharide (O-antigen). While the structure of the inner core is rather conserved (two D- manno-2-keto-octulosonic acid residues and two L-glycero-D-manno-heptose residues), significant variability can be detected in both the Lipid A moiety and in the O-antigen.

The lipid A domain is diglucosamine biphosphate backbone with O- and N-linked primary and secondary fatty acids that anchors the LPS in the bacterial outer membrane. The main structural differences are observed in the number, position, nature of the linked acyl groups, and the modification or type of substituent of the phosphate groups [42].

Two different types of O-antigen have been characterized: A-band LPS is a homopolymer of D-rhamnose which elicits a weak antibody response; B-band LPS is a heteropolymer with three to five distinct sugars in its repeat units with a strong antibody response and is the chemical basis for serotyping [43]. *P. aeruginosa* strains lacking the O-antigen result in a "rough" colony morphology compared to the smooth ones producing it, while others, identified as ‘semi-rough’, substitute the lipid A and core with only one O-saccharide unit [44].

B3.3 Motility-associated factors

Pseudomonas aeruginosa synthesizes a single polar flagellum to swim in liquid media and to swarm over solid surfaces. During swarming, either the number of flagella at the pole increases and/or the single polar flagellum is augmented by an alternative swarming-specific stator complex [45], [46], [47].

Flagella biosynthesis is organized in a Class I-IV hierarchy where Class I is represented by the master regulator protein FleQ, classes II and III encode the hook-basal body genes, and Class IV encodes flagellin and the chemotaxis proteins [48].

Pseudomonas aeruginosa also uses type IV pilus (T4P)-based twitching motility to move over solid surfaces. This form of bacterial movement was hypothesized to result from the repeated extension, tethering and retraction of long protein fibers (T4P) [49]. It was shown that the shortening of T4P after attachment of pilus-specific bacteriophages resulted in trafficking of phage particles to the bacterial cell surface. Like other kinds of pili and fimbriae, T4P provide the ability to adhere to chemically diverse surfaces and promote bacterial cell aggregation involved in micro-colony formation and virulence. However, T4P are unique in mediating flagellum-independent motility [50]. There are two major subfamilies of T4P, type IVa and type IVb (Figure B.5).

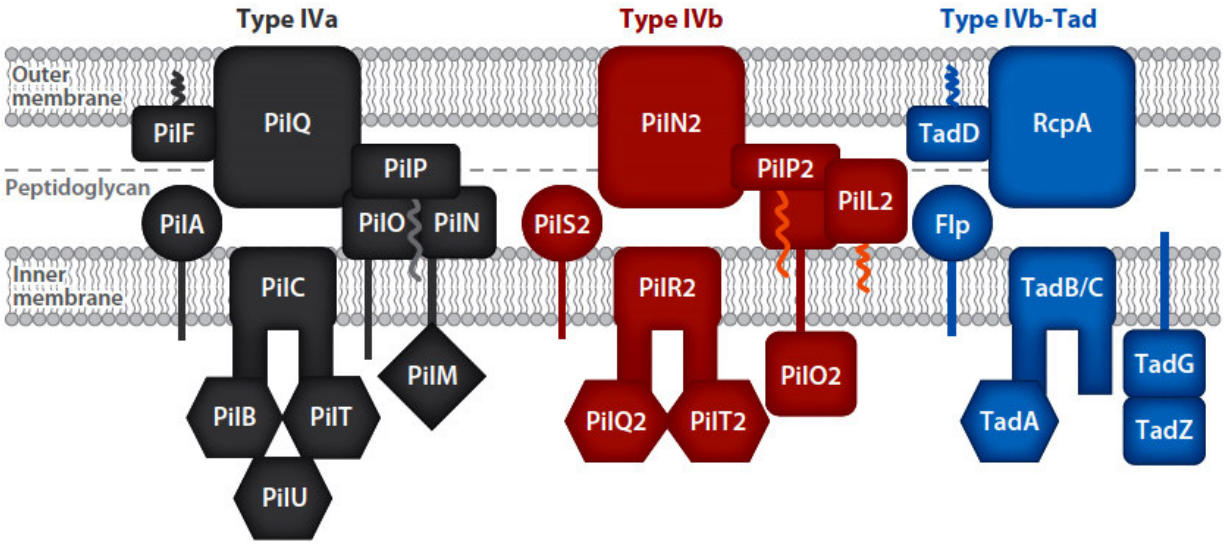

Figure B.5. Type IV pili assembly systems in *Pseudomonas aeruginosa*. Putative organization in the cell envelope of potentially functionally equivalent components of the three assembly systems in *P. aeruginosa*. PilA, PilS2, and Flp are the major pilin subunits; PilQ, PilN2, and RcpA are the secretins; PilF and TadD are putative pilotins; PilC, PilR2, TadB and TadC are putative platform proteins; PilB, PilQ2 and TadA are putative pilin polymerases; and PilT, PilU, and PilT2 are putative pilin depolymerases. (Image from Lory L. Burrows, Annu. Rev. Microbiol. 2012).

Although twitching motility is the best-characterized type of movement associated with T4aP, a number of other T4aP-dependent modalities have been described for *P. aeruginosa*, including swarming, walking, and slingshot motilities. Swarming motility is a complex phenotypic adaptation [51] that affects a number of traits on medium that is less viscous than standard twitching motility medium (0.4–0.7% agar versus 1% for twitching). Swarming motility is characterized by the formation of elaborate dendritic patterns by the swarming colony [52] (Figure B.6). Under canonical nutrient conditions, swarming motility requires T4aP [45], [53], but provision of specific carbon sources including glutamate, glucose or succinate restores swarming in pilin mutants [54]. Swarming is controlled in part by the pilus-related chemotaxis system, indeed point mutations in the chemosensory protein ChpA decrease or modulate swarming [55].

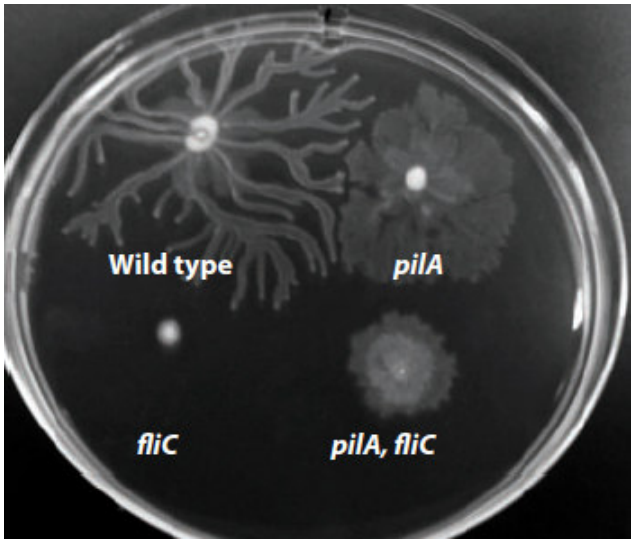

Figure B.6. Role of Type IVa-pili (T4aP) in swarming and sliding motility. On a medium of intermediate viscosity, wild-type *Pseudomonas aeruginosa* is capable of swarming motility, forming elaborate dendritic colonies (top left). Piliated mutants lacking flagella (fliC) are non-motile under these conditions (bottom left), whereas flagellated mutants lacking type IV pili (T4P) (pilA) show aberrant swarming motility (top right). Mutants lacking both T4P and flagella (pilA, fliC) can move by sliding motility (bottom right), suggesting that expression of T4P hampers sliding in the fliC background. (Image from Lory L. Burrows, Annu. Rev. Microbiol. 2012).

If the environment is unsuitable, however, a means of escape is required. In collaboration with flagella, T4aP are crucial to the dispersal of bacteria from surfaces in a coordinate series of events called “a launch sequence” [56].

Pseudomonas aeruginosa requires cell motility to form biofilm, but unregulated motility during its formation destabilizes the biofilm structure. Cells that are enhanced for swarming motility form a flat and featureless biofilm, while cells that are deficient for swarming motility form a more structured biofilm with large tower-like aggregates [54]. Thus, a decrease in motility during biofilm formation may promote and stabilize complex three-dimensional architectural development. Consistent with motility antagonizing biofilms, *P. aeruginosa* variants (colony morphology) that inherently form more robust biofilms, have a decrease in motility compared with their parental strains [57]. Thus, the proper timing of motility control is required for forming wild-type biofilm architecture, but motility *per se* can be bypassed entirely when cells are genetically locked in a biofilm-proficient state.

B3.4 Pyocyanin

Pyocyanins are redox-active phenazines which heterocyclic nitrogen-containing compounds are secreted by some bacteria including *Pseudomonas* species [58]. Phenazines are synthesized from chorismic acid. The two last steps of pyocyanin synthesis from its precursor phenazine-1-carboxylic acid are mediated by S-adenosyl methionine-dependent N-methyltransferase, PhzM, and by flavin-dependent hydroxylase, PhzS (Figure 7a) [58]. Pyocianin is a blue pigment produced and secreted into the medium by stationary phase cultures (Figure B.7b) and it is responsible for the blue-greenish colour.

Pyocyanin can be easily purified from the culture medium by repeated chloroform-distilled water extraction steps resulting in a pure solution of the blue pigment (Figure B.7b). The colour and the characteristic absorption spectrum of pyocyanin is pH-sensitive, as its strong blue colour detected at neutral pH turns red at acidic pH (Figure B.7c).

Originally pyocyanin was considered a metabolic waste product of *Pseudomonas* species, secreted without any other biological function. Later on, it was discovered that pyocyanin is a redox-active compound capable of accepting and donating electrons. Since pyocyanin can cross biological membranes easily, it serves as a mobile electron carrier for *P. aeruginosa*. Under aerobic conditions pyocyanin's main electron acceptor is molecular oxygen. Thus, pyocyanin helps *P. aeruginosa* to survive under oxygen-poor conditions by accepting and transporting electrons produced in respiration away from the bacterium to acceptors found at remote places. In a biofilm setting, pyocyanins creates a biofilm electrocline, a redox potential gradient that reaches hundreds of micrometers beyond the biofilm surface. The pyocyanin appears to enhance the bioavailability of iron, which is essential for biofilm formation [59]. Pyocyanin is required for full Pseudomonas virulence in animal models and most likely in human airway infections as well [60]. Pyocyanin has a wide range of effects on different host cells but the root for its diverse toxicities relies on the production of reactive oxygen species (ROS). ROS are short-lived reactive derivatives of oxygen, which are important in several cell functions, but when produced in excess they can perturb normal cell metabolism. Pyocyanin can cross host cell membranes easily and oxidize their intracellular reduced nucleotides, NADH and mainly NADPH, to produce superoxide anions and downstream ROS exposing the host cell to oxidative stress [60].

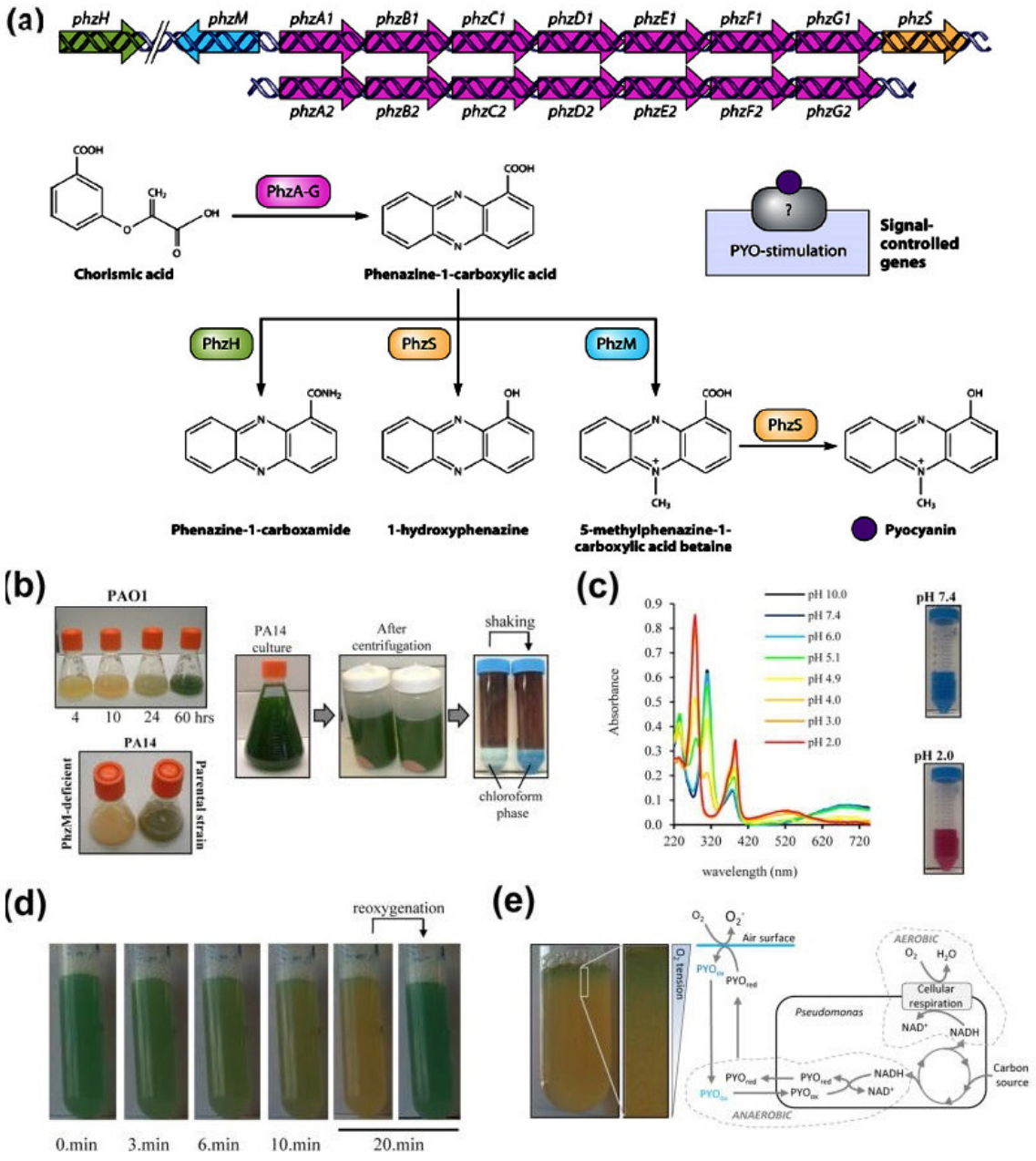

Figure B.7. Pyocyanin (PYO) is a redox-active exotoxin of *Pseudomonas aeruginosa*
(a) Chorismic acid is transformed via the PhzA to -G proteins into phenazine-1-carboxylic acid, which is subsequently converted into different phenazines by the enzymes PhzH, PhzS, and PhzM. The product of the latter, is transformed by PhzS into pyocyanin (PYO). (Image from Wim J. Quax et al, Microbiology and Molecular Biology Reviews 2012). (b) PYO is produced by stationary phase cultures of *P. aeruginosa* PAO1. The PhzM-deficient *P. aeruginosa* PA14 strain fails to turn the medium dark and produce pyocyanin. PYO can be purified from stationary phase Pseudomonas cultures by repeated chloroform–distilled water extraction cycles. (c) The absorption spectrum of PYO is pH-dependent. At low pH values PYO solutions are red, whereas at higher pH values it turns blue. (d) Well-oxygenated stationary phase cultures of PA14 are dark green due to high oxidized PYO concentrations (left). In standing cultures the medium loses its dark colour as reduced PYO accumulates over time (3–20 min). Upon re-oxygenation oxidized PYO turns the culture medium green again (right, 20 min). (e) Standing cultures (anaerobic conditions) show a characteristic green-yellow gradient near the air-medium surface because PYO shuffles electrons between oxygen-poor parts of the culture and the air surface (Image from Rada et al., 2013, Trends. Microbiol.).

B3.5 Toxic exoproducts

P. aeruginosa secretes various toxic exoproducts (Table B.1).

Exoproducts	Locus ID, PA number	Effect on host	Secretion type	Regulation system
Exotoxin A	*toxA*, PA1148	Antiphagocytic, cytotoxic	Type II	(LasR-LasI quorum sensing)
Exoenzyme S	*exoS*, PA3841	Antiphagocytic, cytotoxic	Type III	ExsA-activated type III system
Elastase (LasA, LasB)	*lasA*, PA1871	Elastolytic activity	Type II	LasR-LasI quorum sensing
	lasB, PA3724			
Alkaline proteinase	*aprA*, PA1249		Type I	LasR-LasI quorum sensing
Phospholipase C	*plcH*, PA0844	Disturbance of membrane lipid metabolism	Type II	Inorganic phosphate
	plcN, PA3319			

Table B.1. The major toxic exoproducts of *Pseudomonas aeruginosa*. (Table from Sawa Journal of Intensive Care 2014).

Exotoxin A is a major virulence factor of *Pseudomonas aeruginosa*. This toxin binds to a specific receptor on animal cells, allowing endocytosis of the toxin. Once in endosomes, the exotoxin can be processed by furin to generate a C-terminal toxin fragment that lacks the receptor binding domain and is retro-gradely transported to the endoplasmic reticulum for retro-translocation to the cytosol through the Sec61 channel. The toxin blocks protein synthesis of elongation factor 2 by ADP ribosylation, thereby triggering cell death [61].

The production of Exotoxin A is regulated by exogenous iron. Exotoxin A appears to mediate both local and systemic disease processes caused by *Pseudomonas aeruginosa*. It has necrotizing activity at the site of bacterial colonization and contribute to the colonization process. Toxinogenic strains cause a more virulent form of pneumonia than non-toxinogenic strains. In terms of its systemic role in virulence, purified Exotoxin A is highly lethal for animals including primates.

Exoenzyme S (ExoS) was characterized as an ADP-ribosylating enzyme [62] and has been also proposed to act as an antiphagocytic factor [63] thus enabling the bacteria to evade the host immune system. After its translocation into human epithelial cells by the contact-dependent type III secretion (TTS) apparatus, the ADP-ribosyltransferase (ADPRT) activity of ExoS targets multiple substrates, including the low-molecular-weight G (LMWG) proteins Ras, RalA, certain Rab proteins, Rac1, and Cdc42 [64], [65]. More recently, ExoS was found to also include a GTPase-activating protein (GAP) activity that targets the LMWG proteins Rho, Rac1, and Cdc42, which affect eukaryotic cell cytoskeletal structure [66].

TTS-translocated ExoS can exert complex effects on eukaryotic cell function, including inhibition of DNA synthesis, alterations in cell morphology, microvillus effacement, and loss of

cellular adherence, in addition to its anti-phagocytic or anti-invasive effects [67], [68]. LasA and LasB are both two elastases which are regulated by the las quorum-sensing system and secreted via type 2 secretion apparatus [69]. LasA, also known as staphylolysin, is a 20 kDa serine protease able to cleave the pentaglycine bonds in the peptidoglycan of *Staphylococcus aureus* [70]. On the contrary, it has been observed that LasB show more specific elastolytic abilities and is capable to degrade a number of connective tissue proteins, including elastin, fibrin and collagen [71]. Although their role in chronic infection remain not well understood, they clearly play a fundamental role in interference with host defence mechanisms [25].

Alkaline protease (AprA) is a type1 secreted zinc protease that degrades several components of the host immune system and host fibronectin [72]. In addition, assists *P. aeruginosa* to screw-on the immune response by degrading free flagellin and so preventing toll-like receptor 5 activation [73].

Phospholipases are ubiquitous enzymes that induce changes in membrane composition, activate the inflammatory cascade and alter cells signaling pathways. *P. aeruginosa* elaborates two known phospholipases C (PLCs), PlcHR (hemolytic) and PlcN (nonhemolytic) [74]. While PlcN has no demonstrated pathogenic activity, PlcHR may be an important virulence factor. Indeed, purified PlcHR causes vascular permeability, end organ damage and death when injected into mice in high doses [75]. The *plcHR* operon consists of the structural gene *plcH* and others two downstream genes, overlapping *plcR1* and *plcR2*, whose products are necessary for secretion and solubility of PlcHR [76]. Because of the induction of PlcHR by phosphate starvation, it appears to function in phosphate-scavenging pathways. This induction seems to be of pathogenic significance, since humans infected with gram-negative pathogens have inorganic phosphate levels reduced to a level suboptimal for bacterial growth [77]. Interestingly, however, both PLCs recognize phospholipids found predominantly on eukaryotic (e.g., phosphatidylcholine and sphingomyelin) rather than prokaryotic membranes. Since metazoan PLCs play a central role in host inflammatory cell signaling, *P. aeruginosa* PLCs may represent the evolution not only of nutritional enzymes, but also of secreted products which specifically alter the host's immune response to the bacterium.

B3.6 Exotoxins secretion systems

Gram-negative bacteria, which have inner and outer bacterial membranes, use dedicated secretion systems to transport proteins synthesized to the outside environment. The secretion systems of gram-negative bacteria can be classify into six subtypes [78]. The type I secretion system is relatively simple and consist of only a few proteins. Unlike proteins secreted by the type II secretion system, proteins secreted by the type I contain no signal sequence at their amino termini; instead, they contain domains at their carboxyl termini necessary for recognition by the type I secretion complex. The type II system is responsible for the so-called *sec* dependent secretion [79]. Proteins secreted by the type II system possess amino-terminal signal sequences of 16–26 residues. The type III and IV secretion systems have been more recently defined (Figure B.8).

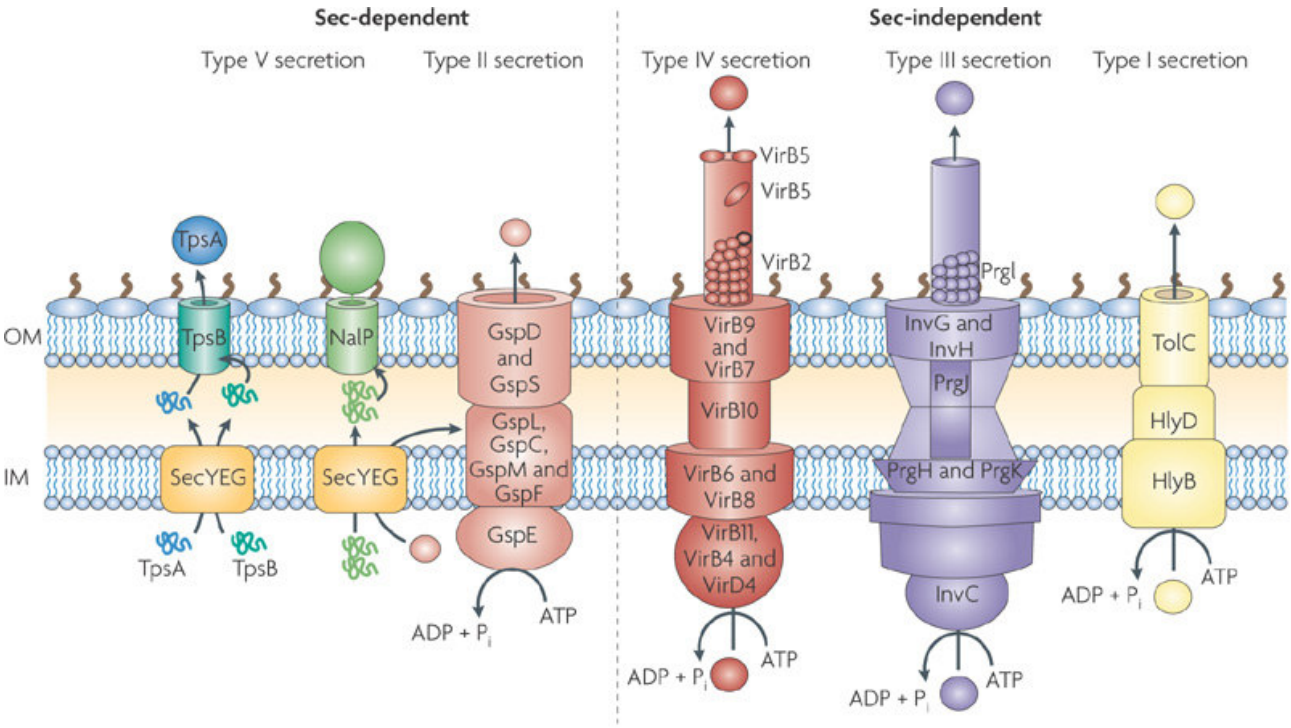

Figure B.8. Gram-negative bacterial protein secretion system. Type I secretion systems (T1SSs), exemplified by the haemolysin secretion system in *Escherichia coli*. Type II secretion systems (T2SSs) are multicomponent machines that use a two-step mechanism for translocation. As an example, the general secretion pathway (Gsp) system is shown. Type III secretion systems (T3SSs), also called injectisomes, mediate a single-step secretion mechanism, illustrated by the *Salmonella Typhimurium* system, which uses the invasion (Inv) and Prg proteins. Type IV secretion systems are versatile systems that secrete a wide range of substrates, from single proteins to protein–protein and protein–DNA complexes, in figure is shown the *Agrobacterium tumefaciens* VirB/D system. Type V secretion systems (T5SSs) include autotransporters and two-partner secretion systems which translocate substrates in two steps such as NalP from *Neisseria meningitides*. (Image, Nature Reviews Microbiology 7, 703-714, 2009).

Using secretion systems, bacteria directly deliver proteins into the cytosol of target eukaryotic cells [80]. Evolutionarily, TTSS derived from flagella, while the type IV system derived from a conjugational system [81], [82]. All TTSSs studied till date share an important feature: the genes encoding this system are upregulated by direct contact between bacteria and host cells, with consequent direct delivery of bacterial virulence products (type III secretory toxins or effector molecules) into the host cell via the secretion and translocation apparatus [83].
P. aeruginosa has a variety of secretion systems of which at least four likely play a role in virulence (Type I, II, III, and VI): particularly, type III (T3SS). The T3SS is a complex macromolecular machinery, encoded by 36 genes on five operons, spanning the inner bacterial membrane, the periplasmic space, the peptidoglycan layer, the outer bacterial membrane, the extracellular space, and finally reaching the host cell membrane (Figure B.9).

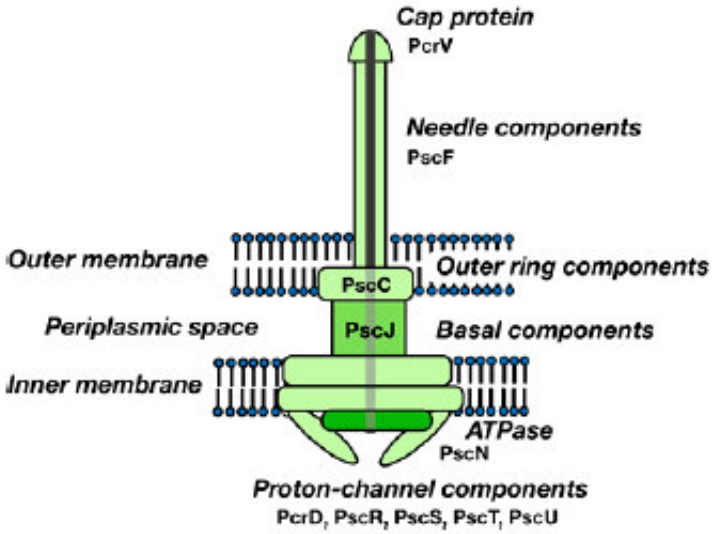

Figure B.9 ***Pseudomonas aeruginosa*** **type III secretory apparatus: the needle complex or injectisome**. The type III secretory apparatus comprises many protein components: a cap component, PcrV; a needle component, PscF; an outer ring component, PscC; and basal components, including PscJ, ATPase PscN, and others. (Image, from Sawa Journal of Intensive Care 2014).

This secretion apparatus is able to deliver effector proteins from the bacterium directly into the host cell through a pore formed in the membrane. Despite extensive characterization by a number of laboratories, so far, only four effector toxins have been identified (ExoY, ExoS, ExoT, ExoU) a number much lower compared to other bacterial pathogens, such *Salmonella* (≈13) or *Shigella* (≈ 25) [84].

B3.7 Iron uptake

Iron is essential for bacteria since is present in proteins with [Fe-S] clusters and in heme, making it essential for many metabolic activities, such as in enzymes of the TCA cycle, catalases, cytochromes [85]. However, aerobic microorganisms are confronted with the poor solubility of Fe^{3+}, with as consequence, the need for the development of elaborate high-affinity iron uptake systems [85]. Another problem caused by iron is its reactivity that can generate dangerous reactive oxygen species, which means that its uptake has to be tightly controlled [85]. Gram-negative bacteria adopted two strategies to take up iron under aerobic conditions: via the uptake of heme or via the uptake of iron-siderophore complexes. In both cases, a TonB-dependent outer membrane receptor (porin) plays a critical role recognizing the iron-loaded complex. Once inside the cell, iron is removed by a non-destructive reduction of the complexed Fe^{3+} to Fe^{2+} leaving the iron chelator (apo-siderophore or tetrapyrrole ring) intact, allowing recycling, or by an enzymatic destruction of the siderophore or heme molecule by esterases or heme oxygenases [85] [86].

In *Pseudomonas aeruginosa*, two heme uptake systems have been described, Phu and Has [87]. In the Phu system, hemoproteins bind directly the receptor and heme is extracted, while in the Has system a hemophore protein HasAp, is secreted via a type I secretion system and takes heme from hemoproteins, bringing it to the HasR receptor [88] [87]. Once in the cytoplasm of *P. aeruginosa* heme is degraded by a heme oxygenase encoded by hemO, liberating biliverdin and Fe^{2+} [89] [90] [91]. Since free heme is toxic, it is bound in the cytoplasm to the PhuS protein which delivers it to the heme oxygenase HemO [91].

Another important mechanism of iron uptake is the ferri-pyoverdine-based, in which the iron is taken up via the FpvA receptor, and, once in the periplasm, reductive release of iron is occurring, followed by rapid recycling and excretion of apo-pyoverdine [92] (see below). Recycling and efflux of pyoverdine involves the tripartite efflux pump PvdT, PvdR, and OpmQ pyoverdine [92] (Figure B.10). So far, no evidence for a ferri-pyoverdine-binding protein in the periplasm was reported, suggesting that only iron and not ferripyoverdine is transported into the cytoplasm. The cellular organization of PVDI biosynthesis is such as to protect against disruption of iron or other metal homeostasis. In other words, PVDI storage in the bacterial periplasm suggests that siderophores may even have a wider function than just feeding bacteria with iron: they may have as well a key role in iron and other metal homeostasis and bacterial metal tolerance. Being a ubiquitous microorganism, *P. aeruginosa* is induced to overcame a huge competition from other microorganisms in the iron uptake and for that

reason possess many genes encoding TonB-dependent receptors for siderophores produced by other bacteria and fungi (xenosiderophores) [93]. Some of these receptors, as an example FpvB, are used to take up pyoverdines produced by other members of the genus.

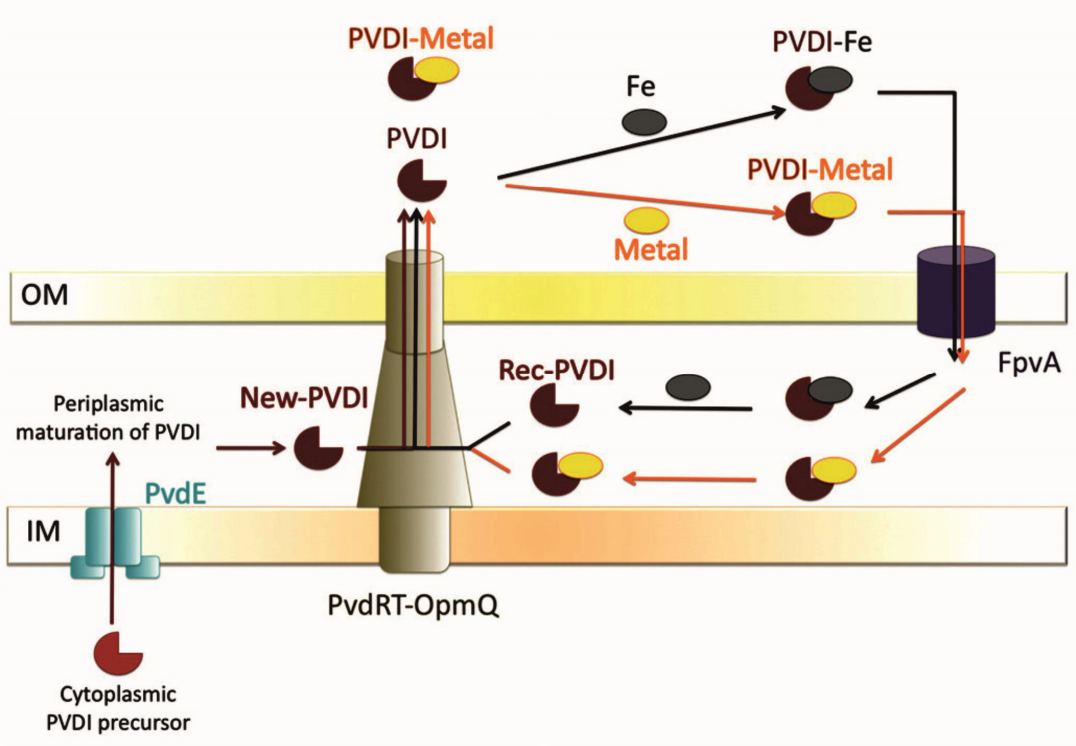

Figure B.10. Secretion of PVDI by PvdRT-OpmQ. The ATP-dependent efflux pump PvdRT-OpmQ is involved in the secretion of newly synthesized PVDI. Biosynthesis of this siderophore involves assembly of the PVDI backbone in the bacterial cytoplasm and maturation in the periplasm. PVDI that has already transported and released iron ions into the bacterial periplasm is also recycled into the extracellular medium by PvdRT-OpmQ. The chelator picking up iron in the periplasm and transporting it across the inner membrane into the cytoplasm has not been identified yet. Any unwanted PVDI–metal complexes imported into the periplasm by the outer membrane transporter FpvA are expelled as a PVDI–metal complex to the extracellular medium by PvdRT-OpmQ without any metal release from the siderophore. For all these different PVDI forms, PvdRT-OpmQ collects the siderophore from the periplasm and transports it across the outer membrane to the extracellular medium. OpmQ is predicted to be a porin-like b-barrel located in the outer membrane with a large periplasmic extension, PvdT an ATP-dependent inner membrane protein, and PvdR a periplasmic adaptor protein. (Image from Isabelle J. Schalk, Review, 2013, Env. Microb.).

Pyoverdines are siderophores composed of three parts: a dihydroquinoline-type chromophore responsible for their fluorescence, a strain-specific peptide comprising 6–12 amino acids; and a side-chain bound to the nitrogen atom at position C-3 of the chromophore. In most cases, this side-chain is a di-acid of the Krebs cycle, such as succinic, malic or a-ketoglutaric acid or one of their amide derivatives. The peptide chain and the side-chain are connected to the carboxyl group and to the NH_2 group of the chromophore respectively. The sequence of the peptide moiety differs substantially between species and even between strains of the same species. This peptide chain may contain unusual amino acids, such as D-isomers, and amino acids which are not usually found in biomolecules.

Three distinct pyoverdine types can be produced by *P. aeruginosa* (PVDI, PVDII and PVDIII) each characterized by a different peptide chain [94]. Pyoverdines have been shown to be important or even essential for the colonization of host tissues and for virulence in plant and animal pathogens, and in the case of *P. aeruginosa* shown to be necessary for the establishment of mature biofilms [95].

P. aeruginosa produces another siderophore of lower affinity, pyochelin. Recently it has been described a pyochelin-induced ATCs (secondary metabolite, 2-alkyl-4,5-dihydrothiazole-4-carboxylates) able to bind iron as well [96].

Like in many other bacteria, Fur is the major iron-responsive general regulator in pseudomonads and it is essential in *P. aeruginosa* since the fur gene could not be inactivated in this bacterium [97] [98]. Fur is a classical prokaryotic aporepressor that requires iron (co-repressor) in order to bind a target sequence (Fur box) in the promoter region of iron-regulated genes blocking their transcription when the level of intracellular iron (Fe^{2+}) reaches a certain threshold [99]. Some genes are directly under the control of Fur, responding to the intracellular concentration of iron, but the majority of genes, including those involved in the uptake of ferrisiderophores are indirectly regulated (Figure B.11). Fur regulates the expression of several extra-cytoplasmic sigma factors (ECF-σ) which are needed for the transcription of different genes, including the pyoverdine biosynthesis genes (PvdS), the ferripyoverdine receptor gene *fpvA* (FpvI), and other genes encoding TonB-dependent receptors for xenosiderophores.

The availability of the iron source (the ferrisiderophore) is the signal triggering the expression of the receptor via the ECF-σ factor. Finally, two Fur-regulated small RNAs, PrrF1 and PrrF2, act as mRNA anti-sense post-transcriptional regulators, resulting in up-regulation by Fe–Fur of genes involved in resistance to oxidative stress, iron storage and intermediary metabolism [100] [101] (see Small RNA B4.3).

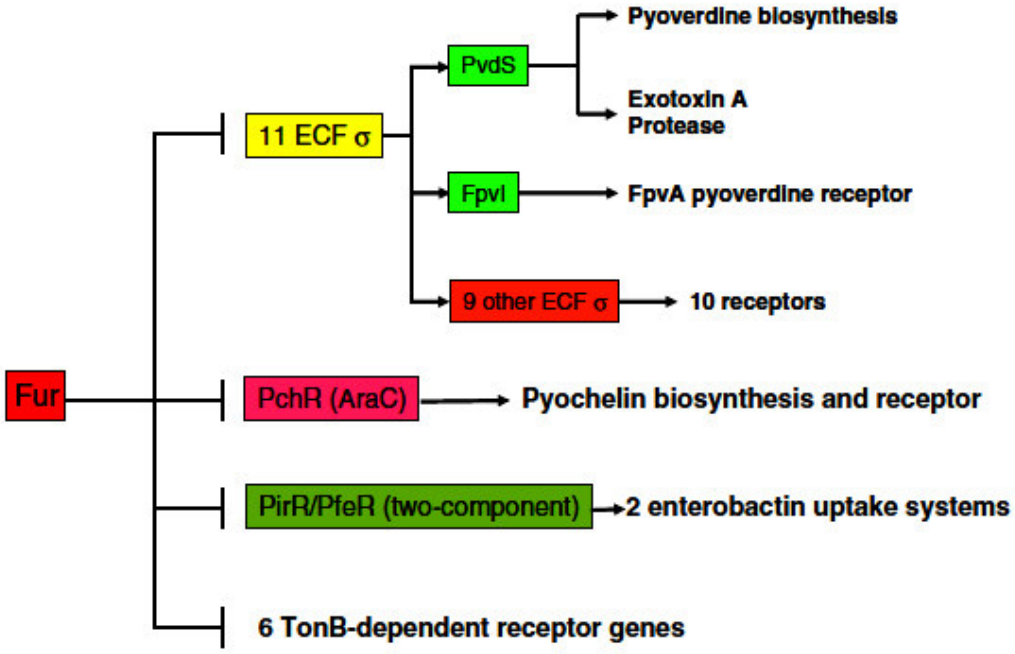

Figure B.11 Regulation of iron uptake via TonB-dependent receptors in *P. aeruginosa*. Fur represses the expression of 11 genes encoding ECF sigma factors, among which PvdS (for the transcription of pyoverdine genes, exotoxin A, and PrpL protease) and PvdI for the transcription of the ferripyoverdine gene fpvA. All ECF sigma factors are themselves controlled by anti-sigma factors which are released when the cognate ferrisiderophore interacts with the receptor (Cornelis et al. 2009). Fur also controls the AraC regulator PchR which activates the transcription of genes for the biosynthesis and uptake of ferripyochelin (Michel et al. 2005). Fur also controls the expression of two component systems for the uptake of ferrienterobactin, PfeS/PfeR via the PfeA receptor and a similar system via the second enterobactin receptor, PirA (Dean et al. 1996; Ghysels et al. 2005). (Image, Iron uptake and metabolism in pseudomonads Pierre Cornelis, Appl Microbiol Biotechnol 2010).

B4. Virulence regulatory systems

The signaling network of *Pseudomonas aeruginosa* is perhaps one of the most complex systems known. It consists of multiple interconnected signaling layers that detect and react to endogenous and environmental molecules, triggering massive changes in genetic expression.

B4.1 Two Component systems

P. aeruginosa controls its lifestyle (free-living and biofilm) and the production of multiple virulence factors via two-component signal transduction systems. These systems act through phosphorylation cascades that induce conformational changes in regulatory proteins, resulting in global changes in gene expression. Over 60 two-component systems have been found in the genome of *P. aeruginosa*; among them, the GAC system (global activator of antibiotic and cyanide synthesis) is perhaps the most interesting.

The GAC system consists of a transmembrane sensor kinase GacS (LemA), which, upon autophosphorylation, transfers a phosphate group to its cognate regulator GacA, which in turn upregulates the expression of the small regulatory RNAs RsmZ and RsmY. Binding of RsmZ and RsmY to the small RNA-binding protein RsmA activates the production of genes involved in biofilm formation and represses multiple genes involved in acute virulence and motility (Figure B.11).

As a consequence, mutation in RsmA reduce colonization during the initial infection stages but ultimately favored chronic infection in mouse model of acute pneumonia [102].

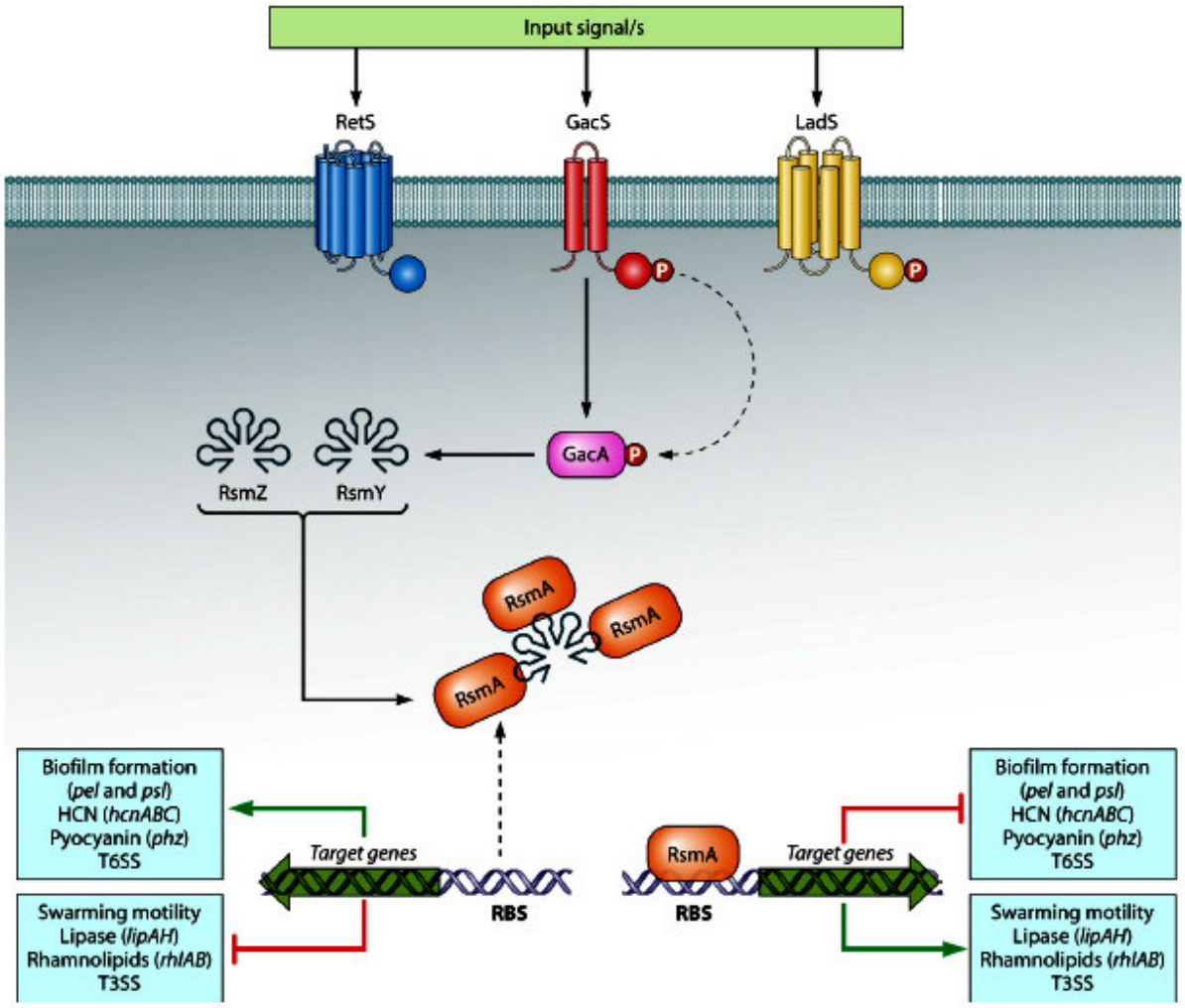

Figure B.11. The GAC system network in *P. aeruginosa* controls the reversible transition from acute to chronic infections. The small regulatory protein RsmA binds to the promoters of multiple genes, enhancing bacterial motility and activating the production of several acute virulence factors while repressing the production of virulence factors associated with chronic infections. GacA phosphorylation via GacS stimulates the production of the small RNAs RsmZ and RsmY, which bind to the RsmA protein, releasing the repression of virulence factors associated with chronic infections and repressing the production of acute infection-associated factors. The sensor kinase LadS works in parallel to GacS, activating RsmZ and RsmY production, while the sensor kinase RetS acts in an opposite manner to LadS and GacS, forming a protein-protein complex with GacS that blocks RsmY and RsmZ production. (Image Pol Nadal Jimenez et al. Microbiol. Mol. Biol. Rev. 2012).

Two further sensor kinases, LadS and RetS, have been found to modulate gene expression via GacA. LadS (lost adherence sensor) acts in parallel to GacS, positively controlling the expression of the pel operon, which increases biofilm production and repressing the expression of genes involved in the T3SS [19]. The third sensor kinase involved in this pathway, RetS (regulator of exopolysaccharide and type III secretion), controls GacA in an opposite manner to GacS and LadS, promoting acute infection and repressing the expression of genes associated with biofilm production. The GAC system also has a control on the AHL system via RsmA, by negatively controlling the synthesis of C4-HSL and 3-oxo-C12-HSL and of extracellular virulence factors controlled by AHLs.

B4.2 Quorum sensing systems

Quorum sensing (QS) is a bacterial cell-to-cell communication process that involves the production, detection and response to extracellular signaling molecules called auto-inducers (AIs). AIs accumulate in the environment as the bacterial population density increases, and bacteria monitor this information to track changes in their cell numbers and collectively alter gene expression. Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation and virulence factors secretion [103] [104]. Three basic principles are shared to all known QS systems. First, bacterial cells produce AIs. At low cell density (LCD), AIs are not able to reach the threshold concentration required for detection. At high cell density (HCD), the cumulative production of AIs leads to a local high concentration, enabling detection and response [105]. Second, AIs are detected by cell receptors in the cytoplasm or in the membrane. Third, in addition to activating expression of genes necessary for cooperative behaviors, detection of AIs results in activation of AI production [106].

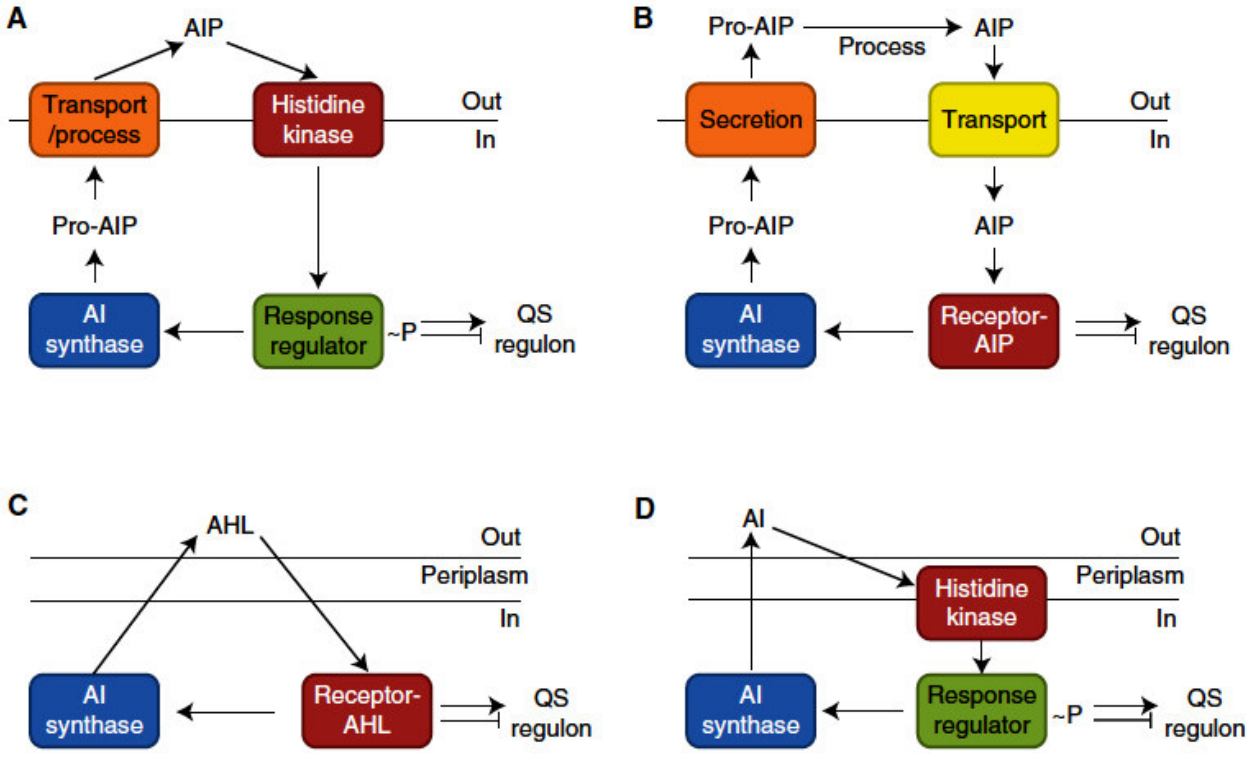

Figure B.12. Canonical bacterial quorum-sensing (QS) circuits. Autoinducing peptide (AIP) QS in Gram-positive bacteria by (A) two-component signaling, or (B) an AIP-binding transcription factor. Small molecule QS in Gram-negative bacteria by (C) a LuxI/LuxR-type system, or (D) two-component signaling. (Image source Steven T. Rutherford1 and Bonnie L. Bassler1,2Cold Spring Harb Perspect Med 2012).

Gram-positive and Gram-negative bacteria use different types of QS systems (Figure B.12) Gram-positive bacteria use peptides (auto-inducing peptides, AIPs), as signaling molecules that are processed and secreted. When the extracellular concentration of the AIP is high it binds to a cognate membrane-bound two-component histidine kinase receptor. Usually, binding activates the receptor's kinase activity, it auto-phosphorylates and passes phosphate to a cognate cytoplasmic response regulator. The phosphorylated response regulator activates transcription of the genes in the QS regulon (Figure B.12 A). In some cases of Gram-positive bacterial QS, AIPs are transported back into the cell cytoplasm where they interact with transcription factors to modulate the transcription factor's activity and, in turn, modulate gene expression changes (Figure B.12 B).

Gram-negative bacteria communicate using small molecules as AIs. These are either acylhomoserinelactones (AHLs) or other molecules whose production depends on S-adenosylmethionine (SAM) as a substrate [107]. The AI-bound receptors regulate expression of the genes in the QS regulon (Fig. B.13 C). In some AIs are detected two-component histidine kinase receptors that function analogously to those of Gram-positive bacteria (Fig. B.13 D).

B4.2.1 Quorum sensing in *Pseudomonas aeruginosa*

P. aeruginosa harbors three QS systems: two LuxI/LuxR-type QS circuits that function in series to control expression of virulence factors as well as a third, non-LuxI/LuxR-type system called the Pseudomonas quinolone signal (PQS)system (Figure B.13). In the first circuit, the LuxI homolog LasI synthesizes 3-oxo-C12-homoserinelactone (3OC12HSL) that is detected by the cytoplasmic LuxR homolog of LasR [108] [109] [110] [111] [112]. The LasR–3OC12HSL complex activates transcription of target genes including those encoding virulence factors such as elastase, proteases, and exotoxin A (see B3.5/.6 sections). One of the LasR–3OC12HSL targets is lasI, which establishes an auto-inducing feed-forward loop [113]. Another target is RhlI that synthesizes a second AI, butanoyl homoserinelactone (C4HSL) [114] [115]. At high concentrations, this AI binds to RhlR, a second LuxR homolog. RhlR–C4HSL activates target genes, including those encoding elastase, proteases, pyocyanin, and siderophores [116] [117]. Among its targets is rhlI, leading to auto-induction of this second QS circuit.

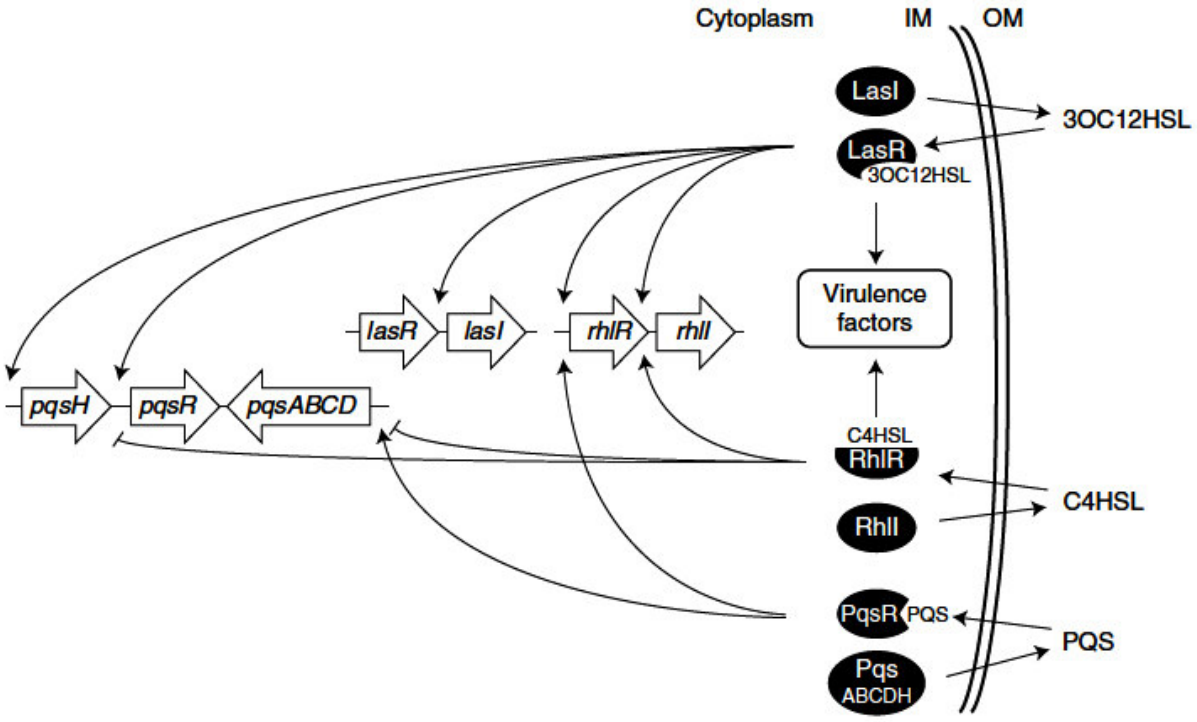

Figure B.13. *P. aeruginosa* QS circuits. The three AI sythases, LasI, RhlI, and PqsABCDH, produce the AIs 3OC12HSL, C4HSL, and PQS, respectively. The AIs are detected by the cytoplasmic transcription factors LasR, RhlR, and PqsR, respectively. Each transcription factor regulates expression of its corresponding AI synthase as well as additional targets as indicated by the arrows (image Steven T. Rutherford1 and Bonnie L. Bassler, Cold Spring Harb Perspect Med 2012).

P. aeruginosa uses an additional non-LuxI/ LuxR QS system to control virulence factor gene expression, the PQS-based system (see B4.2.3).

P. aeruginosa QS-activated virulence factors include elastase, proteases, pyocyanin, lectin, swarming motility, rhamnolipids and toxins.

It is interesting to note that up to 10% of the *P. aeruginosa* genome is controlled by QS [118], thus it is not surprising that there are additional levels of regulation impinging on the QS circuits.

A hierarchical relationship exists between the Las and Rhl systems: the Las system controls the Rhl system, as the 3-oxo-C12-HSL–LasR complex directly upregulates rhlR transcription [119].Thus, activation of the LasI/R system allows the later activation of the RhlI/R system (Fig. B.13). There are, however, notable exceptions to this observation. *P. aeruginosa* isolates from the lungs of chronically infected cystic fibrosis patients often carry lasR mutations but are known to produce virulence factors regulated by RhlR and PqsR [120] [121]; also laboratory strains grown with limited phosphate seem to be able to induce RhlR- and PqsR-dependent genes without activation of LasR [122]. Recently was identified a non-ribosomal peptide synthase gene called ambB that, when knocked out, impairs the LasR-independent

activation of RhlR and PqsR under phosphate-limited conditions [123]. In varied models of *P. aeruginosa* infection, including nematode, mouse and fruit fly, ambB mutations result in almost as much attenuation of virulence as lasR mutations. This provides a new explanation for the occurrence of LasR mutants in certain persistent human infections such as the lung infections of people with cystic fibrosis [124] in which active production of QS-dependent virulence products might result from IQS signaling.

Due to this broad range control on *P. aeruginosa* genome, particularly focused on virulence factors, QS circuits make attractive targets for novel antimicrobials. This is especially critical in the treatment of persistent infections in cystic fibrosis patients given the resistance of many *P. aeruginosa* isolates to available antibiotics. Because LasR sits at the top of the QS cascade, diverse LasR inhibitors have been studied i) molecules that contain modifications to the native 3OC12HSL ligand [125] [126] [127], ii) natural products (furanones , patulin and their synthetic modified versions) that inhibit QS by antagonizing LasR [128] [129].

A potential complication in targeting LasR is that, as mentioned, some clinical *P. aeruginosa* isolates possess defective LasR proteins [130]. Nonetheless, establishment of the initial infection is known to be LasI/LasR-dependent, suggesting that LasR inhibitors could be used at least as prophylaxis.

A final approach is to target regulators that affect both the LasI/LasR and RhlI/RhlR QS systems, in fact by agonizing QscR, it could be possible to diminish the overall QS response and thus prevent or delay expression of virulence factors. Interestingly, some non-natural AHLs that target QscR also inhibit LasR, raising the intriguing possibility of a compound that can act broadly to target all of the *P. aeruginosa* QS systems [131].

B4.2.2 AHL-mediated systems

N-Acyl homoserine lactones (AHLs) were the first broadly bacterial cell-to-cell signals to be discovered and, to date, remain the most-studied communication molecules in bacteria [132] [133] [134] [135].

In general, AHLs consist of fatty acids, varying in length and substitution, linked via a peptide bond to a homoserine lactone moiety. AHLs are synthesized by members of the LuxI family of proteins and are sensed by members of the LuxR family of transcriptional regulators [136] [137]. After a certain concentration of AHLs (the threshold correlating to a certain bacterial cell density) a complex with the cognate LuxR transcriptional regulator will be formed, enabling binding to DNA, thereby altering the expression of multiple virulence genes.

Two different AHL systems coexist in *P. aeruginosa*: the Las and Rhl systems (Figure B.14). The Las system produces and responds to N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL), which is produced by the LasI synthase and recognized by the transcriptional regulator LasR [138] [139]. The Las system controls the production of multiple virulence factors involved in acute infection and host cell damage, including the LasA and LasB elastases, exotoxin A and alkaline protease.

The second AHL system, the Rhl system, produces and responds to N-butanoyl homoserine lactone (C4-HSL) [115]. This molecule is generated by the RhlI synthase and sensed by the transcriptional regulator RhlR. The RhlR-C4-HSL complex induce the expression of several genes, including those responsible for the production of rhamnolipids, and repressing those responsible for assembly and function of the type III secretion system (T3SS).

In addition to RhlR and LasR, *P. aeruginosa* possesses several putative LuxR-type homologues lacking a LuxI-type cognate partner; these homologues have been designated orphan LuxR homologues [140].

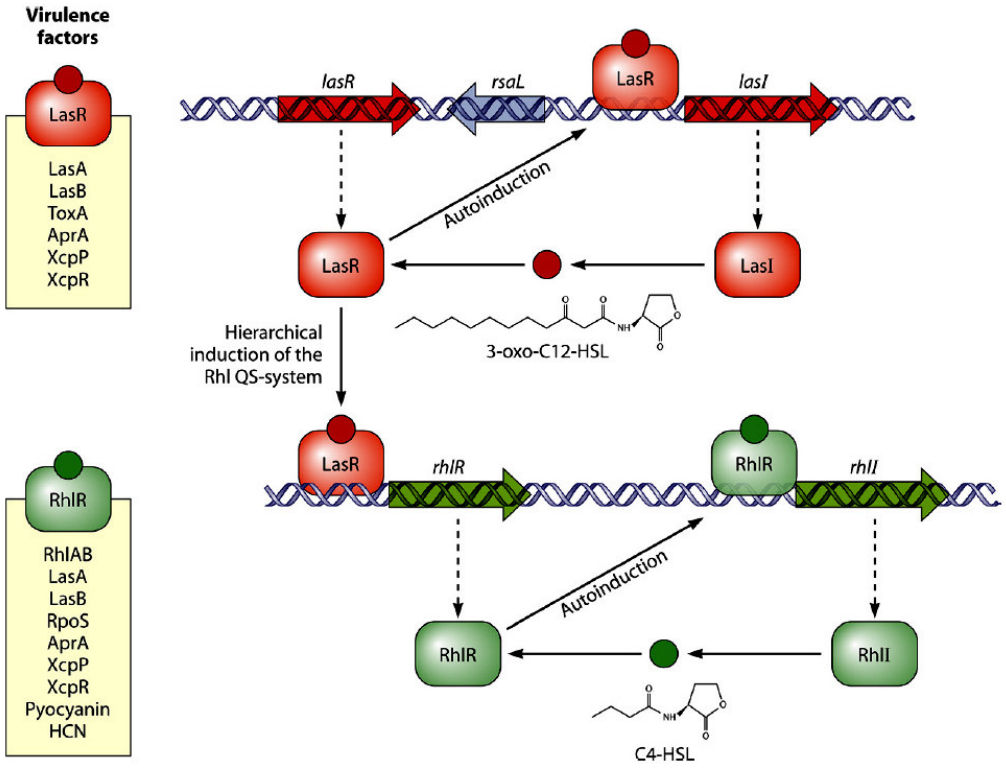

Figure B.14. Virulence regulation and interaction between the two AHL quorum-sensing systems in *P. aeruginosa.* After a threshold concentration of 3-oxo-C12-HSL is produced, the 3-oxo-C12-HSL–LasR complex binds the promoter regions of multiple genes, activating or repressing their transcription. Among the genes upregulated by this complex are *lasI*, which enhances the production of 3-oxo-C12-HSL (autoinduction effect), and *rhlR*, which increases the production of the *rhl* response regulator RhlR, activating the second AHL pathway at an earlier stage. Virulence factors regulated by each respective receptor-ligand complex are detailed on the left. (Pol Nadal Jimenez, 2011, Microb. And Molec. Boil. Reviews).

B4.2.3 4-Quinolones mediated systems

The molecule 2-heptyl-3-hydroxy-4-quinolone, termed the Pseudomonas quinolone signal (PQS), is synthesized from anthranilate and α-keto-fatty acid by the products of the pqs biosynthesis genes *pqsABCD* [141] [142] (Figure B.15). These enzymes synthesize the precursor molecule 2-heptyl-4(1H)-quinolone (HHQ) which is finally converted into PQS by PqsH. When a certain threshold concentration of PQS in the extracellular medium is reached, this molecule binds to its cognate receptor PqsR. The resulting complex activates the expression of the *pqsABCDE* and *phnAB* operons, increasing PQS and pyocyanin production [143] [144] [145].

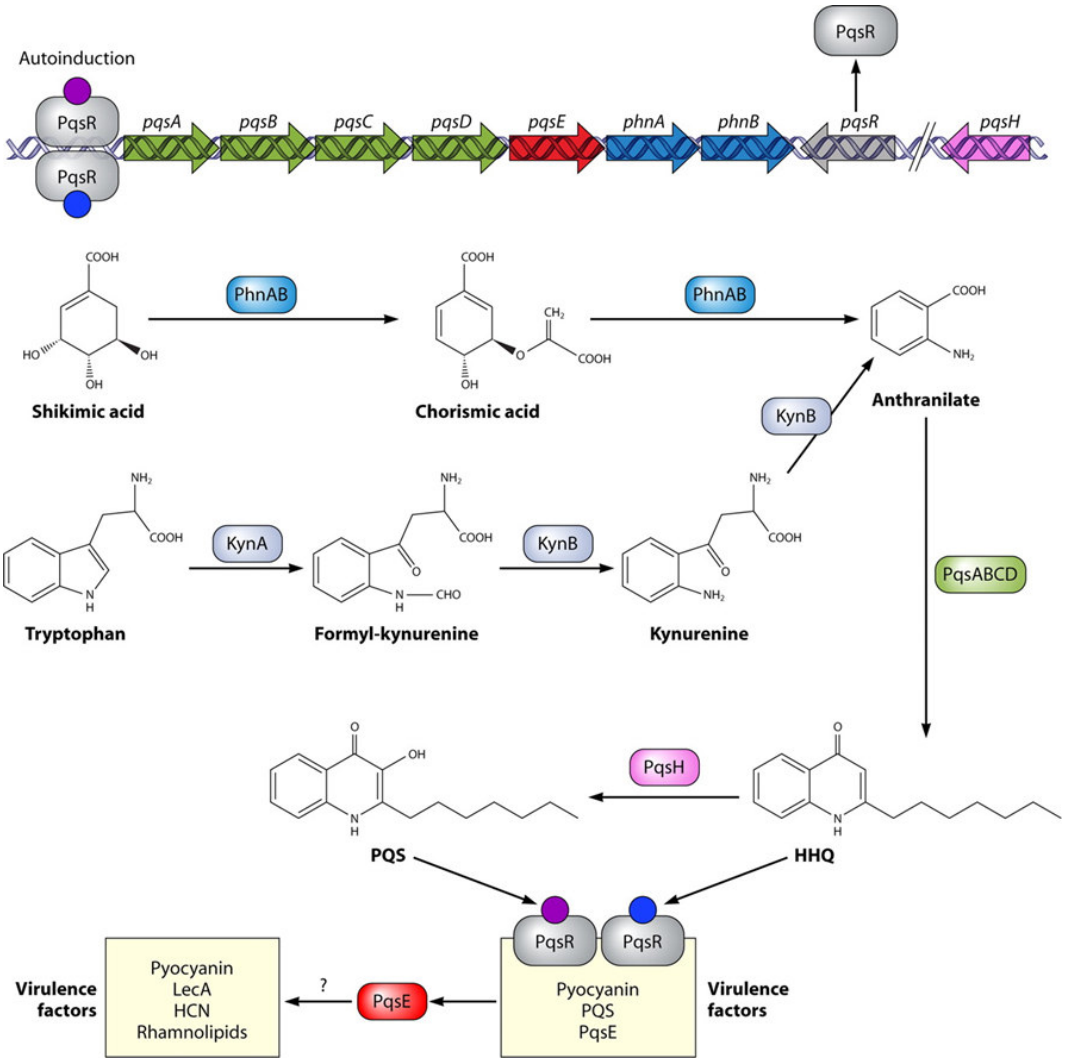

Figure B.15 Biosynthesis, autoinduction and virulence regulation by 4-alkyl-quinolones in *P. aeruginosa*. Biosynthesis of PQS starts with the conversion (by the PqsABCD proteins) of anthranilate (which originates from either the kynurenine pathway or the PhnAB anthranilate synthase) into HHQ, which is finally converted into PQS by the PqsH monooxygenase. Both HHQand PQS bind the PqsR regulator, and the complex activates the transcription of the pqsABCDE and phnAB operons, increasing the levels of PQS (auto-induction) and pyocyanin production. Additionally, transcription of the PQS operon results in an increase in the levels of PqsE, an enzyme of uncharacterized function that increases the levels of pyocyanin, lectin, HCN, and rhamnolipids. (Image Pol Nadal Jimenez et al. Microbiol. Mol. Biol. Rev. 2012).

The increase in production of PQS resulting from the PQS-PqsR complex binding to the *pqsA* promoter region constitutes an auto-induction mechanism similar to that observed in AHL quorum sensing systems. Two additional regulators, MvaT and its homologue MvaU, are found in several *Pseudomonas spp*. and may also be involved in PQS production in *P. aeruginosa*. In addition to the four genes involved in PQS biosynthesis, the *pqsABCDE* operon contains a fifth, *pqsE,* encoding a protein that is not required for PQS synthesis [146] [147]. Despite the recent elucidation of the crystal structure of PqsE [148], little is known about its function and natural substrate. PqsE is the major virulence effector in the 4-alkyl-quinolone (4-AQ) system, controlling the production of several virulence factors, such as pyocyanin, lectin, rhamnolipids and HCN [147], which are all implicated in toxicity and acute infection (Figure B.15). The 4-quinolone signaling system is linked in a hierarchical manner to the AHL signaling systems of *P. aeruginosa*, as LasR (positively) and RhlR (negatively) control the levels of PQS by binding to the promoter region of the PqsR regulator [149]. Additionally, PqsE alone is sufficient to regulate its virulence target genes via the rhl QS system intrinsically linked to RhlR [146]. Mutations in either *pqsA* or *pqsE* significantly reduce *P. aeruginosa* virulence in plant and animal infection models [150]. Although PQS is the major 4-quinolone signaling molecule produced by *P. aeruginosa*, approximately 50 structurally related 4-quinolones are also produced by the PqsABCD proteins. One of them, the PQS precursor HHQ, has clearly been demonstrated to also act as a cell-to-cell signal [151].

B4.3 Small RNAs

Small RNAs (sRNAs) of prokaryotes are 40–600 nucleotides in length and most have regulatory functions. To date, close to 100 sRNAs have been reported in *Escherichia coli* and more than 150 sRNAs in prokaryotes altogether [152]. More than 200 novel sRNA have been detected in *P. aeruginosa*; however, just about 10 has been characterized for its functions [153] [154] [155]. These regulators mostly act as coordinators of adaptation processes in response to environmental changes, integrating environmental signals and controlling target gene expression, primarily at posttranscriptional levels [156] [157].

Post-transcriptional gene regulation mediated by small regulatory RNAs (sRNAs) is commonly found in both prokaryotic and eukaryotic kingdoms. Small RNAs in these systems act to down-regulate target genes by decreasing translation and/or increasing mRNA turnover [158] [159]. Eukaryotic microRNAs (miRNA) or small interfering RNAs (siRNA) are assembled into ribonucleoprotein complexes known as RNA-induced silencing complex (RISC). These complexes modulate stability and/or translation of multiple target mRNAs [158]. RISCs are composed of a variety of proteins such as RNA-binding proteins, RNA helicases, and nucleases. These characteristics are reminiscent of bacterial sRNAs and RNA-binding protein Hfq, both of which form ribonucleoprotein complexes with the endoribonuclease RNase E [160].

In bacterial pathogens small RNAs (sRNAs) are now recognized as important components of the regulatory networks involved in the pathogen-host interaction [161] [162] [163] [157] [152] [164]. Regulatory RNAs can modulate transcription, translation, mRNA stability and DNA maintenance / silencing [152]. They can act using diverse mechanisms, including changes in RNA conformation, protein binding, base pairing with others RNAs and interaction with DNA. There are different classes of sRNAs classified according to their mechanism of action.

One of the classes of RNAs is represented by those that act through binding with proteins (Figure B.16) activating (CsrA) [165], inhibiting (CsrB-CsrA) [166] or modifying (6S-RNAP) [167], the protein activity. One of the most studied example is the *E. coli* 6S RNA, which binds to the housekeeping form of RNA polymerase (σ70-RNAP) modifying its activity [167].

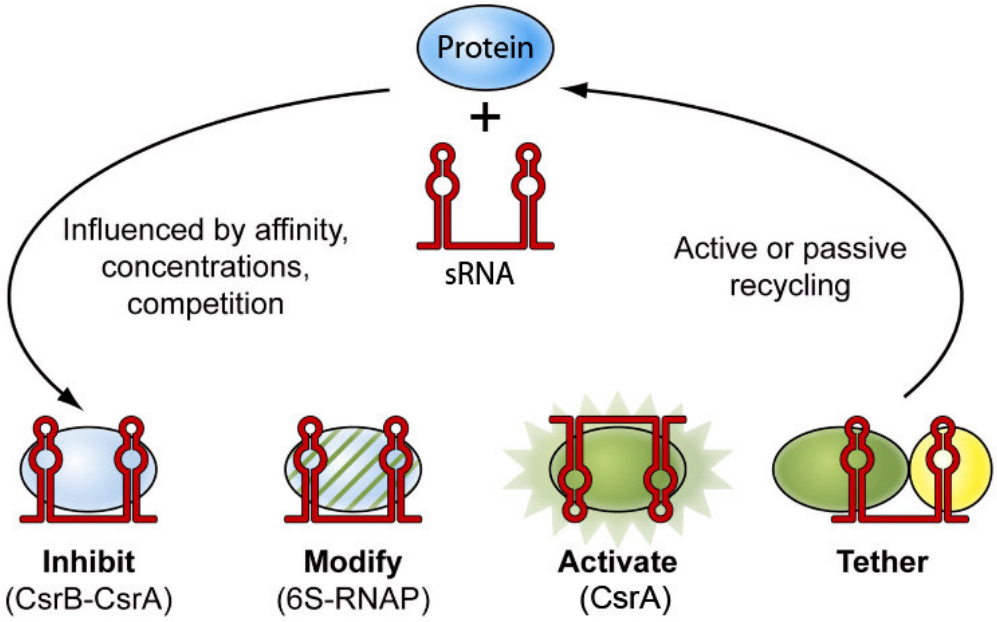

Figure B.16 General Properties of sRNAs that Modulate Protein Activity
Bacterial sRNA binding to proteins has been demonstrated to inhibit and/or modify protein activities. It is proposed that sRNA binding to proteins also can activate or to bring two or more proteins into together. The association of sRNA and proteins is likely to be influenced by many different factors, and the disassociation can be actively or passively controlled (Image from Storz, 2011, Molecular Cell Review, 2011).

Another group of RNA regulators is represented by CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems, an adaptive immune system in bacteria and archaea that provides protection from invading viruses and mobile elements. The CRISPR-Cas system is characterized by two features: the CRISPR locus and a set of CRISPR-associated (cas) genes. Both components need to be expressed for defense against invasive genetic elements. The CRISPR loci contain short repeated sequences separated by short unique spacers usually acquired from phages or other mobile genetic elements [168] [169] [170]. The CRISPR is transcribed and the RNA is processed by Cas proteins into CRISPR RNA (crRNA) [171] [172], crRNAs use sequence complementarity to guide Cas proteins to target DNA for degradation [173], although other functions have also been described [174].

The largest and most studied group of sRNA acts through base pairing with mRNAs, modulating their translation and stability. This class of regulatory RNAs can be subdivided in two distinct broad classes: the *cis* enconed sRNAs, that have a perfect complementarity with their targets and the *trans* encoded, which undergo base-pairing through regions of partial complementarity and may have distinct mRNA targets. The cis and trans acting sRNA belongs to the major class of sRNA regulators as mRNA translation modulators, they can act with different mechanism such as: i) blocking translation by direct binding to the ribosome-binding

site (RBS) in the 5′ untranslated region of target mRNAs (Fig. B.17 A), ii) sRNA-mediated mRNA rapid degradation by inducing the RNase E attacks after base-pairing with sRNA, as a result of the loss of protection conferred by translating ribosomes (Fig. B.17 B, passive nucleolytic repression), iii) According to the other pathway, recruitment of RNase E on the target mRNA triggers formation of a sRNA/Hfq/RNase E complex that favors RNase E attacks; this complex then becomes a specialized RNA decay machine (Fig. B.17 C, active nucleolytic repression), iv) binding in the 5′-UTR the inhibitory sequence of RBS that becomes available, allowing initiation of translation (Fig. B.17 D).

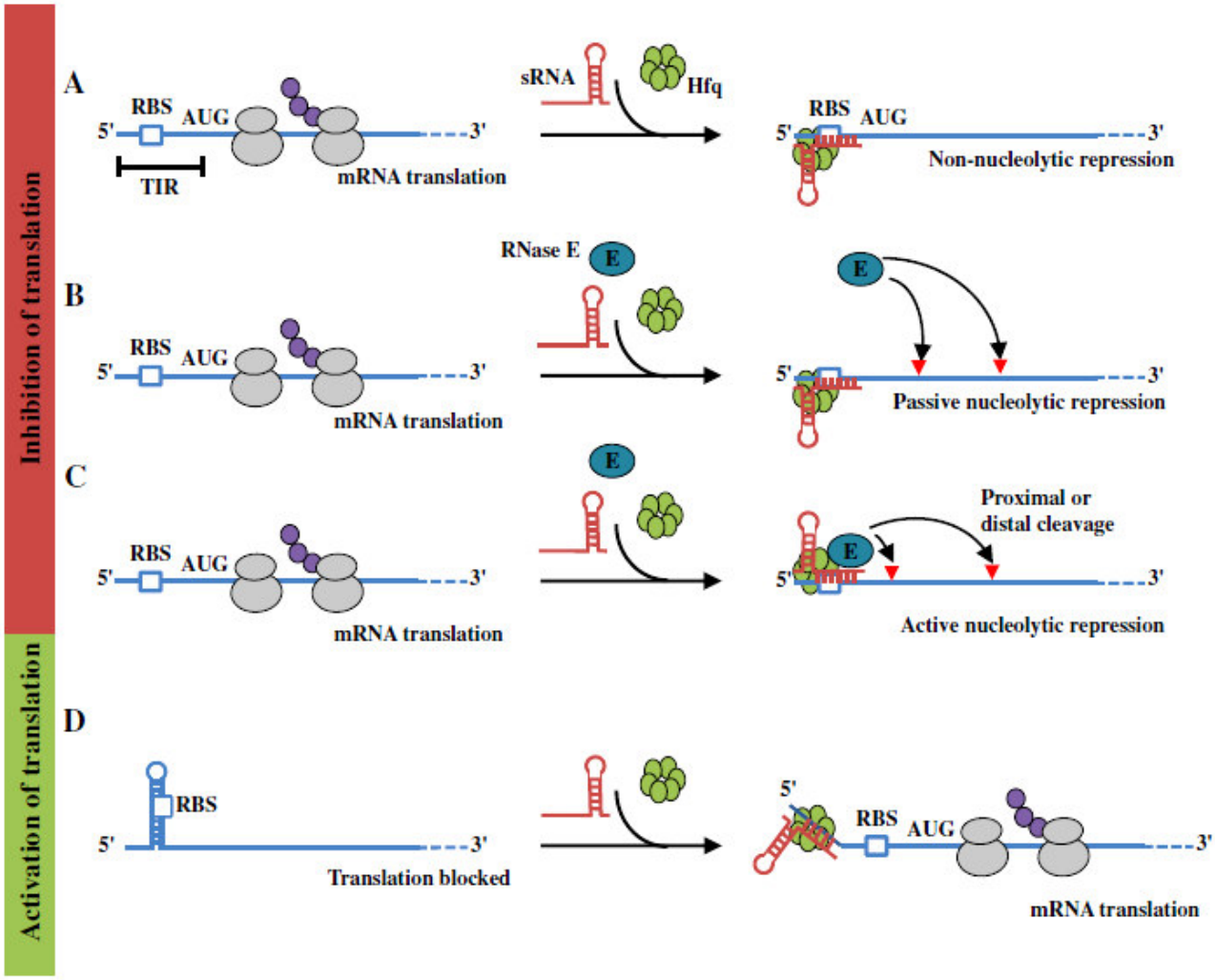

Figure B.17 Small RNA-based regulatory mechanisms of mRNA expression.
Bacterial sRNA act on mRNA targets throught different mechanisms A) Binding to the RBS in the 5' untranslated region causing the Non-nucleolytic repression, B) Binding to the RBS inducing the RNAse E attacks causing the Passive nucleolytic repression, C) Formation of sRNA-Hfq-RNaseE complex inducing the RNA decay machine, D) Binding in the 5'-UTR the inhibitory sequence of RBS that becomes available activatibìng the translation. (Image from D. Lalaouna, Biochimica et Biophysica Acta, Review, 2013).

The group of *cis*-encoded sRNAs are synthesized from the strand complementary to the mRNA they regulates, on the other hand *trans*-encoded sRNAs are synthesized at a different genomic location respect to the mRNA target. A widespread cis-acting RNA element in mRNAs regulation is the metabolite-sensing riboswitch [175], 5' mRNA sequences that can adopt different conformations in response to environmental changes, [176] regulating in this way the expression of the coding sequence.

Typically, the riboswitch feedback regulates the associated genes, which are typically involved in the uptake or metabolism of the sensed metabolite [175] [177]. According to sequence and structure conservation [178], riboswitches seem underrepresented in Gram-negative pathogens but may regulate almost 2% of the genes in Gram-positive bacteria such as *Staphylococcus aureus* [179] and *L. monocytogenes* [180]. Another major class of cis-acting regulatory RNA elements in mRNAs responds to temperature. These RNA thermometers are usually characterized by irregular stem-loop structures (ROSE, FourU motifs) that sequester the Shine-Dalgarno (SD) region of the associated mRNA [181] [182]. 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 [183].

The majority of trans-encoded sRNAs regulation is negative [184] [157], in fact base pairing with the target mRNA usually leads to repression of protein levels through translational inhibition, mRNA degradation or both [185] [186]. However, sRNAs can also activate expression of their target mRNAs. Base pairing of the sRNA disrupt an inhibitory secondary structure, which sequesters the ribosome binding site [187] [188] [189] or stabilizing the mRNA target [190] [191]. Frequently, the trans-acting antisense class of sRNAs require the RNA chaperone Hfq as a cofactor, which facilitates the interaction between sRNAs and target mRNAs [192] [193]. The binding of Hfq can affect the structure of sRNA and target mRNAs [194] and Hfq has been demonstrated to accelerate strand exchange to facilitate dynamic RNA-RNA interactions [195]; furthermore Hfq protects many sRNAs from degradation, most likely by binding to RNase E cleavage sites within these sRNAs [196].

Those sRNAs for which a function has been elucidated are involved in many different cellular processes including adaptation, resistance to stresses [197], metabolism and homeostasis [198] [199], virulence and pathogenesis [200] [201] [202].

B4.3.1 Small RNAs in *P. aeruginosa* virulence

Successful host infection by pathogenic bacteria largely depends on the coordinated expression of a multitude of virulence factors and genes involved in the infection process [159]. In *P. aeruginosa* just about 10 regulatory RNAs (rgRNAs) were characterized, some of them have been identified as previous missing links in the regulatory pathways that allow bacteria to sense population density, to modulate and modify cell-surface properties, to fine-tune their metabolism during cell growth, and to regulate virulence gene expression [203].

Perhaps the most well characterized system involves the rgRNAs, RsmY and RsmZ involved in the Two Component System response (see B4.3 TC system section) [204]. Another well characterize sRNA is the *Pseudomonas aeruginosa* Crc, an RNA-binding protein that recognizes CA-motifs around the ribosome binding sites of the mRNA of carbon compound catabolism genes. Crc thus represses genes whose products help utilize less preferred carbon sources [205].

The sRNA PhrS plays a role in PQS and pyocyanin expression modulating PQS synthesis during the transition from aerobic to hypoxic conditions [206] [207]. The *phrS* gene is strongly induced by an ANR-dependent promoter when oxygen becomes limiting. This regulation may stabilize PQS production in environments of fluctuating oxygen concentrations. Interestingly, it was shown that PhrS specifically targets a region in the RBS of a small ORF (uof), which is present upstream of PqsR [206]. Translation of pqsR and uof are coupled and PhrS regulates *pqsR* translation by modulating translation of uof [206]. Small RNAs also play a role in regulation of iron uptake and involve base pairing by the sRNAs PrrF1 and PrrF2, which are the *P. aeruginosa* orthologues of *E. coli* RyhB [100] [208]. By a base-paring mechanism, these sRNAs occlude the ribosome binding site of *sodB* (involved in the trichloroacetic acid cycle and anthranilate and cathechol degradation) mRNA and hence inhibit translation initiation [101]. As a result, when iron is replete, the two sRNAs are not expressed and *sodB* translation can proceed. When iron is limiting, the sRNAs are produced and *sodB* mRNA translation is blocked. Thus, PrrF1 and asPrrF2 link carbon metabolism, iron uptake and QS-mediated virulence [100]. Another sRNA PrrH is located in the same locus as PrrF1 and PrrF2. The expression of PrrH is maximal in the stationary phase of growth, similar to PrrF1 and PrrF2, and under iron-deplete conditions [209]. PrrH appears to regulate genes involved in heme, metabolism and homeostasys, an abundant source of iron in the human host [210]. Under conditions of heme starvation, PrrH expression leads to the repression of

achAB and sdhCDAB, which are also targets of the PrrF asRNAs. In addition to these targets, PrrH also represses NirL, a protein involved in biosynthesis of heme.

B4.4 Alternative sigma factors: σ^{22} and σ^{s}

In *Pseudomonas aeruginosa*, as in most bacterial species, the expression of genes is tightly controlled by a repertoire of transcriptional regulators, particularly the so called sigma (σ) factors. In addition to the major housekeeping or vegetative σ factor, σ^{70}, *Pseudomonas aeruginosa* have alternative σ factors that direct the expression of particular subsets of genes [211].

Regulatory factors used to maintain essential functions and cell integrity during environmental fluxes include transcriptional regulators based on alternative sigma factors and two-component regulatory response systems (see B4.1). One alternative sigma factor in *P. aeruginosa* that has been extensively studied is the *algT/algU*-encoded 22kDa σ^{22} (also known as σ^{E}). Among the genes that utilize σ^{22} for expression are those responsible for the production of the exopolysaccharide alginate, an important virulence factor produced in response to envelope stress [212] [213] [214] [215] [216]. During CF lungs colonization, this extracellular polysaccharide preserves *P. aeruginosa* from the consequences of inflammation by inhibiting part of the innate immune response activation and decreasing phagocytosis by neutrophils and macrophages [217]. In general, as the process of infection progresses, a fundamental transition in the gene expression profile occurs in *P. aeruginosa* strain. This switch is mainly governed by the *algU* regulon, which is activated under stress conditions and leads to a coordinated downregulation of central metabolism, motility and virulence, and a simultaneous upregulation of genes affecting membrane permeability, efflux and, as mentioned before, alginate production [218].

The transcriptional factor σ^{22} and its regulators are encoded by the *algTmucABCD* operon.

The cytoplasmic (N-terminal) domain of the anti-sigma factor MucA (Inner membrane protein with the N- and C-terminal regions in the cytoplasm and periplasm), tightly binds σ^{22} and removes it from the cytoplasmic pool of alternative sigma factors competing for core RNA polymerase, thus resulting in low expression of σ^{22} target promoters [219] [220] (see figure B.18). Since alginate genes are regulated by σ^{22}, the loss of MucA-repression results in the constitutive mucoid phenotype. Another negative regulator of σ^{22} was identified just downstream of *mucA* [221] [222] and is named *mucB,* encoding for MucB periplasmic protein

that is able to bind MucA (see figure B.15) [220] [223]. Cell wall stress [224] can activate proteolytic degradation of MucA by regulated intramembrane proteolysis (RIP). RIP is a mechanism that is conserved from bacteria to humans [225] and, responding to environmental conditions such as envelope stress, it is responsible of the cleavage of MucA allowing σ^{22} to initiate transcription of its regulon. Antibiotics that attack peptidoglycan in the cell wall, such as D-cycloserine, result in the activation of σ^{22} and high activity of the alginate biosynthetic operon. D-cycloserine is an analogue of D-alanine, produced by *Streptomyces orchidaceus*, which interferes with bacterial cell wall synthesis by competitively inhibiting L-alanine racemase and D-alanine ligase, thereby impairing peptidoglycan formation necessary for bacterial cell wall synthesis. The therapeutic use of these kind of cell wall-targeting drugs to treat *Pseudomonas* infections may have unintended consequences due to activation of alginate genes that could result in a survival advantage.

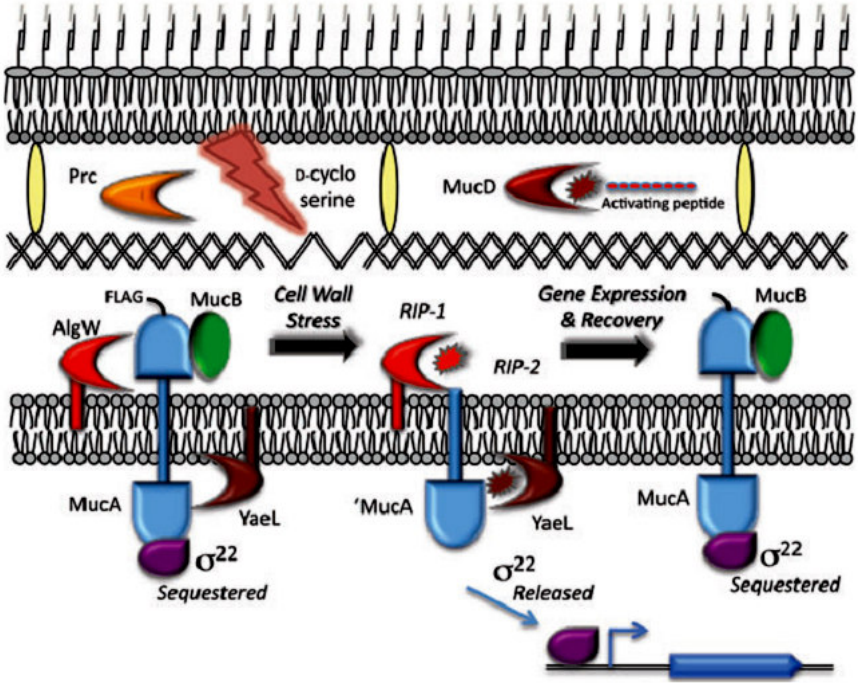

Figure B.18 Model for the RIP degradation of MucA leading to the derepression of σ^{22} activity. Under non-stress conditions σ^{22} activity is low due to its sequestration at the inner membrane by the MucAB complex. In response to cell wall stress brought about by a number of peptidoglycan biosynthesis inhibitors, such as D-cycloserine, the regulated proteolytic destruction of the anti-sigma factor, MucA, is quickly triggered by AlgW cleavage of the MucA C-terminal domain in the periplasm. Periplasmic MucD protease contributes to the destruction of the peptide signal that activates AlgW. YaeL probably cleaves at the MucA-NTD, releasing σ^{22} from post-translational repression and allowing it to complex with core RNA polymerase, thus increasing transcription of target promoters in the σ^{22} regulon, some of which promote recovery from cell wall stress. Since σ^{22}'s negative regulators, MucABD, are also under σ^{22} control, there is a rapid restoration of the σ^{22}–MucAB complex as the cells respond and recover from stress exposure. (Image from Wood et al, 2009, Mol. Micro.)

The σ factor RpoS (also known as σ^{38} or σ^{S}) was originally identified in *Escherichia coli* as an alternative σ factor that activates gene expression in stationary phase when cells are experiencing nutrient starvation. Actually, σ^{S} is considered a master stress response regulator important for adaptation to a variety of conditions including heat, osmotic and oxidative stress [226].

However, due to the differences in the physiology and metabolism, the role of the *rpoS* gene in *Pseudomonas aeruginosa* seems to be different, and linked to production of virulence determinants (extracellular toxins, pyocyanin, biofilms, enhanced resistance antibiotics) and cell-to-cell signalling [227] [228] [229].

Recently RpoS regulon was suggested to be involved in the QS circuit [229] [230], via the modulation of the transcriptional regulators RhlR and LasR [119]. RpoS is effective at the onset of the stationary phase of growth.

It has been reported that RpoS control 800 genes, representing about 14% of the *P. aeruginosa* genome [230]. More than 40% of the genes that were found previously to be controlled by quorum sensing were also controlled by RpoS (conversely, 18% of all RpoS-regulated genes were also regulated by quorum sensing). Most of these genes were induced at the onset of stationary phase, and some were induced later during stationary phase. Thus, RpoS in *P. aeruginosa* seems to be a 'stationary phase' σ factor, as the name had originally implied when proposed for *E. coli* [231] [232]. Moreover, as in *E. coli*, RpoS dependent gene expression in *P. aeruginosa* may also occur in response to many different specific stress conditions [233].

C. Aim of the work

Pseudomonas aeruginosa is endowed with a complex regulatory network that dynamically controls the production of virulence factors thus enabling survival, proliferation/persistence and host damage after infection [203]. In other bacterial pathogens, small RNAs (sRNAs), which are akin to eukaryotic microRNAs in their ability to modulate stability and/or translation of multiple target mRNAs, are now recognized as important components of the regulatory networks involved in the pathogen-host interaction [234]. On the contrary, there is a gap of knowledge on the role of sRNAs in the orchestration of the interaction with host underlying the broad range of diseases caused by *P. aeruginosa.* This project aimed to fill this gap focusing on the functional characterization of novel *P. aeruginosa* sRNAs involved in virulence-associated regulatory networks.

The study of the involvement of sRNAs in pathogen-host interaction regulatory networks is a frontier area of research in the field of the infectious diseases. In fact, sRNAs represent a largely unexploited category of potential targets for virulence-targeting drugs. Illuminating their functional roles in host/pathogen interaction can thus provide the fundamental knowledge for development of next-generation antibiotics.

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D. Preliminary screenings for sRNAs involved in virulence-associated regulatory networks

A recent sRNA sequencing approach in our lab validated 52 novel sRNAs in *P. aeruginosa* [154]. This analysis aimed to compare the sRNA complement of PAO1 and PA14 strains which share the same host range but differ in virulence, PA14 being considerably more virulent in several model organisms. A certain number of the validated sRNAs were conserved in the two strains while others were strain-specific or showed strain-specific expression. To get preliminary hints about the involvement in the regulation of virulence traits, 30 of the 52 validated sRNAs were screened by Northern blot for their responsiveness to infection relevant cues that *P. aeruginosa* can experience in mammalian hosts such as a temperature shift from 20 to 37°C and the oxygen availability that induce the shift from aerobic to anaerobic respiration. The temperature shift was chosen to mimic the change of the *P aeruginosa* life-style from "environmental" to "infection" conditions. Whereas the shift from "aerobic-respiration" to "denitrification" mimics some important virulence-associated behaviors and cues such the host colonization through biofilm formation and the oxygen gradient that is created around cells near the edges of mucous layers in CF lungs [235]. Furthermore, the levels of expression of these 30 sRNAs were assessed in a collection of *P. aeruginosa* mutants in key *quorum sensing* (QS) genes such as *lasR*, *lasI*, *rhlR, rsaL* and *pqsR.* The experimental procedures followed for these approaches are described in the papers attached in Part II. These preliminary screenings generated a list of 15 sRNAs responsive to at least one of the conditions tested. Then, four sRNAs, SPA0012, SPA0021, SPA0084 and SPA0122 were selected from this list for further characterization. SPA0012 and SPA0021 are unique to PA14, while SPA0084 and SPA0122 are conserved in both PAO1 and PA14. All four sRNAs were responsive to both temperature and oxygen availability. Only SPA0084 showed an altered expression pattern in the QS mutants.

D1 Preliminary analysis of SPA0012, SPA0021, SPA0084 and SPA0122

The pathogenesis of *P. aeruginosa* opportunistic infections is multifactorial, as suggested by the large number of cell-associated and extracellular virulence determinants. Some of these determinants help colonization, whereas others facilitate bacterial invasion. Actually, the pathogenesis of *P. aeruginosa* is complex, and the outcome of an infection depends on the virulence factors displayed by the bacteria, as well as the host response. The aim of this preliminary phenotypic analysis was to explore the implication of SPA0012, SPA0021, SPA0084 and SPA0122 in the modulation of virulence factors. The phenotypes assayed were selected in order to assess various *P. aeruginosa* infection phases: initial host colonization, infection and invasion, settlement and dissemination throughout host organism.

D1.1 Generation of sRNA-deletion mutants and construction of sRNA-overexpressing plasmids

The first step of our analysis was the generation of PAO1 and PA14 strains deleted for SPA0012, SPA0021, SPA0084 and SPA0122. Six stable deletion mutants (Tab. D.1) were generated by an enhanced method of marker-less gene replacement [236] with some modifications to adapt it to *P. aeruginosa* [237]. At the same time, to obtain regulatable overexpression, each sRNA was cloned into the shuttle vector pGM931, a pHERD20T [238] derivative carrying the arabinose inducible P_{BAD} promoter and a transcriptional terminator located upstream and downstream, respectively the multiple cloning sites. Chromosomal deletion and arabinose-induced overexpression were validated by Northern blot in each case. All strains that were generated are listed in Table D.1.

Table D.1 PAO1 and PA14 strains generated for the phenotypic analysis

	Strain			
Name	PAO1	PA14	sRNA	sRNA expression level
PA14Δ*spa0084*	-	+	*spa0084*	-
PAO1Δ*spa0084*	+	-	*spa0084*	-
PA14Δ*spa0122*	-	+	*spa0122*	-
PAO1Δ*spa0122*	+	-	*spa0122*	-
PA14Δ*spa0012*	-	+	*spa0012*	-
PA14Δ*spa0021*	-	+	*spa0021*	-
PA14pGM*spa0084*	-	+	*spa0084*	+++
PAO1pGM*spa0084*	+	-	*spa0084*	+++
PA14pGM*spa0122*	-	+	*spa0122*	+++
PAO1pGM*spa0122*	+	-	*spa0122*	+++
PA14pGM931	-	+	-	+
PAOpGM931	+	-	-	+

D1.2 Adhesion and invasion: swarming and swimming motility

Planktonic organism are able to initially colonize a surface by utilizing a flagellum to swim toward the surface and attach via bacterial adhesins, such as type IV pili and flagella [239] [239] [240]. In the particular case of *P. aeruginosa*, it is possible to identify both the swimming, exclusively mediated by the flagellum, and the surface-associated swarming motility, mediated by flagellum and type-IV pili [45]. During the initial colonization phase, the motility could be considered a crucial virulence factor facilitating the *P. aeruginosa* progression towards epithelial contact and its dissemination throughout the host organism [241].

To evaluate the putative SPA0012-, SPA0021-, SPA0084-, SPA0122-mediated modulation of motility, the capacity of PA14 and PAO1 wild type strains to swarm and swim was compared to the swarming and swimming ability of the strains in which the sRNA expression level is unbalanced, i.e. “deletion” *vs* “sRNA overexpression”. As shown in Figure D.1, SPA0021 and SPA0084 appeared to be negative regulators of motility (both swarming and swimming); their deletion resulted in an enhancement of motility compared to the wild type whereas their overexpression was related with decreased motility. Conversely, the capacity of PAO1 and PA14 to swarm and swim appear to be positively regulated by SPA0122 (Figure D.1). For SPA0012, it was possible to observe a slight reduction in the capacity of swarming following

overexpression. Surprisingly, a decrease of swarming was also observed for PA14 *Δspa0012* strain (Fig. D.1).

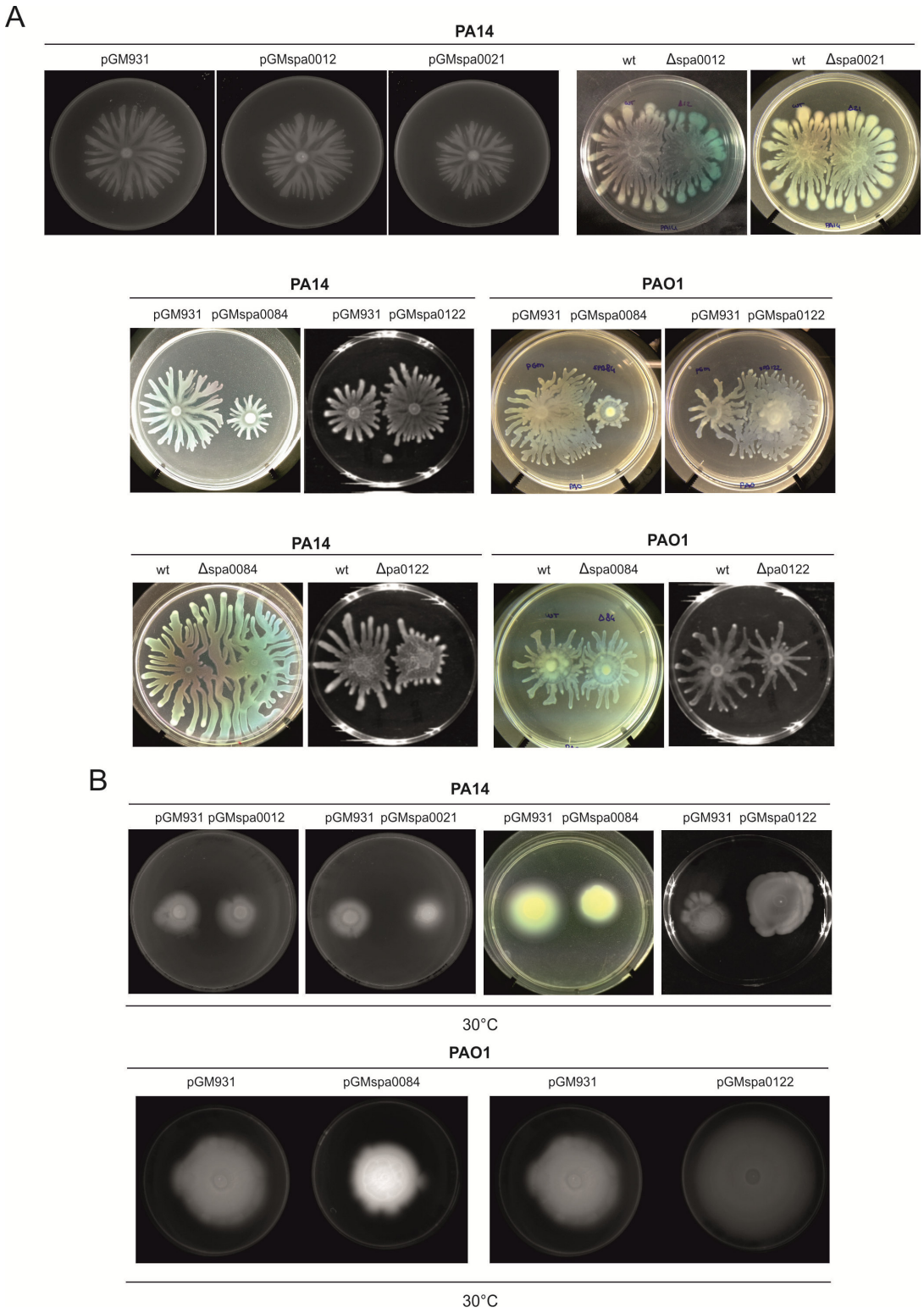

Fig. D.1. Swarming and swimming motility in sRNA-deletion mutants and sRNA-overexpressing strains.
The swarming assays were performed as described previously [242]. For swarming assay 3µl of bacteria culture, diluted at OD_{600}=3, were spotted onto Swarming Plates (5 g/l Bacto Agar, 8 g/l nutrient broth N°2, 28 mM glucose) and grown overnight at 30 or 37°C. For swimming assays, 1.5 µl of culture was spotted onto 'Swimming Plates' (0.3% Bacto Agar Difco added to LB) and grown 18 h at 30°C. **A.** swarming motility assays. **B.** swimming motility assays.

D1.3 Colonization: Biofilm production

Once the bacteria are attached, combinations of specific surface-associated motilities, replication (clonal growth), and/or recruitment of additional planktonic bacteria lead to the formation of aggregates called micro-colonies, that can later lead to mature biofilm development. Biofilm formation and maintenance are tightly regulated in response to environmental cues, conferring to bacteria enhanced resistance against antimicrobial agents and immune defense mechanisms [31]. Surface attachment by *P. aeruginosa* to form micro-colonies has been attributed to type IV pili, flagella, free DNA, alginate and Pel/Psl polysaccharides, although pili, alginate and flagella mutants also form biofilms [243] [244].

Putative SPA0012-, SPA0021-, SPA0084-, SPA0122-mediated modulation of biofilm formation was evaluated comparing PA14 and PAO1 wild type strains with the sRNA-overexpressing counterparts. Biofilm formation was assessed in microtiter plates by Crystal Violet staining assay from cultures grown 48 h in static conditions at 37°C. Adhesion units increased 2.5 fold following SPA0084 overexpression (Figure D.2). These results suggested that SPA0084 could positively regulate both PAO1 and PA14 biofilm formation. On the contrary, adhesion units appeared to decrease twofold following SPA0012 overexpression, suggesting that this sRNA could negatively affect PA14 biofilm formation. Finally, SPA0122 and SPA0021 overexpression did not result in significant changes in biofilm formation.

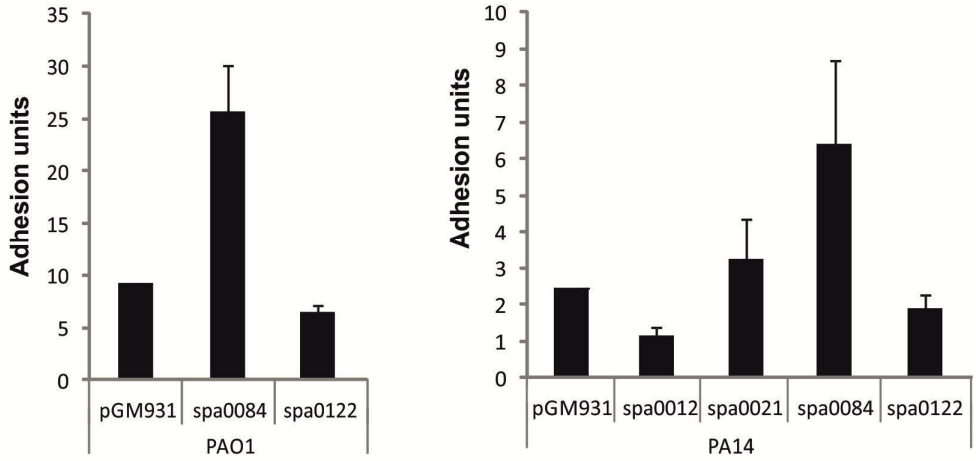

Fig. D.2 Biofilm formation in sRNA-overexpressing strains.
Biofilm formation was assessed in polystyrene 96-well microtiter plates. Cultures were grown in BHI medium supplemented with arabinose for 48h at 37°C. The biofilm production was assessed for PAO1/pGM931 empty vector, PAO1/pGM-*spa0084,* PAO1/pGM-*spa0122*, PA14/pGM931 empty vector, PA14/pGM-*spa0012,* PA14/pGM-*spa0021,* PA14/pGM-*spa0084,* PA14/*pGM-spa0122* strains. "Adhesion units" were calculated using the following formula: [(OD_{600} Crystal Violet stained biofilm cells)/(OD_{600} planktonic cells)].

D1.4 Pyocyanin secretion

Pyocyanin is a redox-active phenazine molecule produced abundantly by *P. aeruginosa* that controls gene expression, community behavior and biofilm thickness [245] [246] [247]. Pyocyanin accepts electrons directly from NADH or NADPH and reduces molecular oxygen, forming reactive oxygen species that can change the redox balance of host cells causing injury and death. Pyocyanin is recognized as a virulence factor in chronic lung infections in cystic fibrosis patients [247]. To test whether SPA0012, SPA0021, SPA0084 and SPA0122 could regulate pyocyanin secretion, PAO1 and PA14 wild type strains were compared with the sRNA-overexpressing counterparts. As shown in Figure D.3, the amounts of secreted pyocyanin increased following SPA0084 overexpression. These results suggested that SPA0084 could positively regulate pyocyanin secretion in both PAO1 and PA14. Surprisingly, SPA0122 overexpression inhibited pyocyanin production in PAO1 and enhanced it in PA14, suggesting a different regulation mechanism for this sRNA in the two strains. Pyocyanin secretion seemed to be unaffected following both SPA0012 and SPA0021 overexpression.

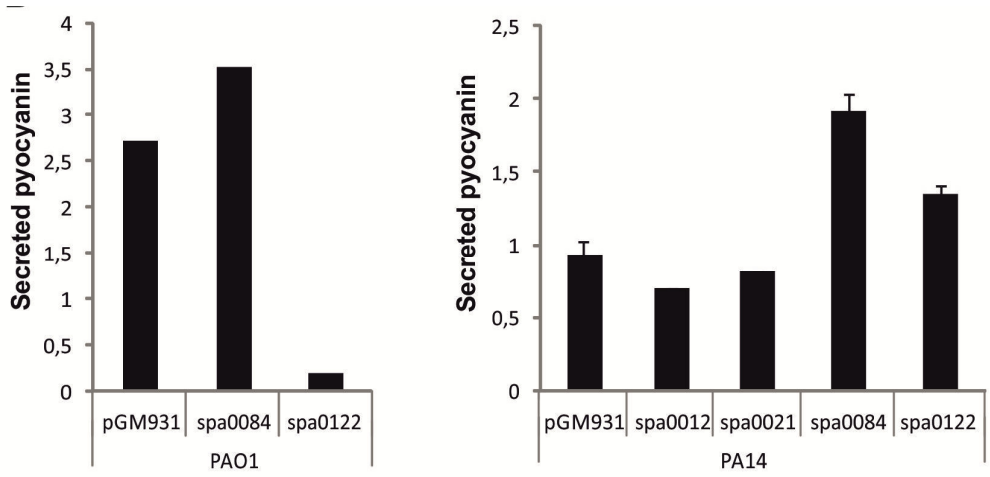

Fig. D.3 Pyocyanin secretion in sRNA-overexpressing strains.
Pyocyanin secretion was assessed in 5 ml of culture supernatants after 18 h of growth at 37°C until OD_{600}≃4.5 in 20 ml of LB supplemented with antibiotics and arabinose. Pyocyanin concentration was measured from supernatants of PAO1/pGM931 empty vector, PAO1/pGM-*spa0084,* PAO1/pGM-*spa0122*, PA14/pGM931 empty vector, PA14/pGM-*spa0012,* PA14/pGM-*spa0021,* PA14/pGM-*spa0084,* PA14/*pGM-spa0122* strains. The amount of pyocyanin was calculated using the following formula: $[(A_{520} \times 2)/A_{600} \times 17.072] \times 0.66$ = µg of pyocyanin per ml, modified from [248].

D1.5 Hemolytic activity

It is well known that *P. aeruginosa* persists in lungs of CF patients despite the heavy accumulation of granulocytes in the airway walls and lumen. This suggests that *P. aeruginosa* may elaborate substances which suppress neutrophil activation enabling it to survive despite inflammatory cell recruitment, i.e. expression of phospholipases C (PLCs, hemolytic PlcHR , non-hemolytic PlcN) [74], [249]. The *P. aeruginosa* PLCs may represent the evolution not only of nutritional enzymes, but also of secreted products which specifically alter the host's immune response. The secretion of phospholipase C PlcHR, by *P. aeruginosa* cells results in a strong hemolytic activity [74]. To test whether SPA0012, SPA0021, SPA0084 and SPA0122 could modulate hemolytic activity, PAO1 and PA14 wild type strains were compared with the sRNA-overexpressing counterparts. To assay hemolytic activity, 2 µl of overnight cultures were spotted on defibrinated sheep blood agar plates and incubated for 24 h. The hemolytic activity was tested by comparison of the halo around and under the cell spots due to complete lysis of red cells. As shown in Fig. D4, SPA0084 and SPA0122 overexpression repressed hemolytic activity both in PAO1 and PA14, while no effects resulted from SPA0012 and SPA0021 overexpression in PA14.

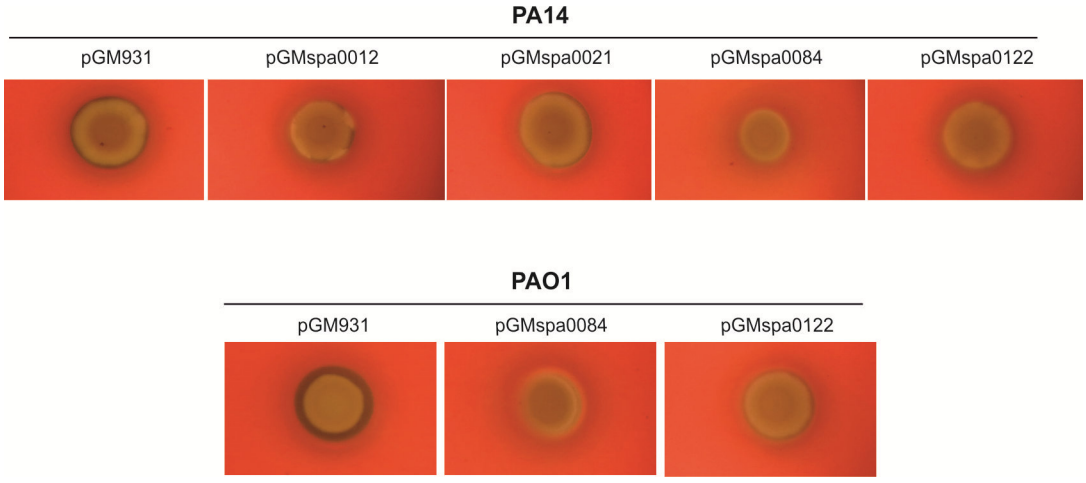

Fig. D.4 Hemolytic activity in sRNA-overexpressing strains.
The hemolytic activity assay was performed on PAO1/pGM931 empty vector, PAO1/pGM-*spa0084,* PAO1/pGM-*spa0122*, PA14/pGM931 empty vector, PA14pGM-*spa0012,* PA14pGM-*spa0021,* PA14pGM-*spa0084,* PA14*pGM-spa0122* strains. Hemolytic activity was assessed by comparison of the halo around spotted colonies grown in aerobic condition 24 h at 37°C on sheep blood agar plates.

D1.6 Pyoverdine secretion

Pyoverdine is the generic name given to a vast family of fluorescent green-yellowish pigments produced by *Pseudomonas* species. When subjected to iron starvation conditions, *P. aeruginosa* synthesize pyoverdines, their primary siderophores, to acquire iron from the extracellular medium [250]. Besides, the role of pyoverdines in *P. aeruginosa* is not only limited to scavenge iron(III) from the bacterial environment. Indeed, these siderophores act as signal molecules for the production of acute virulence factors and are involved in biofilm formation as well [251].

To test whether SPA0012, SPA0021, SPA0084 and SPA0122 could modulate pyovedine secretion, PAO1 and PA14 wild type strains were compared with the sRNA-overexpressing counterparts. As shown in Figure D.5, the amounts of secreted pyoverdine increased following only SPA0084 overexpression. These results suggested that SPA0084 could positively regulate pyoverdine secretion in both PAO1 and PA14.

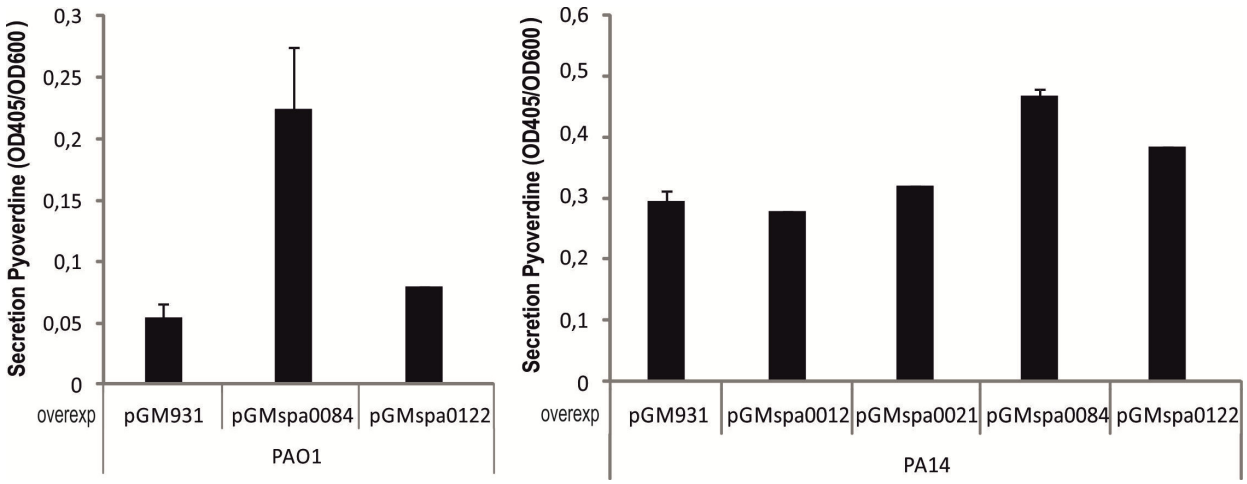

Fig. D.5 Pyoverdine secretion in sRNA-overexpressing strains.
Pyoverdine secretion was assessed in 2 ml of culture supernatants after 18 h of growth at 37°C until $OD_{600}\simeq4.5$ in 20 ml of LB supplemented with antibiotics and arabinose. Pyoverdine concentration was measured from supernatants of PAO1/pGM931 empty vector, PAO1/pGM-*spa0084,* PAO1/pGM-*spa0122*, PA14/pGM931 empty vector, PA14/pGM-*spa0012,* PA14/pGM-*spa0021,* PA14/pGM-*spa0084,* PA14/*pGM-spa0122* strains. The amounts of pyoverdine were determined as ratio between OD_{403} of supernatant and OD_{600} of cell culture.

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PART II

F. Focus on SPA0122 and SPA0084

The aim of the preliminary phenotypic analyses described above was to explore the implication of SPA0012, SPA0021, SPA0084 and SPA0122 in the modulation of virulence factors. As shown in Tab. F.1, SPA0084 and SPA0122 were suggested to be implicated in the modulation of the majority of the virulence traits tested. For this reason, they were further characterized.

Table F.1 Resume of phenotypic assays performed in Part I

Strains		Adhesion and Invasion		Colonization	Toxins		Siderophore
		Swarming	Swimming	Biofilm	Pyocyanine	Hemolysis	Pyoverdine
PAO1	spa0084	↓	↓	↑	↑	↓	↑
	spa0122	↑	↑	=	↓	↓	=
PA14	spa0084	↓	↓	↑	↑	↓	↑
	spa0122	↑	↑	=	=	↓	=
	spa0012	↓	=	↓	=	=	=
	spa0021	↓	↓	=	=	=	=

=, no effect; ↑, suggested positive regulation; ↓, suggested negative regulation

The results obtained during this work of thesis on the further characterization of SPA0122 and SPA0084 are appended below as “published paper” (SPA0122) and “manuscript draft”.

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Post-transcriptional regulation of the virulence-associated enzyme AlgC by the σ^{22}-dependent small RNA ErsA of *Pseudomonas aeruginosa*

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Summary

The small RNA ErsA of *Pseudomonas aeruginosa*, transcribed from the same genomic context of the well-known *Escherichia coli* Spot 42, has been characterized. We show that, different from Spot 42, ErsA is under the transcriptional control of the envelope stress response, which is known to impact the pathogenesis of *P. aeruginosa* through the activity of the alternative sigma factor σ^{22}. The transcriptional responsiveness of ErsA RNA also spans infection-relevant cues that *P. aeruginosa* can experience in mammalian hosts, such as limited iron availability, temperature shifts from environmental to body temperature and reduced oxygen conditions. Another difference between Spot 42 and ErsA is that ErsA does not seem to be involved in the regulation of carbon source catabolism. Instead, our results suggest that ErsA is linked to anabolic functions for the synthesis of exoproducts from sugar precursors. We show that ErsA directly operates in the negative post-transcriptional regulation of the *algC* gene that encodes the virulence-associated enzyme AlgC, which provides sugar precursors for the synthesis of several *P. aeruginosa* polysaccharides. Like ErsA, the activation of *algC* expression is also dependent on σ^{22}. Altogether, our results suggest that ErsA and σ^{22} combine in an incoherent feed-forward loop to fine-tune AlgC enzyme expression.

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Introduction

Small RNAs (sRNAs) have been identified in a wide range of bacteria and have been shown to play critical regulatory roles in many intra- and extracellular processes and pathogenesis (Gottesman and Storz, 2011; Storz *et al*., 2011; Caldelari *et al*., 2013). sRNAs play their regulatory role either by base pairing with mRNAs or by modulating protein activity upon interaction. sRNAs can share extended base complementarity when they are *cis*-encoded on the opposite strand of the target mRNA. Instead, *trans*-encoded sRNAs usually share limited complementarity with mRNA targets and interact with them via short and imperfect base pairing, modulating mRNA translation and/or stability. The RNA-binding protein Hfq has been implicated in the function and/or stability of *trans*-acting sRNAs (De Lay *et al*., 2013) and is required for the efficient stabilization and annealing of sRNAs to some mRNA targets (Vogel and Luisi, 2011).

The expression of most *trans*-acting sRNAs is responsive to environmental stress conditions spanning from iron and oxygen limitation, to oxidative, metabolic/nutrient, pH and cell envelope stresses (Hoe *et al*., 2013). Also, the regulatory networks that *trans*-acting sRNAs orchestrate can be very complex: single targets can be regulated by multiple sRNAs, and a given sRNA can regulate many targets (Storz *et al*., 2011). In bacterial pathogens, *trans*-acting sRNAs have been recognized as key elements of the regulatory networks for the coordinated expression of virulence factors (Caldelari *et al*., 2013).

The bacterium *Pseudomonas aeruginosa*, a major cause of chronic lung infections in cystic fibrosis individuals, as well as of numerous acute infections in injured, severely burned, and immune-compromised patients, produces a large assortment of virulence factors and is endowed with an exquisite ability to form biofilms and to rapidly develop resistance to multiple classes of antibiotics (Wagner *et al*., 2008). The expression of these traits is fine-tuned by a dynamic and intricate regulatory network (Balasubramanian *et al*., 2012), in which more than 50 regulatory proteins play key roles as transcription

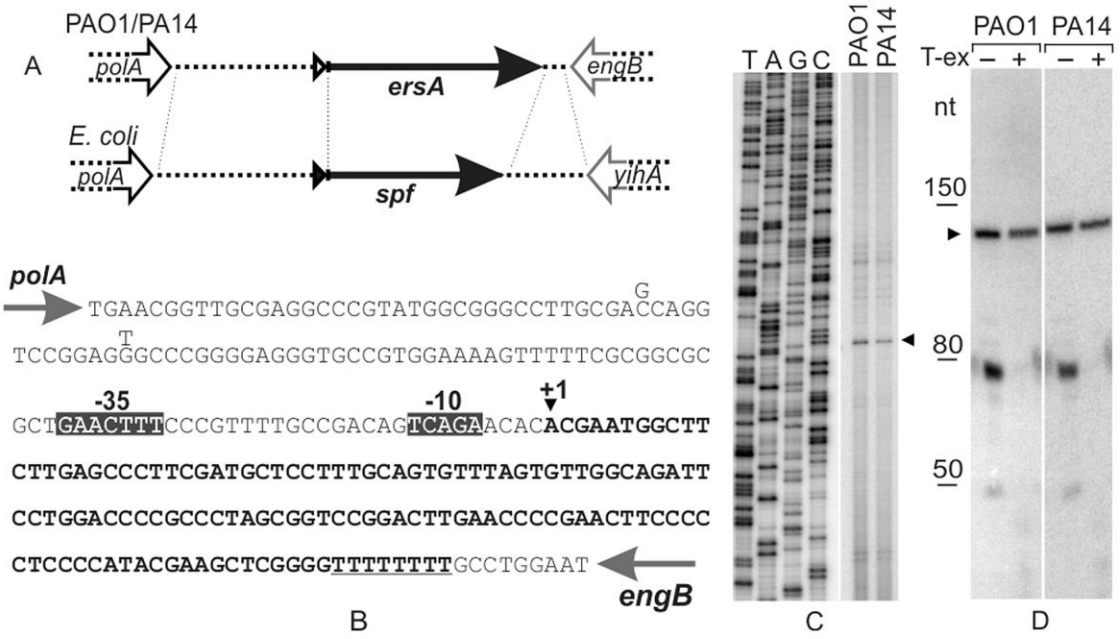

Fig. 1. Genomic context of *E. coli spf* and *P. aeruginosa ersA* genes and analysis of the 5′-end of ErsA RNA.
A. Comparison of the *P. aeruginosa* and *E. coli* intergenic regions between the genes for the highly conserved proteins PolA (DNA polymerase I) and EngB (GTPase of unknown function). The genes *spf* and *ersA* encoding Spot 42 and ErsA, respectively, have a different length and sequence and are transcribed in the same direction. An arrowhead shows the core promoter that is σ^{70}- and σ^{22}-dependent for *spf* and *ersA* respectively.
B. Sequence of the *polA–engB* intergenic region of PAO1 and PA14. At positions with different bases between the two strains, the PA14 alternative is reported above. The *ersA* sequence is in bold. The mapped 5′-end of ErsA is indicated with +1, while the transcription terminator poly-T tail is underlined. Shaded sequences are those matching the –35/–10 consensus sequences GAACTTN$_{16–17}$TCNNA (N: any base; 16–17: base spacing between the –35 and –10 motifs) of the σ^{22}-dependent core promoters (Firoved *et al*., 2002).
C. The 5′-end was mapped by primer extension using 10 μg of total RNA from PAO1 or PA14 extracted at the end of the exponential growth phase and flanked by the TAGC sequencing ladder generated using the same oligo as in primer extension. The arrowhead corresponds to position +1 indicated in (B).
D. Northern blot analysis of ErsA on 10 μg of total RNA extracted as in (C), treated (+) or untreated (–) with terminator 5′-phosphate-dependent exonuclease (T-ex). The arrowhead indicates the primary transcript of ≈ 130 nt.

regulators. However, the number of sRNAs that has been implicated in the regulation of *P. aeruginosa* virulence is low but certainly set to increase (Sonnleitner *et al*., 2012). The most-characterized sRNA-based regulatory system in *P. aeruginosa* include the RNA-binding protein CsrA/RsmA (Lapouge *et al*., 2008; Sonnleitner and Haas, 2011; Romeo *et al*., 2013), which regulate several processes by repressing the translation of target mRNAs. The activity of RsmA is antagonized through sequestration by RsmY and RsmZ, two functionally redundant sRNAs playing a critical role in the switch between acute and chronic infections (Goodman *et al*., 2004; Heurlier *et al*., 2004). Other characterized *P. aeruginosa* sRNAs are CrcZ, the PrrF sRNAs (PrrF1 and PrrF2), PhrS and NrsZ. CrcZ acts as a decoy to abrogate Hfq-mediated translational repression of catabolic genes (Sonnleitner and Blasi, 2014); PrrF1 and PrrF2 are involved in iron homeostasis, central carbon and quorum-sensing regulation (Wilderman *et al*., 2004; Vasil, 2007; Oglesby *et al*., 2008); PhrS regulates the key quorum-sensing regulator PqsR (Sonnleitner and Haas, 2011; Sonnleitner *et al*., 2011); and NrsZ modulates motility by up-regulating translation of *rhlA*, a gene essential for the production of rhamnolipids (Wenner *et al*., 2013).

This study focused on a novel *P. aeruginosa* sRNA that is expressed like the well-characterized *Escherichia coli* sRNA Spot 42 (Beisel and Storz, 2011) from the intergenic region amid the genes for the highly conserved proteins PolA (DNA polymerase I) and EngB (GTPase of unknown function) (Fig. 1A). In the clinical isolate *P. aeruginosa* PAO1 strain, the expression of an sRNA from the same genomic context of the *E. coli* Spot 42 was observed previously (Gottesman *et al*., 2006). In a recent sRNA sequencing approach in *P. aeruginosa* (Ferrara *et al*., 2012), we observed this supposed equivalent of Spot 42 not only in PAO1, but also in the more virulent strain PA14 and gave it the operative name SPA0122. Because Spot 42 and SPA0122 do not share obvious sequence and structural similarities, it is difficult to definitively conclude that they are orthologues.

However, their genes are located in a highly conserved genomic context and have a comparable size and the same transcription direction (Fig. 1A).

Spot 42 plays a broad role in catabolite repression in *E. coli* and is responsible for directly repressing genes involved in both central and secondary metabolism, redox balancing and catabolism of non-preferred carbon sources (Moller *et al*., 2002; Beisel and Storz, 2011). The results presented in this work suggest that *P. aeruginosa* SPA0122 is not linked to the catabolism of carbon sources; instead, SPA0122 is under the control of a critical signal transduction pathway that impacts *P. aeruginosa* pathogenesis. Our results indicate that the transcription of SPA0122 is strictly dependent on the envelope stress-responsive sigma factor σ^{22} (AlgT/U) (Potvin *et al*., 2008), the functional homologue of *E. coli* σ^{E} (Yu *et al*., 1995). *Pseudomonas aeruginosa* σ^{22} 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. σ^{22} controls the *algD* promoter (*PalgD*), which drives the expression of the 12-gene alginate biosynthetic operon (*algD-alg8-alg44-algKEGXLIJFA*) (Sumita and Ohman, 2004). Three additional positive regulatory proteins, AlgB, AlgR and AmrZ, are also under σ^{22} control and required for *PalgD* expression (Wozniak and Ohman, 1994; Ma *et al*., 1998; Baynham *et al*., 2006; Leech *et al*., 2008). Located distant from the alginate biosynthetic operon, another *alg* gene, *algC*, encodes a bifunctional enzyme with phosphomannomutase (PMM) and phosphoglucomutase (PGM) activities; this enzyme provides the sugar precursors for the alginate biosynthetic pathway and for the synthesis of other *P. aeruginosa* polysaccharide exoproducts such as Psl, Pel, lipopolysaccharide (LPS) and rhamnolipids (Deretic *et al*., 1995; Olvera *et al*., 1999; Ma *et al*., 2012). The *algC* gene also belongs to the σ^{22} regulon (Wood and Ohman, 2009), being activated by the AlgR regulator (Lizewski *et al*., 2002). Along these lines, we show that SPA0122 is responsive to envelope stress and is induced by the cell-wall-inhibitory antibiotic D-cycloserine, which is known to activate the regulated intramembrane proteolysis (RIP) of the anti-σ^{22} factor MucA (Wood *et al*., 2006; Wood and Ohman, 2009). Because of this property, we renamed SPA0122 as envelope stress-responsive sRNA A (ErsA). In addition, our analyses indicated that the transcriptional responsiveness of ErsA RNA includes infection-relevant cues that *P. aeruginosa* can experience in mammalian hosts. Finally, we show that ErsA directly operates as a negative post-transcriptional regulator of the *algC* gene in a way dependent on the RNA chaperone Hfq. Together, our results suggest that ErsA and σ^{22} combine as an incoherent feed-forward loop (FFL) to fine-tune AlgC enzyme expression.

Results

ErsA: genomic context and RNA structure

The sRNA ErsA, which was previously termed SPA0122 (Ferrara *et al*., 2012), is transcribed from the same genomic context of the *E. coli* Spot 42 sRNA (Beisel and Storz, 2011) (Fig. 1A). The sequence of the gene-encoding ErsA is identical in PAO1 and PA14 and is highly conserved among the sequenced *P. aeruginosa* strains (Fig. S1). The boundaries of the *ersA* gene in PAO1 and PA14 were defined by primer extension analysis of ErsA RNA (Fig. 1C). The 133 nt between the mapped 5′ end and the poly-T tail of the Rho-independent terminator-like sequence is consistent with the size of the longest transcript detected by Northern analysis (Fig. 1D). Resistance to treatment with terminator 5′-phosphate-dependent exonuclease (Fig. 1D), which preferentially degrades processed transcripts (Sharma *et al*., 2010), indicated that the ≈ 130-nt RNA is a primary transcript. Consequently, we suggest that the 5′-end detected by primer extension is generated by transcription initiation (start site indicated by +1 in Fig. 1B), while the other ≈ 80-nt ErsA transcript that is sensitive to terminator 5′-phosphate-dependent exonuclease can be generated by endonucleolytic cleavage (Fig. 1D). The DNA sequence identity shared by PAO1 and PA14 extends upstream of the + 1 position (Fig. S1), where the –10 and –35 promoter motifs (Fig. 1B) were detected with strong similarity to those recognized by the extracytoplasmic function sigma factor σ^{22} (also known as AlgT and AlgU) (Potvin *et al*., 2008).

The ErsA secondary structure predicted by Mfold (Zuker, 2003) contains three stable stem-loops (SLs), SL1 (nt 2–24), SL2 (nt 59–82) and SL3 (nt 88–126), the latter having a strong resemblance to a Rho-independent transcription terminator. Several alternative unstable secondary structures were predicted by Mfold for the 34-nt U-rich region between SL1 and SL2, suggesting that it was mostly unstructured. ErsA RNA was subjected to structural probing using single-strand specific RNAses T1 and A. As shown in Fig. 2A, the structural mapping defined four (I, II, III and IV) single-stranded RNA domains. I and III coincided with the loops of predicted SL1 and SL2, respectively; IV and II spanned the SL2-3 short connecting region and the 34 nt stretch between SL1 and SL2 respectively (Fig. 2B).

ErsA seems to be unlinked to the catabolism of carbon sources

We constructed and validated PAO1 and PA14 Δ*ersA* derivatives and PAO1 and PA14 strains overexpressing

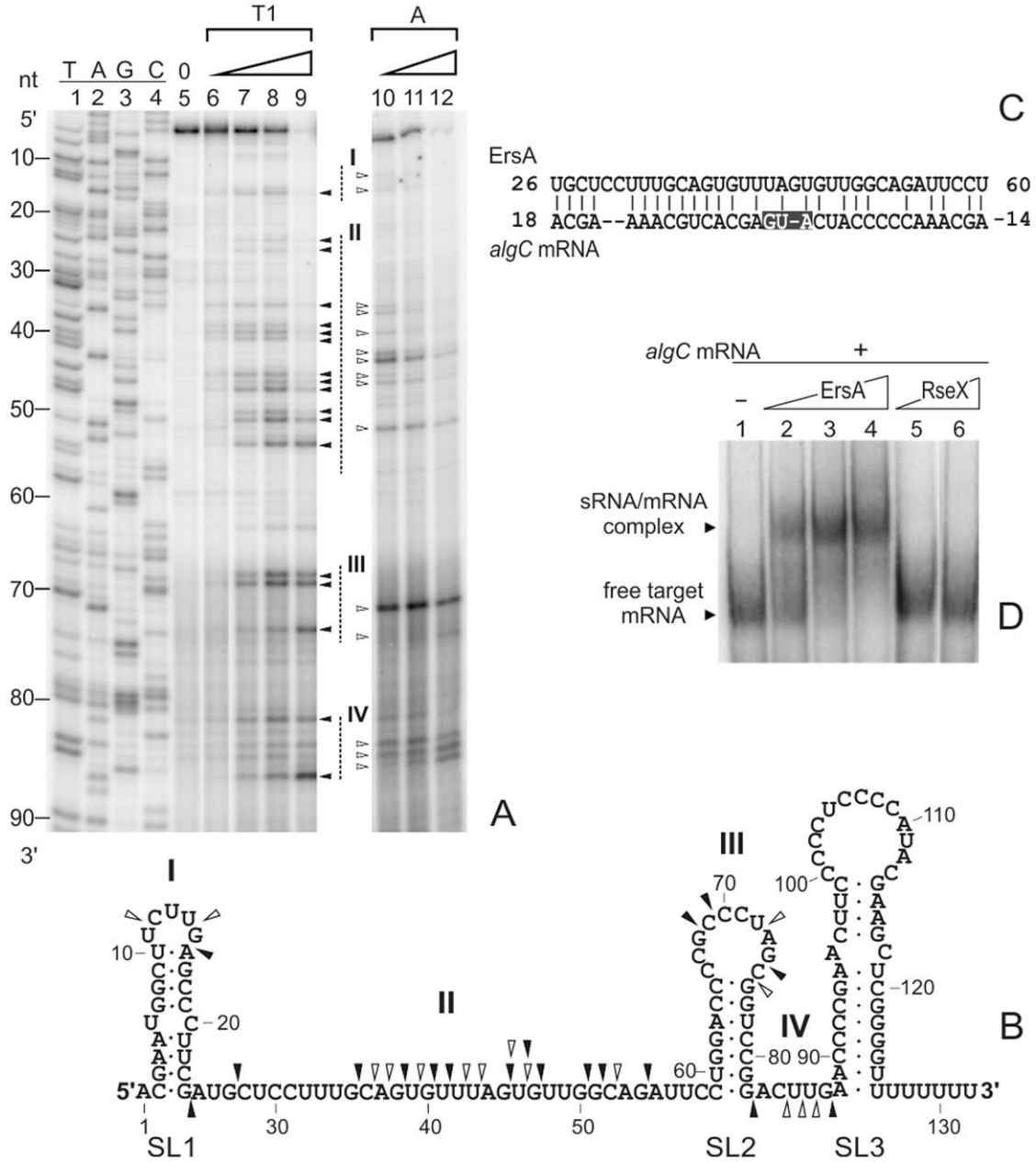

Fig. 2. Secondary structure of ErsA RNA and its interaction *in vitro* with *algC* mRNA.
A. Partial cleavage of ErsA RNA with the single-stranded-specific RNase T1 and RNase A (T1 and A respectively). Digestion reactions were retrotranscribed with a 5′-^{32}P-labelled oligo annealing to the 3′-polyU region and analyzed on sequencing gels. Lanes 1–4: TAGC sequencing ladder generated with the same oligo used for retrotranscription; lane 5: untreated ErsA RNA; lanes 6 and 7: treatment for 15 min with 0.01 and 0.05 U ul^{-1}, respectively; lanes 8 and 9: treatment with 0.1 U ul^{-1} for 5 and 15 min, respectively; lane 10: treatment with 2 pg ul^{-1} for 15 min; lanes 11 and 12: treatment with 10 pg ul^{-1} for 5 and 15 min respectively. Coordinates from the first nucleotide of ErsA RNA are indicated on the left. Clusters of nuclease-susceptible positions (arrowheads) are indicated as I, II, III and IV.
B. Structure of ErsA RNA based on the structural probing with RNase T1 (filled arrowheads) and RNase A (open arrowheads). The I, II, III and IV clusters are indicated as in (A).
C. Prediction by TargetRNA software of the base-pairing interactions between ErsA and *algC* mRNA. Note that ErsA is predicted to interact with *algC* mRNA through the 34-nt U-rich unstructured region between SL1 and SL2 (domain II), while the target region on *algC* mRNA spans the surroundings of the translation start site (highlighted in gray). Sequence coordinates are the same as in (B) for ErsA and refer to the +1 translational start site for *algC* mRNA.
D. *In vitro* interaction between ErsA RNA and *algC* mRNA by an electrophoretic mobility shift assay. Increasing amounts of ErsA 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 radio-labelled *algC* mRNA and loaded onto a native 6% polyacrylamide gel.

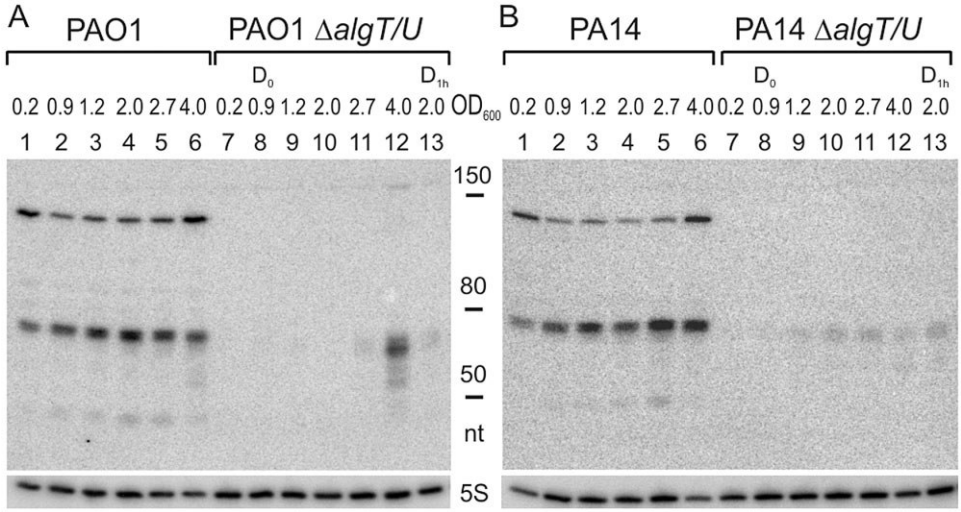

Fig. 3. ErsA expression is induced at the onset of the stationary phase and is dependent on the alternative sigma factor σ^{22}. Wild-type and isogenic markerless *ΔalgT/U* mutant strains of PAO1 (A) and PA14 (B) were inoculated in BHI at an OD_{600} of 0.2 and grown for 20 h at 37°C with agitation. At the indicated OD_{600}, culture samples were taken and processed for total RNA extraction and analysis by Northern blot. For the *ΔalgT/U* mutants, when the cell density reached an OD_{600} of 0.9 (D_0), 400 μg ml^{-1} of D-cycloserine was added to aliquots of cell cultures and incubated for 1 h (D_{1h}).

ErsA from plasmid pGM-*ersA* (Figs S2, S3A and S4A). Neither ErsA deletion nor overexpression influenced the growth patterns of PAO1 and PA14 in the rich medium brain–heart infusion (BHI). By analogy with the regulatory role of *E. coli* Spot 42 in catabolite repression (Beisel and Storz, 2011), we speculated that ErsA could be linked to the regulation of the catabolism of carbon sources in *P. aeruginosa*. In *E. coli*, overexpression of Spot 42 limits growth on specific non-preferred carbon sources (Beisel and Storz, 2011). Therefore, the growth of the PAO1 and PA14 strains overexpressing ErsA was analyzed in M9 minimal medium with preferred (succinate, citrate and malate) and non-preferred (glucose, fructose and glycerol) carbon sources. In addition, we examined in the same set of minimal media the growth of both PAO1 and PA14 and their corresponding Δ*ersA* derivatives. For all tested carbon sources except fructose, neither the lack of ErsA nor its overexpression resulted in alterations in cell growth (Fig. S5). With fructose as the carbon source, ErsA overexpression resulted in enhanced growth of PAO1, the opposite that one would expect in case of ErsA-mediated repression of fructose utilization. This effect seemed to correlate with lower growth rates of PAO1 Δ*ersA* than PAO1 on fructose.

ErsA RNA belongs to the σ²² (AlgT/U) regulon

We aimed to assess the transcriptional wiring of ErsA RNA in both PAO1 and PA14. To begin, we assessed the growth-phase dependency of ErsA expression in the rich medium BHI. As shown in Fig. 3 (lanes 1–6), ErsA displayed identical patterns of expression in PAO1 and PA14. Maximal levels were reached in stationary phase (OD_{600} = 4). Following inoculum with stationary-phase cells diluted to OD_{600} = 0.2, ErsA levels decreased relative to the maximal level in stationary phase and remained constant during exponential phase (OD_{600} = 0.9–2.7). We tested the growth-phase dependency of ErsA expression in cells cultivated in minimal medium with glucose, malate and succinate as carbon sources, and the ErsA expression pattern overlapped with that observed in rich medium (not shown).

Next, we evaluated the dependency of ErsA RNA on σ^{22}, as suggested by the upstream –35/–10 motifs (Fig. 1B), by probing ErsA RNA in Δ*algT/U* strains of both PAO1 and PA14. This analysis showed that ErsA RNA is strictly dependent on σ^{22} because it was undetectable in the *algT/U* mutants (Fig. 3, lanes 7–12). We also evaluated whether ErsA RNA levels could increase in response to cell envelope stressors, such as the cell-wall-inhibitory antibiotic D-cycloserine, which is known to trigger σ^{22} activation (Wood *et al*., 2006; Wood and Ohman, 2009). As shown in Fig. 4A (lanes 2 and 6), D-cycloserine treatment strongly stimulated ErsA RNA expression. To test whether ErsA could also respond to D-cycloserine through σ^{22}-independent mechanisms (Wood and Ohman, 2009), we treated the *algT/U* mutants with D-cycloserine. As shown in Fig. 3 (lane 13), ErsA RNA remained undetectable following treatment with D-cycloserine. Together, these results strongly indicated that ErsA RNA belongs to the σ^{22} regulon.

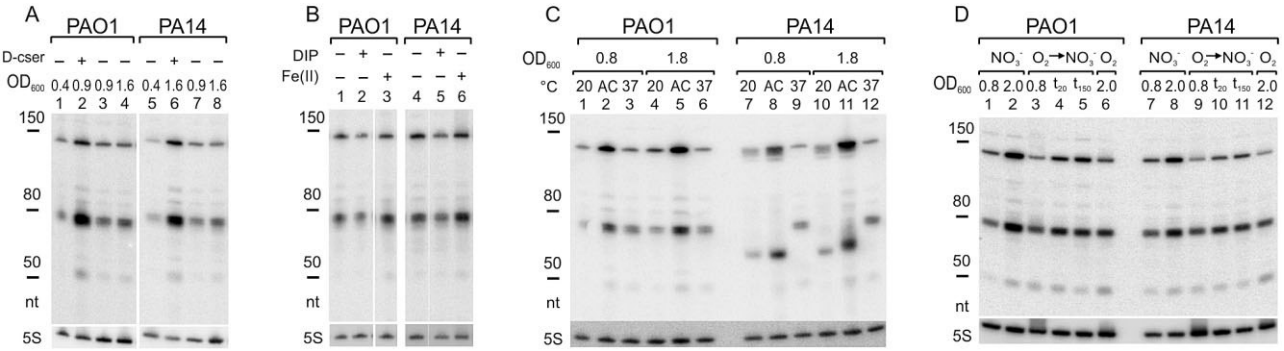

Fig. 4. ErsA expression is induced by cell-wall stress, temperature stress and low availability of oxygen and is repressed in stationary growth phase by low iron availability. Levels of ErsA RNA in:
A. PAO1 and PA14 strains in the absence of D-cycloserine (D-cser; lanes 1, 3, 4, 5, 7 and 8) and following 1 h of incubation, starting from an OD_{600} of 0.4, with 400 and 800 μg ml^{-1} of D-cycloserine in PAO1 and PA14 (lanes 2 and 6) respectively.
B. PAO1 and PA14 strains grown for 20 h in LB (lanes 1 and 4), LB with 200 μM ferrous iron chelator 2,2′-dipyridyl (lanes 2 and 5), and LB with 100 μM $FeCl_3$ (lanes 3 and 6).
C. PAO1 and PA14 grown in BHI at 20°C (lanes 1, 4, 7 and 10), 37°C (lanes 3, 6, 9 and 12) or following 20 min of acclimation (AC) from 20 to 37°C (lanes 2, 5, 8 and 11). Culture samples were taken at middle (OD_{600} of 0.8) and late (OD_{600} of 1.8) exponential growth phase.
D. PAO1 and PA14 grown in BHI anaerobically (NO_3^-; lanes 1, 2, 7 and 8), aerobically (O_2) (lanes 6 and 12) and aerobically until an OD_{600} of 0.8 and then shifted to anaerobic conditions ($O_2 \rightarrow NO_3^-$; lanes 3, 4, 5, 9, 10 and 11). 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.

ErsA responds to physicochemical conditions relevant to host infection and colonization

Our analysis of the transcriptional responsiveness of ErsA RNA spanned also some physicochemical conditions that *P. aeruginosa* can experience in mammalian hosts, including limited iron availability, temperature shifts from environmental to body temperature in the early stages of infection and reduced oxygen conditions, such as those of cystic fibrosis airways. We tested whether the addition of an iron chelator or extra iron could influence the pattern of ErsA RNA expression during growth in the rich medium Luria–Bertani broth (LB). No influence on ErsA RNA expression was observed with low and high iron concentrations during exponential growth. In contrast, iron chelation down-regulated ErsA in stationary phase (Fig. 4B). The addition of extra iron had no effect on ErsA RNA expression in stationary phase.

Temperature sensitivity was tested by probing ErsA 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 shown in Fig. 4C, ErsA levels at 20°C were comparable with those at 37°C for both time points. However, the shift from 20 to 37°C caused a strong up-regulation of ErsA.

We tested whether a decrease in oxygen concentration influenced the ErsA expression pattern during growth in BHI with aeration. ErsA was probed in both PAO1 and PA14 strains grown under anaerobic conditions in BHI with nitrate to sustain anaerobic respiration. In addition, bacterial cells were grown in BHI with aeration until middle-exponential phase ($OD_{600} = 0.8$); then, oxygen was excluded from cultures. ErsA levels were assessed immediately before oxygen exclusion and 20 and 150 min from the start of anaerobic conditions. In both PAO1 and PA14, ErsA levels were higher in anaerobic than aerobic conditions in middle- and late-exponential phase ($OD_{600} = 2$) (Fig. 4D). Remarkably, following the shift from aerobic to anaerobic conditions, ErsA levels increased progressively.

ErsA RNA targets the gene encoding the bifunctional enzyme AlgC

We speculated that ErsA had the potential to act as a *trans*-encoded base-pairing sRNA. To predict target genes, we used the full-length ErsA RNA sequence as input in the web tool TargetRNA (Tjaden, 2008). This tool predicted that the whole ErsA unstructured region II between SL1 and SL2 (Fig. 2B) could be engaged in base pairing with a target mRNA region spanning positions −14 to +18 of the *algC* gene (Fig. 2C) encoding the bifunctional enzyme AlgC. This region is identical in PAO1 and PA14. To assess this predicted ErsA–*algC* mRNA interaction, ErsA RNA and the *algC* mRNA region spanning −75 to +83 were produced *in vitro*, mixed and analyzed on native polyacrylamide gels. As shown in Fig. 2D, the two RNAs specifically formed a complex.

To test the effects of the ErsA–*algC* mRNA interaction, we generated a reporter plasmid carrying the 5′ untranslated region (UTR) and first 28 codons of *algC* mRNA fused with the superfolder variant gene of the green fluorescent protein (sfGFP) under the control of the

heterologous constitutive promoter $P_{LtetO-1}$. GFP activity was assayed in the absence and presence of ErsA overexpression from pGM-*ersA*. In PAO1 (Fig. 5A), there was an approximately twofold reduction in GFP activity with plasmid pGM-*ersA* compared with the empty control vector pGM931. This was a specific effect because overexpression of SPA0167 (see Fig. S3D for overexpression validation from pGM-*spa167*), a *P. aeruginosa* sRNA (Ferrara *et al*., 2012) that is not predicted to interact with *algC* mRNA, did not result in reduced GFP activity (Fig. 5A). Furthermore, spurious outside interactions of ErsA with the GFP open reading frame were controlled using an alternative reported plasmid carrying exclusively the *gfp* gene. As shown in Fig. 5A, ErsA overexpression caused no significant repression of the *gfp* gene alone. These results strongly suggested the negative regulation of ErsA over the *algC* gene. To determine if endogenous ErsA levels could cause similar effects on *algC* expression, the reporter plasmid carrying the *algC::gfp* fusion and the control plasmid pGM931 were transferred into a PAO1 Δ*ersA* mutant strain. As shown in Fig. 5A, *algC::gfp* expression was ≈ 1.8-fold higher in the Δ*ersA* background than in the wild-type (wt) strain.

To test whether the ErsA unstructured region II between SL1 and SL2 was involved in the interaction with *algC* mRNA, we generated a 5′ deletion of 53 nt in ErsA RNA (ErsA Δ1/53; see Fig. S3C for overexpression validation) and overexpressed it in combination with the reporter plasmid carrying *algC::gfp*. As shown in Fig. 5A, ErsA Δ1/53 overexpression resulted in GFP levels that were similar to the empty vector and the control with SPA0167.

We evaluated whether the ErsA-mediated repression of *algC* observed in PAO1 could be influenced by the activity of the RNA chaperone Hfq. To address this, we tested the *algC::gfp* fusion expression in an *hfq* background. As

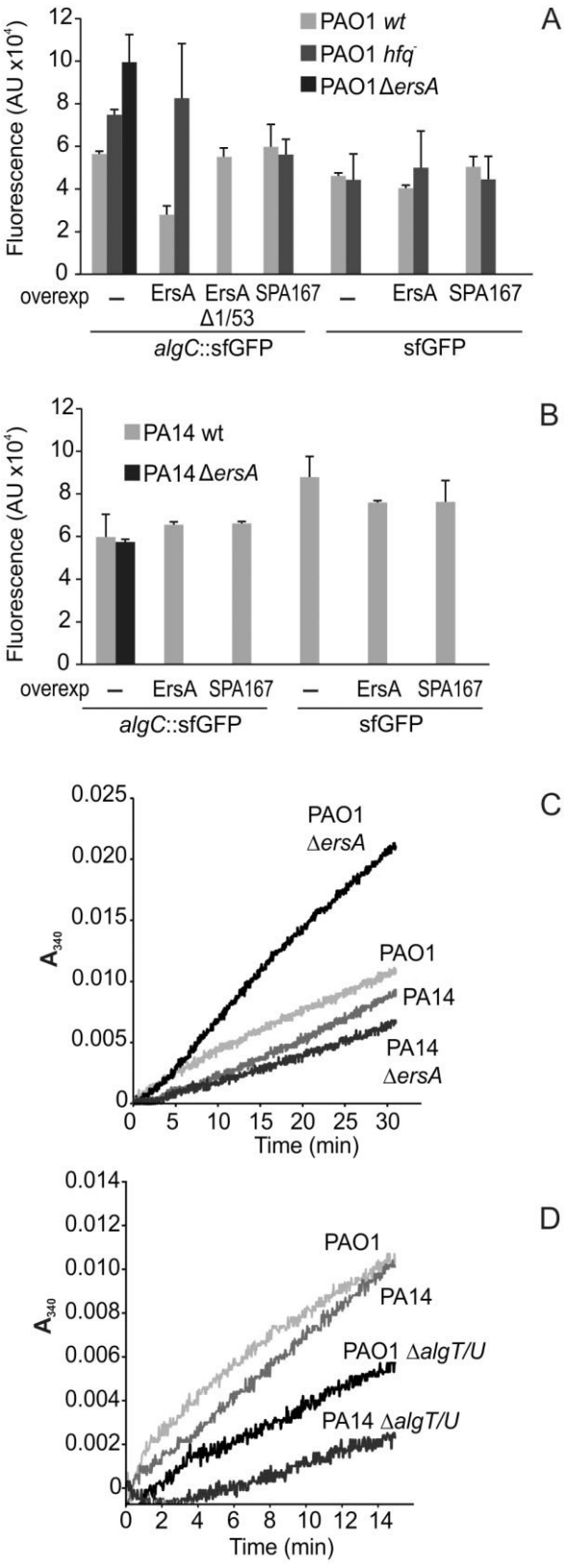

Fig. 5. ErsA negatively regulates the expression of the *algC::gfp* translational fusion in PAO1 and its deletion results in higher levels of AlgC protein in PAO1. Comparison of the fluorescence polarization expressed in arbitrary units (AU) resulting from the translational fusion *algC*::*gfp* or the *gfp* control combined with the control vector pGM931 (–), the plasmids pGM-*ersA* (ErsA), pGM-*ersAΔ1/53* (ErsA Δ1/53) or pGM-*spa0167* (SPA0167) in PAO1, PAO1 *hfq*⁻ and PAO1 Δ*ersA* (A) or in PA14 and PA14 Δ*ersA* (B). (C) Levels of AlgC protein were evaluated by measurements of the AlgC-associated phosphoglucomutase (PGM) activity in 600 μg of protein extract obtained from PAO1 or PA14 cells with wild-type and Δ*ersA* backgrounds. (D) Levels of AlgC protein were evaluated by measurements of the PGM activity in 900 μg of protein extract obtained from PAO1 or PA14 cells with wild-type and Δ*algT/U* backgrounds. PGM activity was determined by measuring the increase in Abs_{340} caused by the reduction of NADP to NADPH due to the activity of glucose 6-phosphate dehydrogenase on glucose-6-phosphate generated by PGM from the substrate glucose-1-phosphate.

shown in Fig. S3B, native ErsA levels are lower in an *hfq* background (lanes 2–4) than wt (lane 1). This correlated with higher *algC::gfp* expression in the PAO1 *hfq* strain (Fig. 5A). However, the overexpression of ErsA at levels similar to those of PAO1 wt (Fig. S3B; lane 1 versus lanes 6 and 7) showed no effects on *algC::gfp* synthesis in the PAO1 *hfq* strain (Fig. 5A). These results suggest that Hfq contributes not only to ErsA levels, but also to ErsA/*algC* mRNA interaction.

Surprisingly, the behaviour of the *algC::gfp* fusion in PA14 was different. ErsA overexpression did not significantly repress GFP activity, and the *algC::gfp* expression was comparable in Δ*ersA* and wt backgrounds (Fig. 5B; see Fig. S4A and B for overexpression validation of ErsA and SPA0167 in PA14).

Finally, we checked whether ErsA targeting of *algC* influenced *algC* mRNA levels by quantitative reverse transcription polymerase chain reaction analysis (RT-PCR) of wt and Δ*ersA* in PAO1 and PA14. No significant differences in *algC* mRNA levels were observed in Δ*ersA* and wt backgrounds.

AlgC expression is deregulated in an ΔersA *background*

The results presented above strongly suggested that ErsA negatively influences the translation of *algC* mRNA. This effect was expected to impact the levels of AlgC protein. To test this, we evaluated the effects of ErsA deletion by measuring the AlgC-associated PGM activity in crude protein extracts from cells with wt and Δ*ersA* backgrounds. As shown in Fig. 5C, we observed a twofold increase of PGM activity in PAO1 Δ*ersA* extracts relative the wt counterpart. PGM assays were also performed in PA14 and PA14 Δ*ersA* strains. A slight decrease of PGM activity was observed in PA14 Δ*ersA*. Finally, we evaluated the effects of σ^{22} deletion on AlgC levels by measuring PGM activity in *ΔalgT/U* strains of PAO1 and PA14. As expected, the PGM activity in *ΔalgT/U* strains was significantly reduced (Fig. 5D).

Discussion

We studied the novel *P. aeruginosa* sRNA ErsA, which is transcribed from the intergenic region between the genes for the highly conserved proteins PolA (DNA polymerase I) and EngB (GTPase of unknown function) as the *E. coli* sRNA Spot 42. Because ErsA does not share obvious sequence or structural similarities with Spot 42, it is unknown if they originated from a common or an otherwise unrelated ancestor. The results presented in this study strongly suggest that ErsA behaves differently from Spot 42. The first striking dissimilarity consists of their transcriptional regulation. Spot 42 is under the control of a promoter that is dependent on the house-keeping σ^{70} and is repressed by the cyclic adenosine monophosphate receptor protein CRP (Polayes *et al*., 1988), while ErsA is strictly dependent on σ^{22}, the major player in the envelope stress-signalling pathway like *E. coli* σ^{E}. Remarkably, no σ^{22}-dependent sRNA was previously known in *P. aeruginosa*. Even though the σ^{22} dependency of ErsA is here shown specifically in PAO1 and PA14 strains, we suggest this role to occur broadly in *Pseudomonas*. In fact, we found high sequence conservation of the −35/−10 motifs recognized by σ^{22} upstream of the ErsA transcription start site in all sequenced *P. aeruginosa* strains, other *Pseudomonas* species, and *Azotobacter vinelandii* (Fig. S1). In a recent report (Park *et al*., 2014), the ErsA counterpart in *P. syringae* was shown to be under control of σ^{22}.

Second, our results indicate that the transcriptional control of Spot 42 and ErsA reflects a functional divergence. Unlike Spot 42, ErsA does not seem to be involved in the regulation of carbon source catabolism but is linked to anabolic functions for the synthesis of exoproducts from sugar precursors. Specifically, we show that in PAO1 strain ErsA directly exerts a negative post-transcriptional regulation on the virulence-associated *algC* gene for the bifunctional enzyme AlgC (Goldberg *et al*., 1995). AlgC plays a central role in the exopolysaccharide biosynthesis because it generates the sugar precursors mannose-1-phosphate (Man1P) and glucose-1-phosphate (Glu1P) for the synthesis of alginate, Pel, Psl, LPS and rhamnolipids, key components of the biofilm matrix (Mann and Wozniak, 2012). The effects that we observed during PAO1 growth on fructose when ErsA is overexpressed or deleted can be explained as follows. In *Pseudomonas*, fructose is the only carbohydrate that is known to enter the cell through a phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) that couples fructose transport to its phosphorylation to fructose-1-phosphate (Fru1P) (Rojo, 2010). Fru1P is then funnelled to the Entner–Doudoroff sugar metabolic pathway via 6-phosphogluconate. Two key anabolic precursors for exopolysaccharides can form at this stage, fructose 6-phosphate (Fru6P) and glucose-6-phospate (Glu6P). Glu6P is a direct substrate of AlgC PGM activity for the production of Glu1P, while Fru6P is converted to mannose-6-phospate (Man6P) prior to becoming the substrate of AlgC PMM activity that generates Man1P. Therefore, AlgC may be placed at the crossroad between catabolic and anabolic pathways that stem from fructose uptake via the PTS system. Down-regulation of AlgC following ErsA overexpression might favour fructose catabolism at the expense of polysaccharide biosynthesis and thus enhance growth. On the other way round, up-regulation of AlgC following ErsA deletion is expected to redirect precursors toward

exopolysaccharide biosynthesis and in turn to disadvantage growth on fructose.

The biosynthetic pathways for the exopolysaccharides alginate, Psl, Pel and LPS were suggested to compete for the common sugar precursors provided by AlgC (Ma *et al*., 2012). In this scenario, AlgC was proposed to be a checkpoint enzyme, whose expression level/activity influences the sugar precursor pool, ultimately coordinating the total amount of exopolysaccharide biosynthesis (Ma *et al*., 2012). The regulation of *algC* described so far is at the transcriptional level in the context of the σ^{22} network. Actually, *algC* is positively regulated by the transcription factor AlgR whose expression is σ^{22} dependent (Martin *et al*., 1994; Wozniak and Ohman, 1994). Consistent with being a member of the σ^{22} regulon, *algC* is up-regulated following cell-wall stress induced by D-cycloserine (Wood and Ohman, 2009). The role of ErsA that we show here is an additional σ^{22}-dependent regulation converging on AlgC, but it is exerted negatively at the post-transcriptional level.

One regulatory motif recurring frequently in bacteria is the FFL (Alon, 2007). The prototype of this motif has three elements, X, Y and Z, in which X and Y co-regulate Z and X regulates Y. Therefore, an FFL has two regulatory arms converging on Z: a direct arm where X regulates Z and an indirect arm where X regulates Z through Y. In principle, both arms could be indirect when a fourth element W is regulated by X and regulates Z. When the two arms act in opposition on Z, the FFL is termed incoherent (I-FFL). We propose that the expression of AlgC (Z) is subjected to envelope stress regulation via an I-FFL involving σ^{22} (X), from which two indirect opposite arms originate (Fig. S6). The activating arm is mediated by AlgR (Y) at the transcriptional level, while the repressing arm is mediated by ErsA (W) at the post-transcriptional level.

Besides being triggered by cell-wall stress, our results indicate that ErsA responds to infection-relevant cues that *P. aeruginosa* can experience in mammalian hosts. A large body of data support the notion that *P. aeruginosa* σ^{22} activation depends on a RIP mechanism homologous to that of *E. coli* σ^{E} (Qiu *et al*., 2007; Wood and Ohman, 2009; Damron and Goldberg, 2012). RIP can be triggered by external stresses such as heat leading to the accumulation of misfolded periplasmic and outer membrane proteins (Walsh *et al*., 2003; Qiu *et al*., 2007). The up-regulation of ErsA following the shift from 20 to 37°C could result from heat-triggered RIP that, in turn, activates σ^{22}. Instead, we suggest that the responses of ErsA to iron depletion and to low-oxygen conditions are independent of this mechanism. In this respect, we speculate that ErsA belongs to the regulon of the ferric uptake regulator Fur (Cornelis *et al*., 2009) and, as PhrS (Sonnleitner and Haas, 2011; Sonnleitner *et al*., 2011), to the regulon of the oxygen-sensing regulator ANR (Zimmermann *et al*., 1991).

These ErsA regulations are expected to contribute to the fine modulation of *algC* in the context of the above I-FFL led by σ^{22} (Fig. 6). ErsA-mediated regulation of *algC* could impact the multistage process of biofilm development during infection. Every stage of biofilm formation includes modulation of the extracellular matrix. In this context, we speculate that the fine-tuned regulation exerted by ErsA on AlgC could influence the dynamics of exopolysaccharide biosynthesis underlying the development of biofilm matrix.

We suggest that the repressive role of ErsA on *algC* occurs prevalently at the translational level. Actually, we observed no significant differences in *algC* mRNA levels between Δ*ersA* and wt backgrounds. Therefore, it is conceivable that the higher AlgC levels in the Δ*ersA* background result from a better translatability of *algC* mRNA. This is consistent with the view that ErsA pairs with the region of the *algC* mRNA that includes RBS and the first six codons, a location expected to hinder mRNA translation as in the majority of characterized cases.

We also showed that the repressive role of ErsA on *algC* is missed in the absence of Hfq protein. This strongly suggests that the interaction between ErsA and *algC* mRNA is mediated by Hfq. This interaction involves a 34-nt internal ErsA region that appears unstructured. This agrees with evidence that the use of unstructured regions for base pairing is one of the requirements for target selection by Hfq-binding sRNAs (Beisel *et al*., 2012). The 34-nt unstructured region is significantly U-rich compared with the rest of the ErsA sequence. It might include U-rich site(s) required for stable binding to Hfq and subsequent sRNA-mediated regulation as in other characterized cases (De Lay *et al*., 2013).

Finally, our analysis was conducted in PAO1 and PA14, which share the same host range but differ in virulence, PA14 being considerably more virulent in several model organisms. As highlighted in other PAO1 versus PA14 comparative studies, the strain-specific background can influence the regulatory outputs (Mikkelsen *et al*., 2011). Identical features of ErsA were observed in both strains. Despite this, the ErsA-mediated regulation of *algC* seems different in the two strains. We suggest that additional regulatory factors can be involved in the ErsA-mediated regulation of *algC* mRNA. Such elements might be differentially expressed in the two strains. This could explain the dissimilar response of *algC* to the ErsA regulatory activity.

In summary, we characterized the first *P. aeruginosa* sRNA that appears to be embedded in the envelope stress response, a critical signal transduction pathway for bacteria that, in *P. aeruginosa*, also impacts pathogenesis. It is conceivable that ErsA has a broad set of target

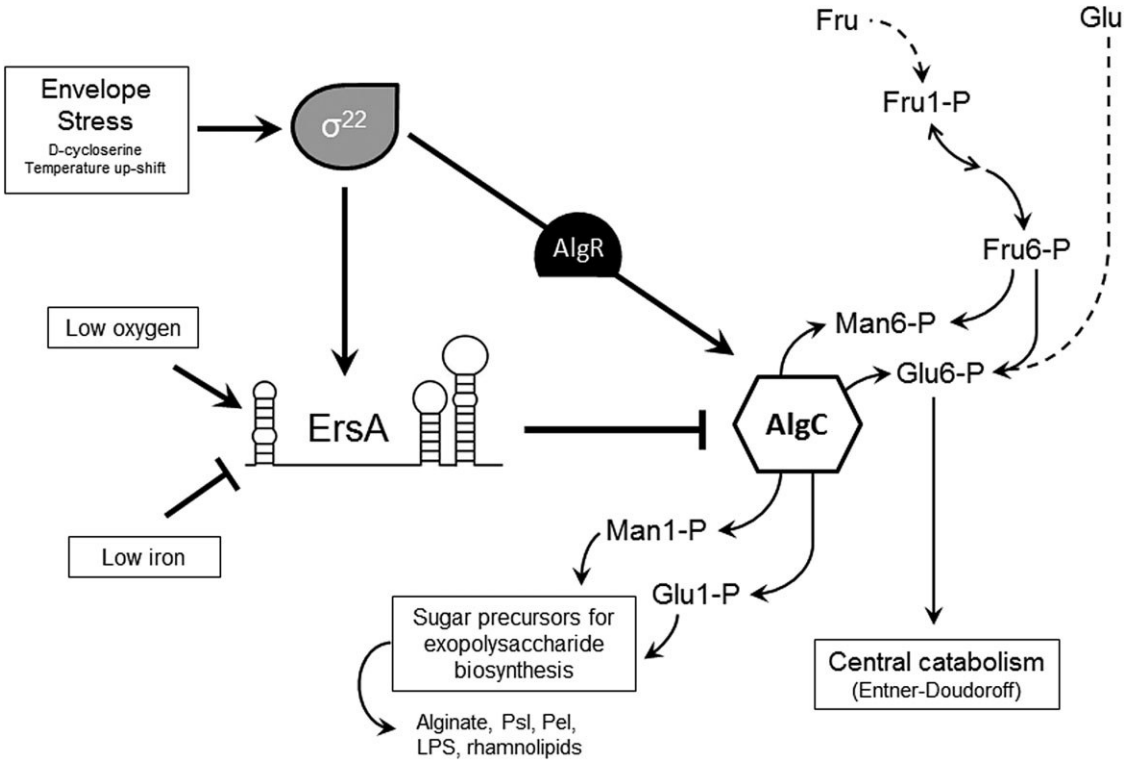

Fig. 6. Schematic representation of the regulatory loop for the ErsA-mediated modulation of AlgC. The incoherent feed-forward loop involved in AlgC regulation (Fig. S6) is depicted along with AlgC role in the generation of sugar precursors for exopolysaccharide biosynthesis. Our data showed that either envelope stress, through σ^{22}, or low oxygen concentrations induce ErsA expression while iron unavailability leads to opposite effects. As an example, fructose (Fru) and glucose (Glu) are indicated as carbon and energy sources (dashed arrows). AlgC, endowed with phosphomannomutase (PMM) and phosphoglucomutase (PGM) activities, is placed at the crossroad between anabolic pathways for exopolysaccharide biosynthesis and the central catabolism represented by the Entner–Doudoroff sugar metabolic pathway via 6-phosphogluconate (not indicated). The negative ErsA-mediated regulation on AlgC acts in opposition to that exerted by the transcription regulator AlgR whose expression is also σ^{22}-dependent. AlgC was proposed to be a checkpoint enzyme, whose expression level/activity influences the sugar precursor pool, thus ultimately coordinating the total amount of biosynthesis of *P. aeruginosa* polysaccharide exoproducts such as Psl, Pel, lipopolysaccharide (LPS) and rhamnolipids (Ma *et al*., 2012). The opposite σ^{22}-dependent regulatory signals converging on AlgC and establishing the incoherent feed-forward loop are thought to fine-tune the AlgC levels (e.g. favouring steady-state accumulation in a given condition) and to govern the coordination of exopolysaccharide biosynthesis. Network motifs configuring as incoherent feed-forward loops were also suggested to have the dynamical function of response accelerators (Alon, 2007). Therefore, steady-state levels of AlgC could be reached rapidly following envelope stress. Fru1-P, fructose-1-phosphate; Fru6-P, fructose 6-phosphate; Glu1-P, glucose-1-phospate Glu6-P, glucose-6-phospate; Man1-P, mannose-1-phospate; Man6-P, mannose-6-phospate.

genes whose functions go beyond the biosynthesis of exopolysaccharides. The role of ErsA in the I-FFL for *algC* gene regulation is reminiscent of that reported for the two *E. coli* sRNAs MicA and RybB at level of the σ^E regulon, i.e. to endow a transcriptional factor restricted to gene activation (σ^E) with the opposite repression function (Gogol *et al*., 2011). In this model, the σ^E response configures as a multi-output I-FFL with protein-mediated activator and sRNA-mediated repressor arms that act primarily to survey and maintain cell envelope homeostasis (Gogol *et al*., 2011). ErsA might be part of a sRNA-mediated repressor arm of the *P. aeruginosa* σ^{22} response to survey and maintain cell envelope homeostasis (Wood and Ohman, 2012). The higher sensitivity to D-cycloserine of both PAO1 and PA14 Δ*ersA* strains that we detected in preliminary experiments would suggest this role of ErsA.

Finally, the set of ErsA targets might include virulence-associated genes such as *algC*. In this respect, ErsA is expected to resemble another *polA*-linked sRNA, sX13 of *Xanthomonas campestris pv. vesicatoria* 85-10, which is a multifaceted regulator of virulence (Schmidtke *et al*., 2013).

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* strains were routinely grown in LB at 37°C. *Pseudomonas aeruginosa* strains were grown at 37°C in BHI-rich medium or in LB at 120 r.p.m. unless otherwise indicated. Carbenicillin and gentamicin were added at 300 and 20 μg ml^{-1}, respectively, unless

otherwise indicated. To induce P_{BAD} in plasmid pGM931, arabinose was added to BHI medium at a final concentration of 10 mM. To generate envelope stress, D-cycloserine was added at a final concentration of 400 and 800 μg ml^{-1} for PAO1 and PA14 respectively.

For *P. aeruginosa* growth in minimal medium, overnight cultures in LB were washed twice and inoculated to an optical density at 595 nm (OD_{595}) of 0.1 in M9 minimal medium supplemented with a carbon source at the following final concentration: 10 mM arabinose, 7 mM citrate, 10 mM fructose, 40 mM glycerol, 11 mM glucose, 20 mM malate and 60 mM succinate. Aliquots (200 μl) of inoculated medium were distributed in triplicate in a 96-Well Cellstar® Suspension Culture Plate (Greiner Bio-One) and incubated for 26 h in a Sunrise microplate reader (Tecan Group) at 37°C with constant orbital shaking and real-time OD_{595} measurements every 15 min.

Anaerobic batch cultivations of *P. aeruginosa* were performed in a Biostat-Q system bioreactor (B-Braun) at 37°C with a stirring speed of 500 r.p.m. in a working volume of 800 ml of BHI supplemented with 100 mM KNO_3 to allow anaerobic respiration. Oxygen was excluded from the bioreactor by flushing nitrogen (< 3 ppm O_2) with a flow of 0.1–0.31 l min^{-1}. Norprene tubes were used to minimize the diffusion of oxygen into the bioreactor. The dissolved oxygen concentration was measured by a Mettler Toledo polarographic oxygen probe. To assess the ErsA response to a shift from aerobic to anaerobic conditions, bacterial cells were grown aerobically in BHI supplemented with 100 mM KNO_3 with a stirring speed of 500 r.p.m. until an OD_{600} of 0.8. Then, oxygen was excluded from the bioreactor by flushing nitrogen, and cultivation continued until stationary phase.

Plasmid construction and mutant generation

To construct plasmids pGM-*ersA* and pGM-*spa0167*, the *ersA* and *spa0167* genes were amplified by polymerase chain reaction (PCR) from PAO1 genomic DNA with oligos 12 and 13 and oligos 14 and 15, respectively; to construct pGM-*ersAΔ1/53*, the *ersAΔ1/53* gene was amplified with oligos 40 and 13. The PCR products were digested with *Nco*I-*Pst*I and cloned into the shuttle vector pGM931, a pHERD20T (Qiu *et al.*, 2008) derivative carrying a transcriptional terminator downstream of the multiple cloning site.

Plasmid pBBR1-*algC::gfp*, expressing the *algC::gfp* fusion protein under the control of $P_{LtetO-1}$, was constructed as follows. A DNA fragment including the 246-nt UTR (Zielinski *et al.*, 1991) and the first 28 codons of the *algC* open reading frame was amplified with oligos 16 and 17, digested with *Nsi*I-*Nhe*I, and cloned into the sfGFP reporter vector pXG10-SF, giving rise to plasmid pXG10-*algC::gfp*. The DNA fragment spanning $P_{LtetO-1}$ and the *algC::gfp* translational fusion was amplified from pXG10-*algC::gfp* with oligos 18 and 19, digested with *Cla*I-*Xba*I and cloned into the low-copy number shuttle vector pBBR1-MCS5. To construct the control reporter plasmid pBBR1-*gfp*, a DNA fragment spanning $P_{LtetO-1}$ and the gene for sfGFP was amplified from pXG1-SF with oligos 18 and 19, digested with *Cla*I-*Xba*I and cloned into pBBR1-MCS5.

Pseudomonas aeruginosa mutants were generated by an enhanced method of marker-less gene replacement (Martinez-Garcia and de Lorenzo, 2011) with some modifications to adapt it to *P. aeruginosa*. PAO1 and PA14 mutants in the *algT/U* gene (PA0762 and PA14_54430 in PAO1 and PA14 respectively) were obtained by allelic exchange with an in frame deletion of 176/194 *algT/U* codons as follows. The targeted sequence TS1 region spanning the left 390-bp flanking sequence and the first three codons of *algT/U* was amplified with oligos 33 and 34. The targeted sequence TS2 region spanning the last 15 codons and right 654-bp flanking sequence of *algT/U* was amplified with oligos 35 and 36. Overlap extension (SOE)-PCR (Horton *et al.*, 1989) with oligos 33 and 36 joined TS1 and TS2. The joined TS1-TS2 DNA fragments were digested with *Eco*RI-*Bam*I and cloned in *E. coli* CC118 *λpir* into the delivery vector pSEVA612S, giving rise to pSEVApao1-*ΔalgT/U* and pSEVApa14-*ΔalgT/U*.

PAO1 and PA14 *ΔersA* mutants were generated by allelic exchange with a deletion from −46 to +82 with respect to the *ersA* transcription start site as follows. The TS2 region spanning the left 646-bp flanking sequence of *ersA* was amplified with oligos 29 and 30. The TS1 region spanning the last 50 nt and right 541-bp flanking sequence of *ersA* was amplified with oligos 27 and 28. PCR was performed from both PAO1 and PA14 genomic DNA. SOE-PCR with oligos 30 and 27 was used to join TS1 and TS2. The joined TS1-TS2 DNA fragments were digested with *Sac*I-*Bam*I and cloned in CC118 *λpir* into the poly-linker site of pSEVA612S, giving rise to pSEVApao1-*ΔersA* and pSEVApa14-*ΔersA*.

For both the *algT/U* and *ersA* allelic exchanges, the TS1-TS2–inserted pSEVApao1 and pSEVApa14 derivatives were transferred from *E. coli* CC118 *λpir* to PAO1 and PA14, respectively, with the assistance of the helper *E. coli* strain HB101(pRK600) in a conjugative triparental mating (de Lorenzo and Timmis, 1994). 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. The co-integration process introduces into the recipient chromosome two sites for the I-SceI endonuclease flanking the multiple cloning site of pSEVA612S. Transient I-SceI endonuclease expression from pSW-I stimulates homologous recombination at the integration site of the pSEVA612S derivative and strongly enhances the resolution leading to allelic exchange (Martinez-Garcia and de Lorenzo, 2011). Plasmid pSW-1 was transferred from *E. coli* DH5α to *P. aeruginosa* clones bearing genomic co-integrates of pSEVA612S derivatives 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 37/38 and 31/32 for *algT/U* and *ersA* respectively.

All plasmid constructs and deletion mutants were checked by sequencing with oligos indicated in Table S2.

RNA isolation and analysis

Total RNA was prepared as described previously (Ferrara *et al.*, 2012) from 2 to 10 ml of bacterial cultures. The quality and concentration of the extracted RNA were assessed by a Biospectrometer (Eppendorf). Northern blot analyses were performed as described previously (Ferrara *et al.*, 2012). DNA oligonucleotide probes (Table S2) were 5′-end–labelled with (γ-^{32}P) ATP and T4 polynucleotide kinase (Promega) according to manufacturer's instruction. Oligos 1 and 2 were used to probe ErsA and 5S RNA respectively.

Primer extension analysis to identify ErsA 5′ ends was performed as follows. An annealing mix (10 µl) containing 10 µg of total RNA, 1 U ul^{-1} of RNasin (Promega), 0.5 pmole radio-labelled oligo 5, and 1 × ss-hybridization buffer [300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetraacetic acid (EDTA)] was denatured at 80°C for 4 min and annealed for 2 h at 50°C. Prewarmed 1.25 × reverse transcription-buffer (40 µl; 1.25 mM each dNTP, 12.5 mM dithiothreitol, 12.5 mM Tris-HCl, pH 8, 7.5 mM $MgCl_2$), 5 U RNasin (Promega) and 10 U Superscript III Reverse Transcriptase (Invitrogen) were added to the annealing mix, and reactions were further incubated for 30 min at 55°C. RNA was hydrolyzed with NaOH for 1 h at 55°C. Reactions were neutralized with HCl, precipitated and dissolved in 6 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF).

For structural characterization, 450 ng of synthesized ErsA RNA was used for enzymatic probing with RNase T1 and RNase A (Ambion) according to the manufacturer's instruction. Briefly, ErsA RNA was incubated in the presence of yeast RNA in 1 × RNA Structure Buffer (Ambion) at room temperature. Partial digestion was carried out with 0.01, 0.05 and 0.1 U ul^{-1} of RNase T1 or with 2 and 10 pg ul^{-1} of RNase A for 5 and 15 min. Reactions were stopped by precipitation, resuspended and retrotranscribed with 5′-^{32}P labelled oligo 5 using Superscript III reverse transcriptase (Invitrogen) at 55°C. cDNAs were precipitated and resuspended in 7 µl of Gel Loading Buffer II (Ambion).

Primer extension reactions and cDNAs derived from RNA structural probing were denatured at 95°C for 3 min, loaded into a prewarmed sequencing gel (7 M urea, 6% polyacrylamide, acrylamide-bis ratio 19:1), electrophoresed at 1400 V for 2 h, dried, exposed to a phosphor screen, acquired using a Typhoon 8600 variable mode imager scanner (GE Healthcare BioSciences) and visualized with ImageQuant software (Molecular Dynamics). Sanger sequencing reactions performed with oligo 5 on ErsA DNA amplified with oligos 8 and 9 were used as molecular weight markers.

Treatment with terminator 5′-phosphate-dependent exonuclease was performed in terminator reaction buffer A (Epicentre) according to the manufacturer's instructions. RT-PCR analysis was performed as described previously (Vitale *et al.*, 2008).

In vitro and in vivo assays of sRNA/mRNA interactions

Electrophoretic mobility shift assays to analyze sRNA/mRNA interactions were performed in 10 µl of reactions containing 1 × RNA-binding buffer (10 mM Tris-HCl, pH 7, 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol), yeast tRNA, radioactively labelled *algC* mRNA, and increasing amounts of ErsA RNA or RseX RNA. Binding reactions were incubated at 37°C for 20 min. The reactions were loaded onto a native 6% polyacrylamide gel (acrylamide-bis ratio 29:1) in 0.5 × TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA), electrophoresed at 4°C and 200 V for 90 min, dried, exposed to a phosphor screen, acquired using a Typhoon 8600 variable mode imager scanner (GE Healthcare BioSciences) and visualized with ImageQuant software (Molecular Dynamics).

Pseudomonas aeruginosa strains carrying the reporter pBBR1-*algC::gfp* or pBBR1-*gfp* combined with pGM931, pGM-*ersA* or pGM-*spa0167* were inoculated in 15 ml of tubes at an OD_{600} of 0.1 in 5 ml of LB with carbenicillin, gentamicin and 0.2% arabinose and grown at 37°C in a rotatory shaker. After 3, 6 and 24 h, cell samples were collected by centrifugation, washed twice and resuspended in PBS (10 mM Na_3PO_4, 150 mM NaCl), and 200 µl of aliquots were transferred to black polystyrene 96-well microplates with a clear, flat bottom (Corning). The absorbance (Abs_{595}) and fluorescence polarization ($FP_{485/535}$) were measured in a Tecan Infinity PRO 200 reader using Magellan data analysis software (Tecan). GFP activity was expressed in arbitrary units (AU) as $FP_{485/535}/Abs_{595}$.

RNA synthesis

RNA for structural probing and RNA/RNA interaction assays was prepared by T7 RNA polymerase transcription of gel-purified DNA fragments. DNA fragments for ErsA RNA and *algC* mRNA preparations were amplified from *P. aeruginosa* PAO1 genomic DNA with oligo pairs 3/4 and 6/7 respectively. The DNA fragment for RseX RNA was amplified from *E. coli* C1a genomic DNA with oligos 10 and 11. Each transcription reaction was performed with the Riboprobe® System-T7 (Promega) with 300 ng of DNA template, and 50 Ci (α-^{32}P) cytidine triphosphate (PerkinElmer) was used in the synthesis of labelled RNA probes. Synthesized RNA was loaded on a denaturing polyacrylamide gel, stained with ethidium bromide, electroeluted from a gel slice in a model 422 Electro-Eluter (Biorad), precipitated and resuspended in diethylpyrocarbonate-treated water. Purified RNA was checked by denaturing polyacrylamide gel electrophoresis and quantified using a Qubit Fluorometer and/or Eppendorf Biospectrometer.

AlgC PGM activity

PGM activity was assayed using crude *P. aeruginosa* extracts as described previously (Sa-Correia *et al.*, 1987; Coyne *et al.*, 1994) with some modifications. Crude protein extracts were obtained from 6 ml of cultures grown in 20 ml of LB medium in aerobic conditions until an OD_{600} of 2.7. Cells were collected by centrifugation at 8000*g* for 10 min, washed twice, resuspended in 1.5 ml of 0.05 M Tris-HCl buffer (pH 7.5), and disrupted in a French press device. Cell debris was separated from the soluble protein crude extract by centrifugation at 12 000*g* for 1 h. The soluble protein concentrations were determined by both Qubit Fluorimeter (Invitrogen) and Biosprectrometer (Eppendorf) and ranged from 7 to 10 mg ml^{-1}. PGM activity was determined in a Shimadzu UV-1601 double-beam spectrophotometer by measuring the

Abs_{340} caused by the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH in 1-ml reactions containing 0.05 M Tris-HCl buffer (pH 7.5), 10 μmol $MgCl_2$, 1 μmol NADP, 1 μmol glucose 1-phosphate, 1 U glucose 6-phosphate dehydrogenase of *Leuconostoc* (Sigma) and soluble protein extracts.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Alignment of the intergenic regions between the conserved *polA* and *engB* genes of several *Pseudomonas* species. Eighteen *P. aeruginosa* strains (*PAE*), strains of *P. denitrificans* (*PDE*), *P. fulva* (*PFU*), *P. mendocina* (*PME*), *P. resinovorans* (*PRE*), *P. entomophila* (*PEN*), *P. putida* (PPU), *P. protegens* (*PPR*), *P. brassicacearum* (*PBR*), *P. fluorescens* (*PFL*), *P. stutzeri* (*PST*), and *P. syringae* (*PSY*), and a strain of the *Pseudomonas*-related species *Azotobacter vinelandii* (*AVI*) were considered for the alignment of the intergenic region between the conserved *polA* and *engB* genes including the *E. coli spf* and *P. aeruginosa ersA* genes (Fig. 1). For *P. stutzeri* and *A. vinelandii*, a gene for a type VI protein secretion system component Acp is present in place of *engB* downstream of the *polA* gene. Blue-shaded sequences are those matching the −35/−10 consensus sequences GAACTTN16–17TCNNA (N: any base; 16–17: base spacing between −35 and −10 motifs) of the σ^{22}-dependent core promoters located upstream of the *ersA* gene. The 5′-end of the primary transcript of ErsA, as defined by the experiments of Fig. 2, is indicated by +1. Note that sequence conservation upstream of the σ^{22} motifs is lost for the non-*P. aeruginosa* species.

Fig. S2. Validation of ErsA deletion. PAO1, PAO1 *ΔersA*, PA14 and PA14 *ΔersA* 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 probing ErsA.

Fig. S3. Validation of overexpression levels of ErsA, ErsA Δ1/53 and SPA0167 in PAO1.

A. PAO1 strains harbouring pGM-*ersA* 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 ErsA RNA.

B. PAO1 *hfq* strains harbouring pGM-*ersA* or the control empty vector pGM931 were grown and ErsA levels analyzed as in (A). To compare native ErsA levels between *hfq* and wt backgrounds, total RNA extracted from PAO1 harbouring the vector pGM931 was included in this analysis.

C. PAO1 harbouring pGM-*ersAΔ1/53* was grown and ErsA levels analyzed as in (A) and (B).

D. PAO1 strains harbouring pGM-*spa0167* or the control empty vector pGM931 were grown as above. Culture samples were taken at the indicated OD_{600} and processed for total RNA extraction and analysis by Northern blot probing SPA0167 RNA. Intensities of the bands of ErsA, ErsA Δ1/53 and SPA0167 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.

Fig. S4. Validation of overexpression levels of ErsA and SPA0167 in PA14.

A. PA14 strains harboring pGM-*ersA* 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 ErsA RNA.

B. PA14 strains harbouring pGM-*spa0167* or the control empty vector pGM931 were grown as above. Culture samples were taken at the indicated OD_{600} and processed for total RNA extraction and analysis by Northern blot probing

SPA0167 RNA. Intensities of the bands of ErsA and SPA0167 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.

Fig. S5. Growth curves of *P. aeruginosa* PAO1 and PA14 strains on different preferred and non-preferred carbon sources.

A. Wild-type and *ΔersA* strains were grown overnight in LB medium, washed and diluted to an OD_{595} of 0.1 in M9 medium with the indicated carbon sources.

B. *P. aeruginosa* strains transformed with the empty vector pGM931 or with pGM-*ersA* were grown overnight in LB medium, washed, diluted to an OD_{595} of 0.1 in M9 medium with the indicated carbon sources, and induced with 10 mM arabinose. Aliquots of 200 µl were distributed in triplicate in 96-well culture plates and incubated for 26 h in a microplate reader at 37°C with constant orbital shaking and realtime OD595 measurements every 15 min. Plotted OD_{595} values reflect the average of three independent experiments.

Fig. S6. Model for the incoherent feed-forward loop involved in AlgC regulation. An incoherent feed-forward loop with four elements, X, Y, W and Z, is depicted. Two σ^{22}-dependent regulatory arms with opposite signs converge on AlgC (Z). The activating arm (AA) is mediated by AlgR (Y) at the transcriptional level, while the repressing arm (RA) is mediated bt ErsA (W) at the post-transcriptional level.

Table S1. Strains and plasmids.

Table S2. Oligonucleotides.

Table S1. Strains and plasmids

Strains or plasmids	Genotype or description	Construction	Reference
Pseudomonas aeruginosa			
PAO1	Wild type		(1)
PAO1 *hfq-*	*hfq::aadA*		(2)
PAO1 Δ*ersA*	markerless Δ*ersA*		this work
PAO1 Δ*algT/U*	markerless Δ*algT/U*		this work
PA14	Wild type		(3)
PA14 Δ*ersA*	markerless Δ*ersA*		this work
PA14 Δ*algT/U*	markerless Δ*algT/U*		this work
Escherichia coli			
C1a	*E. coli C*, prototrophic		(4)
TOP10	mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZDM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG		Invitrogen
CC118λpir	D(ara-leu), araD, DlacX174, galE, galK, phoA, thi1, rpsE, rpoB, argE (Am), recA1, lysogenic λpir		(5)
Plasmids			
pGM931	pHERD20T derivative, *araC*/P_{BAD} - t_{Ω}, Ap^r		Delvillani and Briani, unpublished
pGM-*ersA*	pGM931 derivative, *ersA* under P_{BAD}, Ap^r	contains PCR product using oligos 12 and 13	this work
pGM-*ersAΔ1/53*	pGM931 derivative, *ersA Δ1/53* under P_{BAD}, Ap^r	contains PCR product using oligos 40 and 13	this work
pGM-*spa0167*	pGM931 derivative; *spa0167* under P_{BAD}, Ap^r	contains PCR product using oligos 14 and 15	this work
pXG1-SF	sfGFP reporter plasmid; *gfp* under $P_{LtetO-1}$, Cm^r		(6)
pXG10-SF	sfGFP reporter plasmid; *lacZ*::*gfp* under $P_{LtetO-1}$, Cm^r		(7)
pXG10-*algC::gfp*	pXG10-SF derivative; $P_{LtetO-1}$ -->*algC::gfp*, Cm^r	contains PCR product using oligos 16 and 17	this work
pBBR1-MCS5	*lacZ* α, Gm^r		(8)
pBBR1-*gfp*	pBBR1-MCS5 derivative; $P_{LtetO-1}$ -->*gfp*, Gm^r	contains PCR product using oligo 18 and 19	this work
pBBR1-*algC::gfp*	pBBR1-MCS5 derivative; $P_{LtetO-1}$ -->*algC::gfp*, Gm^r	contains PCR product using oligos 18 and 19	this work
pSEVA612S	oriR6K, *lacZ*α; MCS flanked by two I-SceI sites, Gm^r, Ap^r		(9)
pSW-1	oriRK2, *xylS*; *Pm*-->I-SceI, Ap^r		(10)
pSEVApao1-Δ*ersA*	pSEVA612S derivative, Ap^r	contains SOEing-PCR product of the PAO1-amplificates TS1 and TS2 using oligos 27 and 30	this work
pSEVApa14-Δ*ersA*	pSEVA612S derivative, Ap^r	contains SOEing-PCR product of the PA14-amplificates TS1 and TS2 using oligos 27 and 30	this work
pSEVApao1-Δ*algT/U*	pSEVA612S derivative, Ap^r	contains SOEing-PCR product of the PAO1-amplificates TS1 and TS2 using oligos 33 and 36	this work
pSEVApa14-Δ*algT/U*	pSEVA612S derivative, Ap^r	contains SOEing-PCR product of the PA14-amplificates TS1 and TS2 using oligos 33 and 36	this work

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Table S2. Oligonucleotides

Oligo #	Oligo Name	Sequence (5' → 3')[a]	Utilization
1	SPA0122	CCCGAGCTTCGTATGGGG	Northern-blot probe for ErsA
2	PA5SRNA02	GGAGACCCCACACTACCATCGGCGATG	Northern-blot probes for 5S
3	T7_5'SPA0122_f	**CTAATACGACTCACTATAGGG**CGAATGGCTTCT TGAGCC	Amplification of *ersA* in fusion with RNA polymerase T7 promoter for *in vitro* transcription
4	3'SPA0122_r	AAAAAAAACCCCGAGCTTCGTATGGGGAG	
5	3'SPA0122_RT_r	AAAAAAAACCCCGAGCTTCG	Reverse primer on 3' of ErsA for primer extension, sequencing and retrotranscription
6	T7_-75ATG_AlgC_f	**CTAATACGACTCACTATAGGG**CAGAACACCGA CATTCTG	Amplification of the 5' UTR and beginning of *algC* in fusion with RNA polymerase T7 promoter for *in vitro* transcription
7	+83ATG_AlgC_r	CGGTGAGGGTATCGCCTACCACGC	
8	EngB-SPA0122_f	ATCCATGTGCTGATGACCAAG	Amplification of the genomic region from *polA* to *engB* for the generation of the DNA template for *ersA* sequencing
9	SPA0122-PolA_r	GGAGATCCATTCGAAGAACG	
10	T7_RseX_f	**CTAATACGACTCACTATAGGG**TTTTTATTATTCT GTGTCATGATG	Amplification of *rseX* in fusion with RNA polymerase T7 promoter for *in vitro* transcription
11	RseX_r	TAAAAAAAAGCCGGCATCAT	
12	NcoI_5'SPA0122_f	GAccatggCGAATGGCTTGAGCCCTTCGATGCT	Amplification of *ersA* with *Nco*I/*Pst*I ends for cloning in pGM931 vector
13	PstI_3'SPA0122_r	GAActgcagAAAAAAAACCCCGAGCTTCGTA	
14	NcoI_5'SPA0167_f	GAccatggCATTCCAGTGGAATGCAGG	Amplification of *spa0167* with *Nco*I/*Pst*I ends (lowercase) for cloning in pGM931 vector
15	PstI_3'SPA0167_r	GAActgcagAAAAAAACGGGAGTCCCCG	
16	AlgC_pXG10_NsiI_f	GTTTTatgcatGTGCCTAGCCTGCCGGGC	Amplification of the 5' UTR and beginning of *algC* with *Nsi*I/*Nhe*I ends for cloning in frame with *gfp* in pXG10-SF vector
17	AlgC_pXG10_NheI_r	GTTTTgctagcGGTGAGGGTATCGCCTAC	
18	Ptet-O1_ClaI_f	GTTTTatcgatTCCCTATCAGTGATAGAG	Amplification from the $P_{LtetO-1}$ promoter to the stop codon of *gfp* of pXG10-*algC*::*gfp*/pXG1-SF with *Cla*I/*Xba*I ends for cloning in pBBR1-MCS5
19	sfGFP_TAA_XbaI_r	TGATGCCtctagaTTATTTGTAGAGCTC	
20	sfGFP_+96_r	TTGTGCCCATTAACATCACCATC	Reverse primer on *gfp* for verification of constructs
21	16S_f	TGTCGTCAGCTCGTGTCGTGA	Amplification of 16S for real time-PCR analysis
22	16S_r	ATCCCCACCTTCCTCCGG T	
23	AlgC_1062_RT_f	CCAGGATCAGCGTGACAGC	Amplification of *algC* for real time analysis
24	AlgC_1209_RT_r	GATGTTGCCTTCGCCCCATT	
25	pSEVA_fw	TAAAACGACGGCCAGTATAGGG	Forward primer on pSEVA vector for verification of contructs/sequencing of TS1 and TS2
26	pSEVA_rw	CAGCTATGACCATGATTACGCC	Reverse primer on pSEVA vector for verification of contructs/sequencing of TS1 and TS2
27	TS1_engB_SacI_f	GATgagctcCTCCAATGCCGGCAAGTCGA	Amplification of the genomic region TS1 using genomic DNA of either PAO1 or PA14
28	TS1_engB_r	AGGGTGCCGTGGAAAAGTTTGCGGCCGCACTT GAACCCCGAACTTCCCCCT	
29	TS2_polA_f	GCGGCCGCAAACTTTTCCACGGCACCCT	Amplification of the genomic region TS2 using genomic DNA of either PAO1 or PA14
30	TS2_polA_BamHI_r	CGggatccAAGTCTTCGGCGTGCCGCTGGAAGAC GT	
31	spa0122_300up	GATAAAGCGCACACAAAATCC	Forward primer on 300 bp upstream the *ersA* gene for verification of deletion
32	spa0122_300down	GCATCCATACCTCATACCACC	Reverse primer on 300 bp downstream the *ersA* gene for verification of deletion

33	TS1_up-AlgU_EcoRI_f	GCgaattcTGGAGCCCTAGTATATAGAAGGG	Amplification of the genomic region TS1 using genomic DNA of either PAO1 or PA14
34	TS1_AlgU_r	GCTGCAGAGCTTTGTCGATTGCTTCGGTTAGCA TGAAAGCTCCTCT	
35	TS2_ AlgU_f	GAAGCAATCGACAAAGCTCTGCAGC	Amplification of the genomic region TS2 using genomic DNA of either PAO1 or PA14
36	TS2_MucB_ BamHI_r	CGggatccAAGCAAAAGCAACAGGGAGGTGGTG	
37	TS1_up_AlgU_f	ACCAGGGTGTCCTCGACGT	Forward primer on 618 bp upstream the *algT/U* gene for verification of deletion
38	TS2_down_AlgU_r	ACGGGATTCGCCGACCAG	Reverse primer on 1008 bp downstream the *algT/U* gene for verification of deletion
39	pHERD_rev	TGCAAGGCGATTAAGTTGGGT	Reverse primer on pGM931 backbone for verification of constructs
40	NcoI_Spa122_D53f	GAccatggAGATTCCTGGACCCCGCC	In combination with oligo 13, amplification of *ersA Δ1/53* with *Nco*I/*Pst*I ends for cloning in pGM931 vector

[a] Lowercase: sites for restriction enzymes. Bold: T7 promoter sequence.

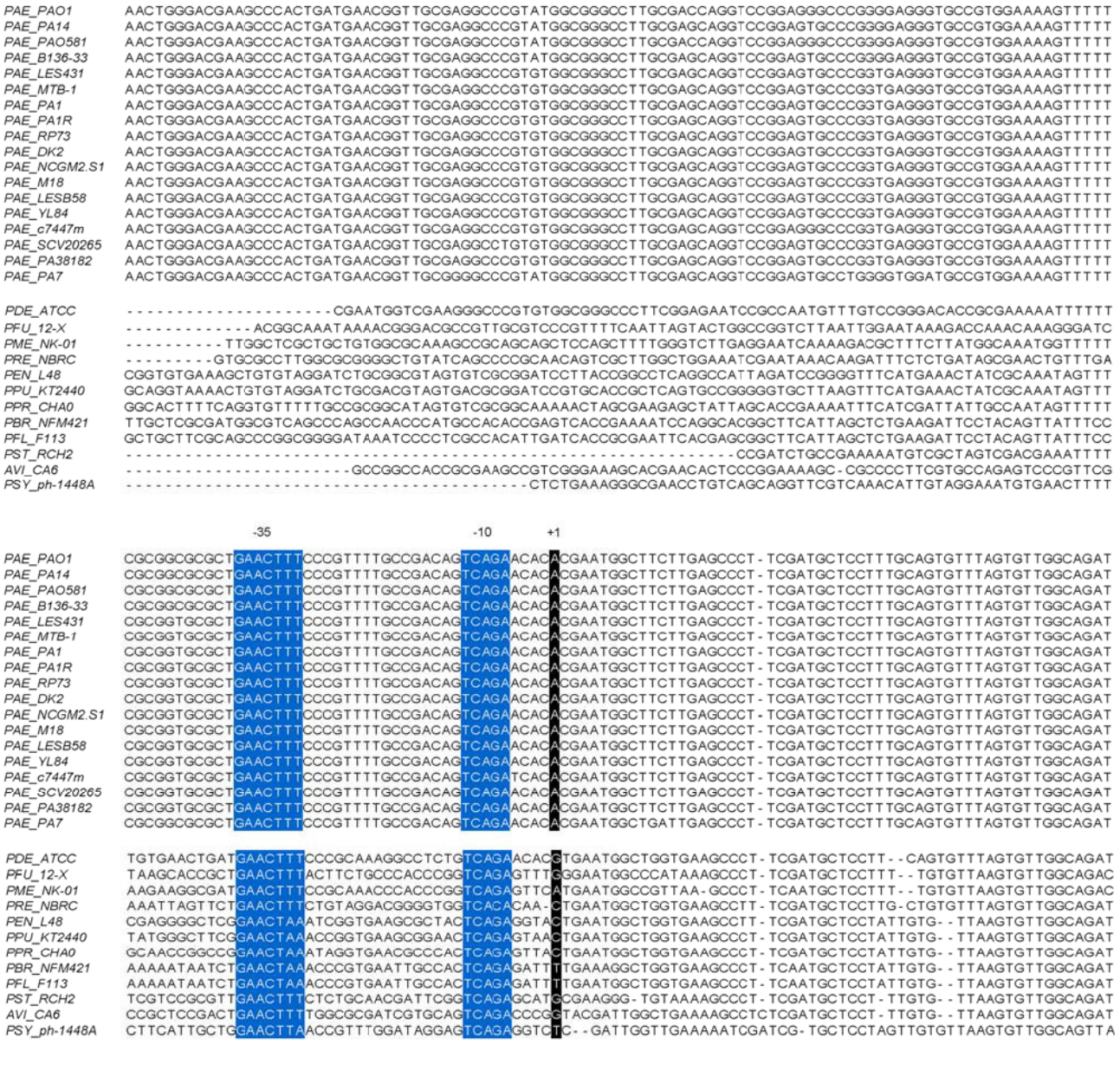
-35
-10
+1

Figure S1 Alignment of the intergenic regions between the conserved *polA* and *engB* genes of several *Pseudomonas* species. Eighteen *P. aeruginosa* strains (*PAE*), strains of *P. denitrificans* (*PDE*), *P. fulva* (*PFU*), *P. mendocina* (*PME*), *P. resinovorans* (*PRE*), *P. entomophila* (*PEN*), *P. putida* (PPU), *P. protegens* (*PPR*), *P. brassicacearum* (*PBR*), *P. fluorescens* (*PFL*), *P. stutzeri* (*PST*), and *P. syringae* (*PSY*), and a strain of the *Pseudomonas*-related species *Azotobacter vinelandii* (*AVI*) were considered for the alignment of the intergenic region between the conserved *polA* and *engB* genes including the *E. coli spf* and *P. aeruginosa ersA* genes (Figure 1). For *P. stutzeri* and *A. vinelandii*, a gene for a type VI protein secretion system component Acp is present in place of *engB* downstream of the *polA* gene. Blue-shaded sequences are those matching the −35/−10 consensus sequences GAACTTN$_{16–17}$TCNNA (N: any base; 16–17: base spacing between −35 and −10 motifs) of the σ^{22}-dependent core promoters (10) located upstream of the *ersA* gene. The 5′-end of the primary transcript of ErsA, as defined by the experiments of Figure 2, is indicated by +1. Note that sequence conservation upstream of the σ^{22} motifs is lost for the non-*P. aeruginosa* species.

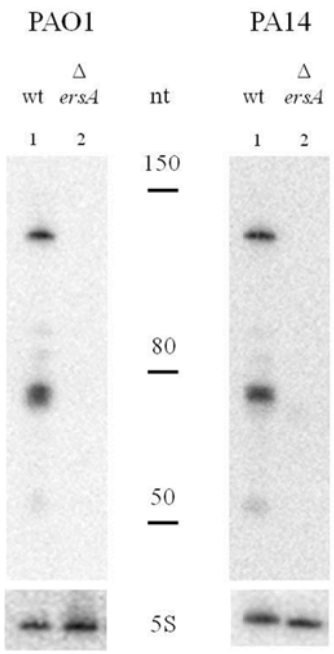

Figure S2 Validation of ErsA deletion. PAO1, PAO1 *ΔersA,* PA14, and PA14 *ΔersA* were grown in BHI medium until an OD_{600} of 2.7. Culture samples were taken and processed for total RNA extraction and analysis by Northern blot probing ErsA.

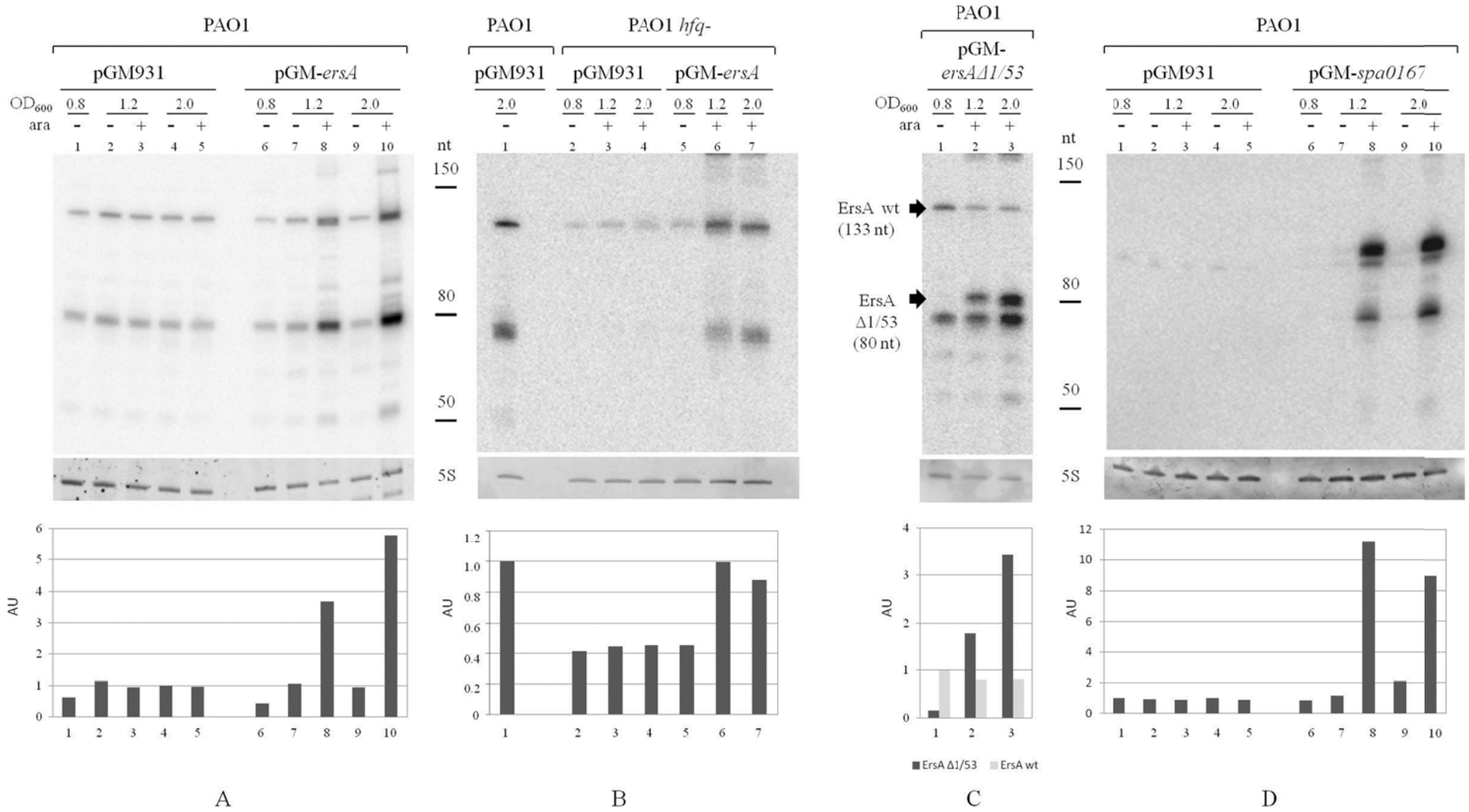
PAO1
pGM931
pGM-ersA
PAO1
PAO1 hfq-
pGM931
pGM931
pGM-ersA
PAO1
pGM-ersAΔ1/53
PAO1
pGM931
pGM-spa0167
OD600
ara
nt
150
80
50
5S
ErsA wt (133 nt)
ErsA Δ1/53 (80 nt)
AU
ErsA Δ1/53
ErsA wt
A
B
C
D

Figure S3 Validation of overexpression levels of ErsA, ErsA Δ1/53 and SPA0167 in PAO1. (**A**) PAO1 strains harboring pGM-*ersA* 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 ErsA RNA. (**B**) PAO1 *hfq* strains harboring pGM-*ersA* or the control empty vector pGM931 were grown and ErsA levels analyzed as in (A). To compare native ErsA levels between *hfq* and wt backgrounds, total RNA extracted from PAO1 harboring the vector pGM931 was included in this analysis. (**C**) PAO1 harboring pGM-*ersAΔ1/53* was grown and ErsA levels analyzed as in (A) and (B). (**D**) PAO1 strains harboring pGM-*spa0167* or the control empty vector pGM931 were grown as above. Culture samples were taken at the indicated OD_{600} and processed for total RNA extraction and analysis by Northern blot probing SPA0167 RNA. Intensities of the bands of ErsA, ErsA Δ1/53 and SPA0167 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.

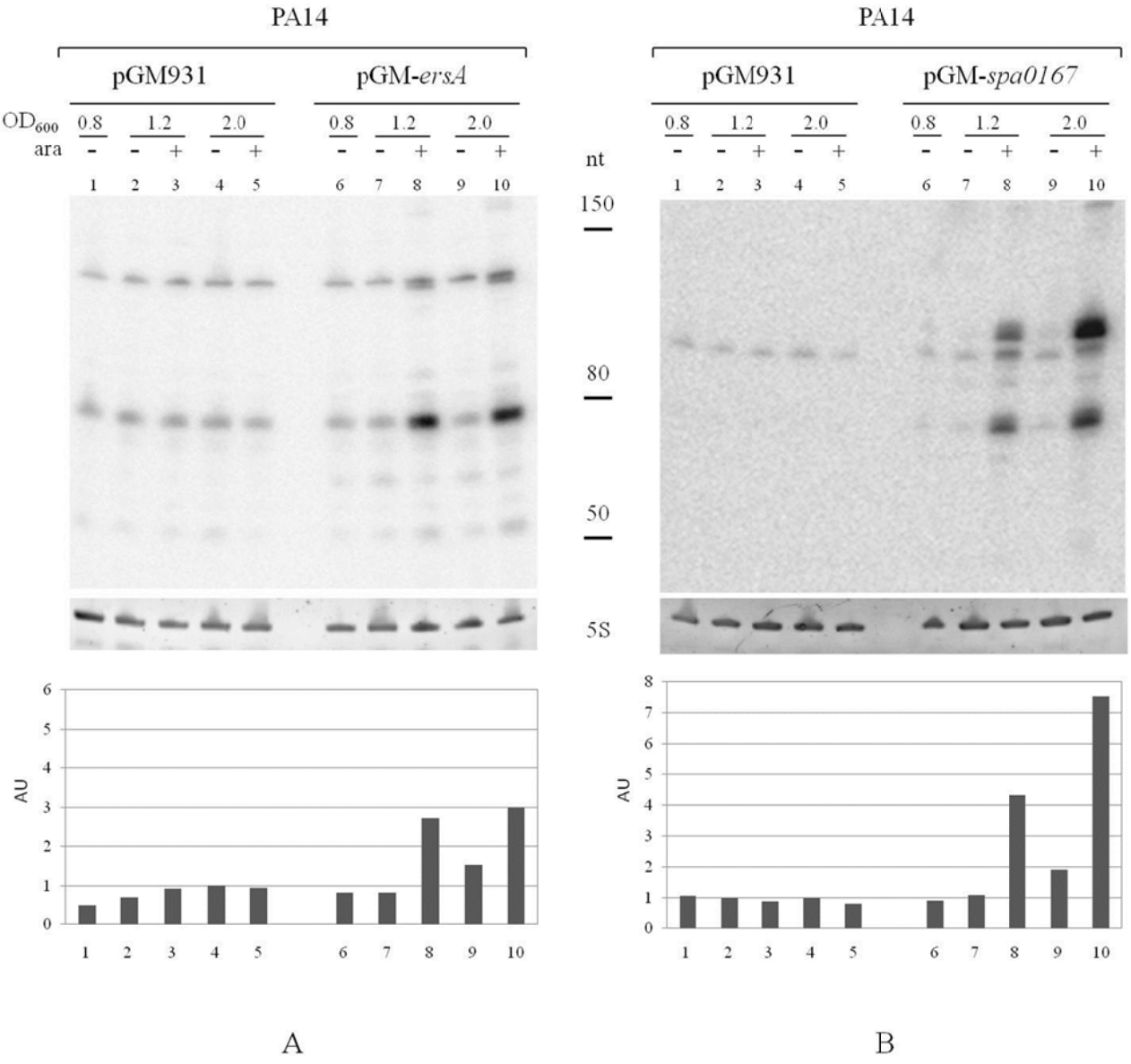

Figure S4 Validation of overexpression levels of ErsA and SPA0167 in PA14. (**A**) PA14 strains harboring pGM-*ersA* or the control empty vector pGM931 were grown in BHI medium with carbenicillin until an OD_{600} of 0.8. Cells were split into two flasks, and 10 mM arabinose (ara) was added to one. Culture samples were taken at the indicated OD_{600} and processed for total RNA extraction and analysis by Northern blot probing ErsA RNA. (**B**) PA14 strains harboring pGM-*spa0167* or the control empty vector pGM931 were grown as above. Culture samples were taken at the indicated OD_{600} and processed for total RNA extraction and analysis by Northern blot probing SPA0167 RNA. Intensities of the bands of ErsA and SPA0167 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.

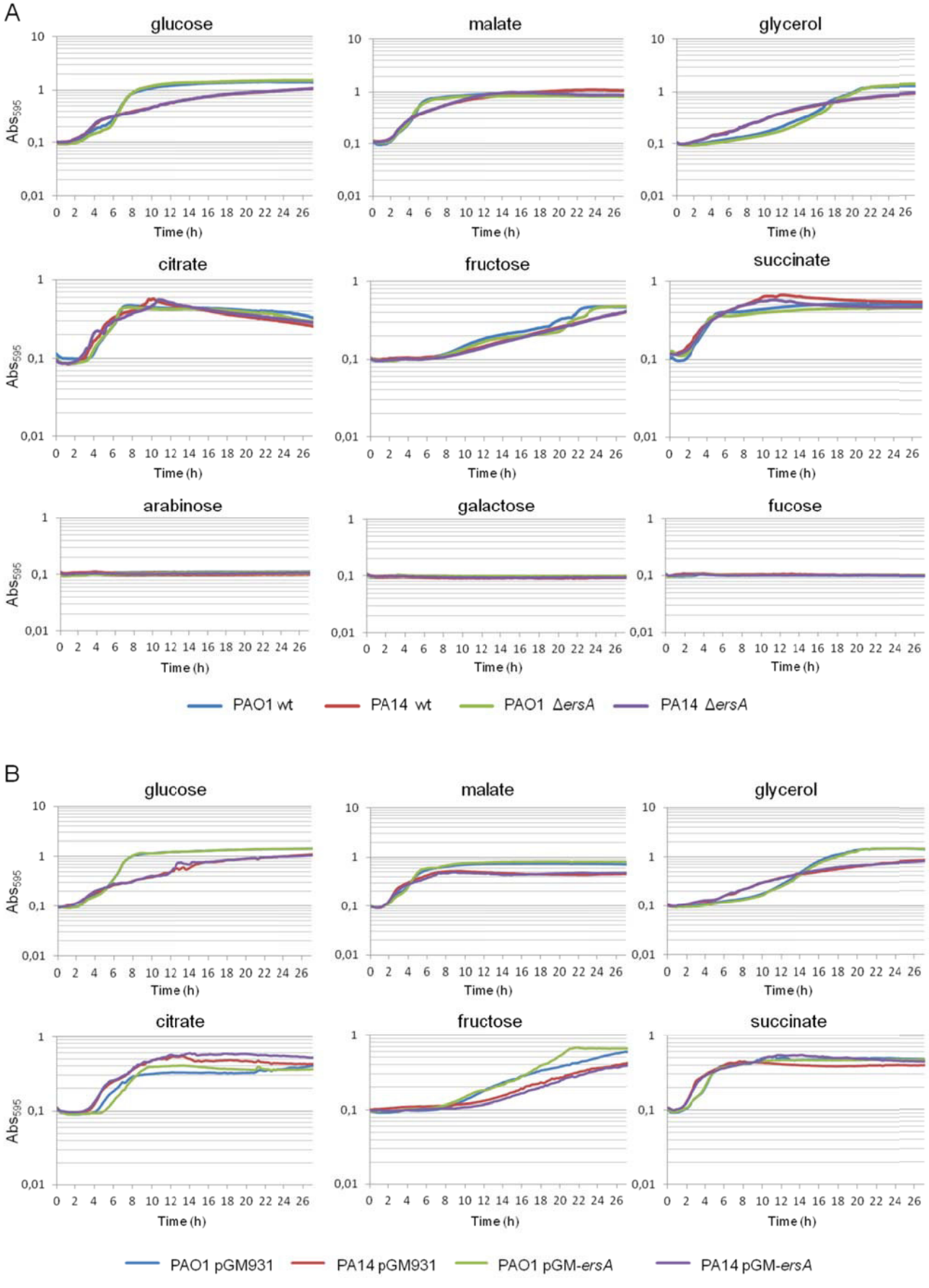
A
glucose
malate
glycerol
citrate
fructose
succinate
arabinose
galactose
fucose
Abs595
Time (h)
PAO1 wt
PA14 wt
PAO1 ΔersA
PA14 ΔersA
B
glucose
malate
glycerol
citrate
fructose
succinate
Abs595
Time (h)
PAO1 pGM931
PA14 pGM931
PAO1 pGM-ersA
PA14 pGM-ersA

Figure S5 Growth curves of *P. aeruginosa* PAO1 and PA14 strains on different preferred and non-preferred carbon sources. (**A**) Wild-type and *ΔersA* strains were grown overnight in LB medium, washed and diluted to an OD_{595} of 0.1 in M9 medium with the indicated carbon sources. (**B**) *P. aeruginosa* strains transformed with the empty vector pGM931 or with pGM-*ersA* were grown overnight in LB medium, washed, diluted to an OD_{595} of 0.1 in M9 medium with the indicated carbon sources, and induced with 10 mM arabinose. Aliquots of 200 µL were distributed in triplicate in 96-well culture plates and incubated for 26 h in a microplate reader at 37°C with constant orbital shaking and real-time OD_{595} measurements every 15 min. Plotted OD_{595} values reflect the average of three independent experiments.

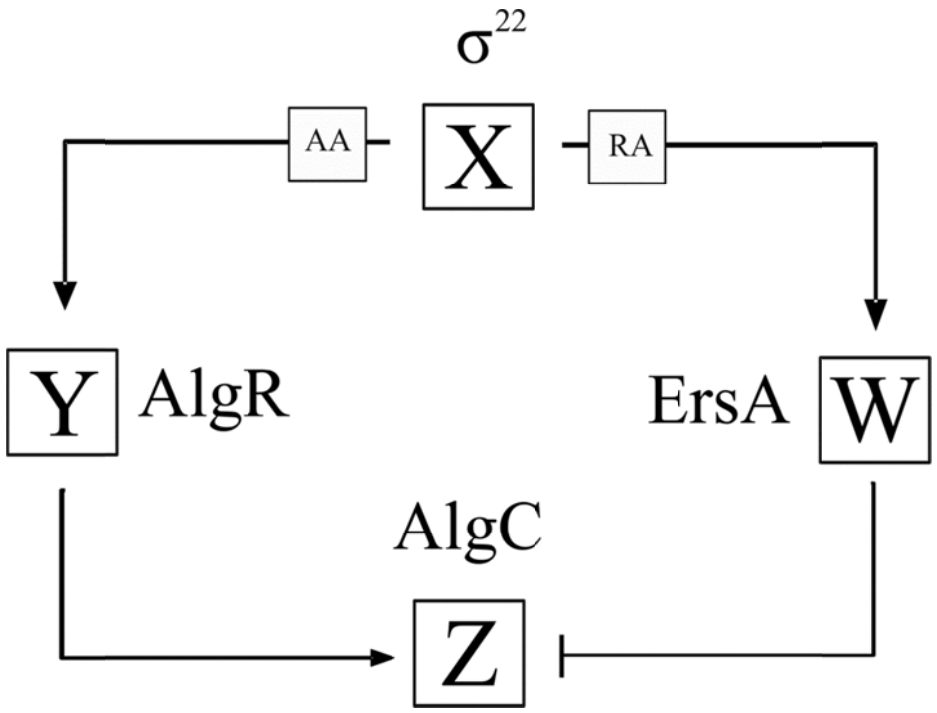

Figure S6 Model for the incoherent feed-forward loop involved in AlgC regulation. An incoherent feed-forward loop with four elements, X, Y, W, and Z, is depicted. Two σ^{22}-dependent regulatory arms with opposite signs converge on AlgC (Z). The activating arm (AA) is mediated by AlgR (Y) at the transcriptional level, while the repressing arm (RA) is mediated by ErsA (W) at the post-transcriptional level.

The small RNA SPA0084: a novel regulatory element embedded in the *Pseudomonas aeruginosa quorum sensing* networks

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Keywords: *Pseudomonas aeruginosa*, small RNAs, virulence, quorum sensing, post-transcriptional regulation

Abstract

Small RNAs (sRNAs) are recognized as important components of the regulatory networks involved in the host-pathogen interaction. In this study, the novel sRNA SPA0084 of the opportunistic pathogen *Pseudomonas aeruginosa* has been characterized. Our results indicate that SPA0084 is embedded in the *P. aeruginosa quorum sensing* (QS) with the role of wiring *las* to *pqs* systems. In fact, we show that SPA0084 responds to the *las* regulator LasR and impacts positively the synthesis of the *pqs* quinolone signal PQS. Our results suggest that the stimulation of PQS synthesis is mediated by a positive post-transcriptional effect of SPA0084 on the *pqsC* gene belonging to the *pqsABCD* cluster involved in the biosynthesis of the PQS-precursor HHQ. We suggest that a fine balancing between the different already known regulatory effects of LasR on PQS synthesis and this one mediated by SPA0084 can influence timing and magnitude of expression of QS-regulated virulence factors. This view is consistent with the evidence that perturbations of SPA0084 levels affect pyocyanin synthesis, biofilm formation and swarming motility, processes that are known to be influenced by PQS synthesis. Besides being regulated by LasR, SPA0084 responds to infection relevant cues that *P. aeruginosa* can experience in mammalian hosts such as temperature and oxygen availability. Furthermore, SPA0084 shows a growth phase-dependent pattern of expression, being up-regulated in stationary phase. Sequence analysis of the *spa0084* promoter region strongly suggests that the growth phase-dependent pattern of SPA0084 expression is due to the activity of the alternative σ factor RpoS whose significance as global factor controlling *quorum sensing* gene expression was shown previously. Together, these SPA0084 regulations are expected to contribute to the fine co-modulation of PQS synthesis.

Introduction

The bacterium *Pseudomonas aeruginosa*, a major cause of chronic lung infections in cystic fibrosis individuals, as well as of numerous acute infections in injured, severely burned, and immune-compromised patients, produces a large assortment of virulence factors, and is endowed with an exquisite ability to form biofilms and to rapidly develop resistance to multiple classes of antibiotics [1]. The expression of these traits is fine-tuned by a dynamic and intricate regulatory network [2], in which more than 50 regulatory proteins play key roles as transcription regulators. However, the number of small RNAs (sRNAs) that has been implicated in the regulation of *P. aeruginosa* virulence is low but certainly set to increase [3].

sRNAs have been identified in a wide range of bacteria and have been shown to play critical regulatory roles in many intra- and extra-cellular processes and pathogenesis [4, 5]. sRNAs play their regulatory role either by base-pairing with mRNAs or by modulating protein activity upon interaction. sRNAs can share extended base complementarity when they are *cis*-encoded on the opposite strand of the target mRNA. Instead, trans-encoded sRNAs usually share limited complementarity with mRNA targets and interact with them via short and imperfect base-pairing, modulating mRNA translation and/or stability. The most-characterized sRNA-based regulatory system in *P. aeruginosa* include the RNA-binding protein CsrA/RsmA [6-8], which regulate several processes by repressing the translation of target mRNAs. The activity of RsmA is antagonized through sequestration by RsmY and RsmZ, two functionally redundant sRNAs playing a critical role in the switch between acute and chronic infections [9, 10]. Other characterized *P. aeruginosa* sRNAs are CrcZ, the PrrF sRNAs (PrrF1 and PrrF2), PhrS and NrsZ. CrcZ acts as a decoy to abrogate Hfq-mediated translational repression of catabolic genes [11]; PrrF1 and PrrF2 are involved in iron homeostasis, central carbon, and quorum-sensing regulation [12-14]; PhrS regulates the key quorum-sensing regulator PqsR [15]; NrsZ modulates motility by up-regulating translation of *rhlA*, a gene essential for the production of rhamnolipids [16]; and ErsA regulates the virulence-associated enzyme AlgC [17].

P. aeruginosa pathogenicity is mainly due to an arsenal of virulence factors that includes extracellular polysaccharides, flagella, type IV pili, proteases, elastase and pyocyanin [18] [19]. Many of the numerous

virulence factors produced by *P. aeruginosa* are under the control of three interconnected quorum sensing systems (QS) namely *las*, *rhl* and *pqs*. They are responsible for the bacterial communication through the production and detection of small diffusible signal molecules, coordinating gene expression as a function of cell density [20]. The *las* system is composed by the transcriptional regulator LasR, its cognate autoinducer molecule *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL) and the 3OC12-HSL synthase LasI. Similarly, the *rhl* system consists of transcriptional regulator RhlR, its cognate autoinducer molecule N-butanoyl-homoserine lactone (C4-HSL) and the C4-HSL synthase RhlI [21]. Finally the *pqs* system consists of transcriptional regulator PqsR, their cognate autoinducer 2-alkyl-4(1*H*)-quinolone (AQ) molecules, being the primary signals 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) and 2-heptyl-4-hydroxyquinolone (HHQ), and the *pqsABCDE* operon responsible for AQs biosynthesis [22]. When a critical threshold concentration of is reached, the autoinducer molecule binds to its cognate transcriptional regulatory protein and triggers the expression of many target genes. Although the *las* and *rhl* systems are two separate QS systems, they are related in a hierarchical manner, with the *las* system being dominant over both the *rhl* and *pqs* systems [20]. In addition, the *rhl* system negatively regulates the *pqs* system [23]. However, under certain conditions, the *rhl* and *pqs* systems can also be activated in the absence of *lasR* gene [24]. The PQS synthesis requires the products of the *pqsABCD* genes. In particular, PqsA is involved in the anthranilate activation, while PqsB, PqsC and PqsD are subsequently involved in the anthranilate conversion to the PQS-precursor HHQ [25]. HHQ is converted to PQS through the action of PqsH monooxygenase [26] [25]. The regulation of the *pqsABCD* genes occurs through PqsR [22] [25]. PqsR binds the promoter of *pqsABCD* and this binding increases dramatically in the presence of HHQ and PQS, suggesting that they are acting as PqsR co-inducers [27] [28].

P. aeruginosa QS regulates a large number of biological functions such as virulence related traits, secondary metabolism and motility [29] [30]. In particular, QS network controls the synthesis and secretion of the blue, redox-active phenazine pyocyanin [31] also through the positive regulation of the *pqs* system [22] [32] [33]. The QS also regulates the biofilm production and motility [34] [35] [36] [37]. In particular, PQS act positively in the increase of biofilm formation [35] [38] and negatively in motility both through PqsR-dependent and PqsR-independent pathways [39].

In this study, we have characterized the novel *P. aeruginosa* sRNA SPA0084, identified in a recent sRNA sequencing approach in *P. aeruginosa* [40] that aimed to compare the sRNA complement of PAO1 and PA14 strains which share the same host range but differ in virulence, PA14 being considerably more virulent in several model organisms. Our results indicate that SPA0084 is embedded in the *P. aeruginosa* QS with the role of wiring *las* to *pqs* systems. In fact, we show that SPA0084 responds to LasR and can impact PQS/HHQ levels likely via positive post-transcriptional regulation of *pqsC* gene.

Results

SPA0084: genomic context, gene structure, and growth-phase dependent-expression.

The sRNA SPA0084 has been identified in a recent sRNA sequencing approach in *P. aeruginosa* [40] that aimed to compare the sRNA complement of PAO1 and PA14 strains which share the same host range but differ in virulence, PA14 being considerably more virulent in several model organisms. SPA0084 RNA is transcribed from a genomic context that is highly conserved among the sequenced *P. aeruginosa* strains (Fig. S1). SPA0084 RNA can be detected in both PAO1 and PA14 by Northern blot with the oligonucleotide probe val0 (Fig. 1A). The resistance to treatment with terminator 5′-phosphate-dependent exonuclease which preferentially degrades processed transcripts indicated that the ~100 nt SPA0084 RNA detected by val0 is a primary transcript both in PAO1 and PA14 (Fig. 1B). From these results, the transcription initiation site (+1) of SPA0084 RNA was suggested to be located ~100 nt upstream the poly-T tail of the Rho-independent terminator-like sequence indicated in Fig. 1A. To validate this, Northern blot analysis was performed with oligonucleotide val1, probing a region located upstream the predicted +1 and not overlapping oligonucleotide val0 (Fig. 1A). No SPA0084 RNA signal was detected by val1 (Fig. 1B). Therefore, we speculated that the region corresponding to val1 and that further upstream included the promoter of SPA0084 RNA transcription. Actually, -10 and -35 promoter motifs could be identified, being the -10 sequence identical to the heptamer consensus CTATACT recognized by the alternative σ factor RpoS [41] (Fig. 1A). Strikingly, these -10 and -35 motifs are strictly conserved among the sequenced *P. aeruginosa* strains (Fig. S1). These results suggested that SPA0084 could be under RpoS control and thus up-regulated starting from the onset of the stationary phase. To test this issue, we analyzed SPA0084 expression during exponential phase (at OD_{600} = 0.9 and 1.8) and at early (OD_{600} =2.7) and late (OD_{600} =4) stationary phase. As shown in Fig. 1C, both in PAO1 and PA14, SPA0084 expression increased in stationary phase reaching, at the last time point, levels about 4-fold higher than those of the exponential phase.

SPA0084 expression is negatively regulated by the QS transcription regulator LasR

Our analysis of the DNA region upstream *spa0084* gene included also virtual screenings for DNA elements bound by known *P. aeruginosa* transcription regulators. This analysis predicted a DNA element overlapping the -35 site and the downstream -35/-10 spacer (Fig. 1A) that matched the *las-rhl* consensus box that was defined for the QS regulators LasR and RhlR [42]. To test whether SPA0084 expression depends on LasR and/or RhlR, we compared the SPA0084 RNA levels in PAO1 and PA14 wt strains with knock-out mutants in *lasR* and *rhlR* genes along with mutants in other key QS regulatory genes, such *lasI*, *rsaL* and *pqsR,* as controls. As shown in Fig. 2A, relative to the corresponding *wt* strains, SPA0084 levels were higher in PAO1 Δ*lasR,* PA14 Δ*lasR* and PA14 Δ*lasI* strains, reduced in PAO1 *ΔrsaL* and comparable in PA14 Δ*rhlR* and PA14 *pqsR*- strains. These results suggested that SPA0084 transcription could be negatively regulated by LasR. The fact that PA14 Δ*lasI* mutant showed SPA0084 levels comparable to PA14 Δ*lasR* pointed to the involvement of 3-oxo-C_{12}-HSL signal molecule in the supposed LasR-mediated negative regulation of SPA0084 RNA. The reduced levels of SPA0084 in PAO1 *ΔrsaL* were also coherent with a 3-oxo-C_{12}-HSL role.

The above changes in SPA0084 levels could most likely reflect differences in the activity of the *spa0084* promoter ($P_{spa0084}$) in the different genetic background. To address this, the region spanning positions -156 to + 65 of *spa0084* gene was transcriptionally fused with the superfolder variant gene of the green fluorescent protein (sfGFP) and transferred into PAO1 Δ*lasR,* PAO1 *ΔrsaL,* PA14 Δ*lasR*, PA14 Δ*lasI,* PA14Δ *rhlR* and PA14 *pqsR*- strains, and into the corresponding wt strains. As shown in Fig. 2B, the $P_{spa0084}$-*gfp* fusion was significantly up-regulated in PAO1 Δ*lasR,* PA14 Δ*lasR*, and PA14 *ΔlasI*, suggesting again a negative regulation of SPA0084 expression by LasR/3-oxo-C_{12}-HSL complex. To further test this in a heterologous genetic background, the $P_{spa0084}$-*gfp* fusion was transferred to an *Escherichia coli* strain along with a vector plasmid expressing LasR, and GFP activity was monitored both in the absence and presence of 3-oxo-C_{12}-HSL. As shown in Fig. 2C, expression of LasR could specifically down regulate the $P_{spa0084}$-*gfp* fusion activity in the presence of 3-oxo-C_{12}-HSL. Altogether, these results strongly suggested a direct negative role of LasR bound to 3-oxo-C_{12}-HSL in SPA0084 expression.

SPA0084 responds to temperature and oxygen availability.

Our analysis of the transcriptional responsiveness of SPA0084 RNA spanned also some physicochemical conditions that *P. aeruginosa* can experience in mammalian hosts including temperature shifts from environmental to body temperature in the early stages of infection, and reduced oxygen conditions, such as those of cystic fibrosis airways. SPA0084 showed to be responsive to temperature and oxygen availability (Fig. 3A and B). Temperature sensitivity was tested in both PAO1 and PA14 strains by probing SPA0084 in early- (OD600 = 0.8) and mid-exponential phase (OD600 = 1.8) at both 20 and 37°C and after 20 min of acclimation following a shift from 20 to 37°C. As shown in Figure 3A, the shift from 20 to 37°C caused a strong up-regulation of SPA0084 that is maintained during growth at 37°C. Then, we tested whether a decrease in oxygen concentration influenced SPA0084 expression pattern during growth with aeration. SPA0084 was probed in both PAO1 and PA14 strains grown under anaerobic conditions in rich medium with nitrate to sustain anaerobic respiration. In addition, bacterial cells were grown with aeration until middle-exponential phase (OD600 = 0.8); then, oxygen was excluded from cultures. SPA0084 levels were assessed immediately before oxygen exclusion and 20 and 150 min from the start of anaerobic conditions. In both PAO1 and PA14, SPA0084 levels were higher in anaerobic than aerobic conditions (Fig. 3B). Following the shift from aerobic to anaerobic conditions, SPA0084 levels increased progressively (Fig. 3B).

SPA0084 influences the expression of *P. aeruginosa* virulence traits linked to QS.

To study the involvement of SPA0084 in the regulation of virulence traits, we constructed the knock-out mutant strain PA14 *Δspa0084* and the plasmid vector pGM-*spa0084*, carrying the *spa0084* gene under the arabinose inducible P_{BAD} promoter, to overexpress SPA0084 in both PA14 and PA14 *Δspa0084* derivative. Our aim was to measure the effects of perturbing SPA0084 levels on the expression of QS-linked virulence traits such as pyocyanin production, biofilm formation and swarming motility. As shown in Fig. 4A, SPA0084 deletion resulted in a decrease of pyocyanin production while SPA0084 overexpression enhanced pyocyanin production in both PA14 wt and PA14 *Δspa0084*. Since pyocyanin production is QS-regulated, we investigated whether the enhancement of pyocyanin levels following

SPA0084 overexpression required functional LasR, LasI, RhlR and PqsR. As shown in Fig. 4A, Δ*rhlR* and *pqsR*- strains showed no response to SPA0084 overexpression in terms of pyocyanin production. On the contrary, the increase of pyocyanin production following SPA0084 overexpression appeared to be LasR-independent. These results suggested that RhlR, PqsR and/or functions which they regulate could be targets of SPA0084-mediated regulation.

The strains above were also tested for biofilm formation and swarming motility. As shown in Fig. 4B, deletion of SPA0084 resulted in a decrease of biofilm production while SPA0084 overexpression stimulated biofilm production in both PA14 wt and PA14 *Δspa0084*. The enhancement of biofilm formation following SPA0084 overexpression seemed to be independent from LasR, since in the *ΔlasR* mutant it was comparable to that observed in both PA14 wt and PA14 *Δspa0084*. In addition, some stimulation of biofilm formation, at lesser extent than PA14 wt, PA14 *ΔlasI* and PA14 *ΔlasR* strains, could be observed in PA14 *pqsR*-. On the contrary, some degree of dependency on RhlR was suggested by SPA0084 overexpression in the *ΔrhlR* strain. In fact, in this case, no significant increase of biofilm formation was observed. Finally, SPA0084 appeared to have opposite effects on swarming with respect to pyocyanin production and biofilm formation. As shown in Fig. 5A, PA14 *Δspa0084* showed enhanced swarming with extensive tendril formation. On the contrary, SPA0084 overexpression strongly repressed swarming in both PA14 wt and PA14 *Δspa0084*. These negative effects became evident also in the *Δlas*R, *ΔlasI* and *pqsR*- strains (Fig. 5C). Surprisingly, in the case of PA14 *ΔrhlR,* which is motility deficient [43], swarming seemed to be slightly increased following SPA0084 overexpression. Overall, these results suggested that SPA0084 plays a positive regulatory role in pyocyanin production and biofilm formation while its role appears to be negative in swarming motility. These effects cannot be unspecifically due to an influence of SPA0084 on growth rate. In fact, neither SPA0084 deletion nor its overexpression affect *P. aeruginosa* growth rate (Fig. S3).

SPA0084 positively regulates the *pqsC* gene.

We speculated that SPA0084 had a potential to acts as a *trans*-encoded base-paring sRNA. To predict target genes, we used the SPA0084 full-length sequence as input in three different web tools: TargetRNA2 [44], RNA Predator [45] and sTarPicker [46]. Coherently, these three tools reported as candidate target with high energy and confidence scores a region in the mRNA of the *pqsABCD* genes responsible of the production of the PQS-precursor HHQ. In particular, the predicted target region was located at the 3' end of *pqsB* mRNA spanning -49 to -28 positions from the translation start site of *pqsC* (Table S3). We named this region RI (Fig. 6A). The interaction of SPA0084 with *pqsABCD* mRNA was further assessed with IntaRNA web tool [47]. This tool predicted again RI as target region. In addition, IntaRNA predicted the interaction of SPA0084 with a region spanning +18 to +28 positions from the translation start site of *pqsC* that we named RII (Table S3; Fig. 6A; Fig. S4). As shown in Fig. 6A, the interaction with RI and RII region involves a SPA0084 region from nt 28 to 63 including the whole stem loop SL2, and the short flanking SL1-2 and SL2-3 connecting regions. To test the effects of the SPA0084-*pqsBC* mRNA interactions, we generated a reporter plasmid carrying a region spanning -94 from *pqsC* translation start site and the first +22 codons of *pqsC* mRNA translationally fused with sfGFP gene under the control of the heterologous constitutive promoter $P_{Ltet0\text{-}1}$. GFP activity was assayed in PA14 *wt* and PA14*Δspa0084* in the absence and presence of SPA0084 overexpression from pGM-*spa0084*. As shown in Fig. 6B, there was an approximately threefold reduction in GFP activity in the PA14 *Δspa0084* background without SPA0084 overexpression. In the presence of pGM-*spa0084* overexpressing SPA0084, both in *wt* and *Δspa0084* backgrounds, GFP activity increased approximately twofold. These results suggested that SPA0084 positively regulates *pqsC* expression. Spurious outside interactions of SPA0084 with the GFP open reading frame were controlled using an alternative reported plasmid carrying exclusively the *gfp* gene. SPA0084 deletion or overexpression caused no significant effects on *gfp* gene alone (Fig. 6B).

SPA0084 RNA stimulates PQS synthesis

The results presented above strongly suggested that SPA0084 positively influences the translation of *pqsC* mRNA. We speculated that this effect could impact positively the levels of the products, in particular HHQ, of the biosynthetic pathway encoded by *pqsABCD* genes. Furthermore, since HHQ is the precursor converted by the product of *pqsH* gene to the signal molecule PQS, SPA0084 regulation could also impact positively PQS levels. To test this hypothesis, AQs molecules were assayed in the supernatants of stationary phase cultures of PA14 *wt* and PA14*Δspa0084* in the absence and presence of SPA0084 overexpression from pGM-*spa0084*. As shown in Fig. 7, SPA0084 deletion resulted in a decrease of PQS levels while SPA0084 overexpression enhanced PQS accumulation in both PA14 wt and PA14 *Δspa0084*.

SPA0084 increases PA14 killing activity in the *G. mellonella* model of infection.

Due to the importance of AQs for regulation of *P. aeruginosa* virulence and contribution to the severity of infection, we performed *G. mellonella* killing assays to assess the effects of both deletion and overexpression of SPA0084. For each strain used, *G. mellonella* larvae were infected by the injection of 10 µl aliquots containing about 20 cells derived from washed overnight cultures. The number of dead larvae was scored from 16 to 24 hours post-infection. As shown in Fig. 8, PA14 *Δspa0084* killing activity was fivefold lower than PA14 wt. Conversely, SPA0084 overexpression resulted in a hypervirulent phenotype. One hundred percent of the larvae were dead in about 21 hours with PA14 *Δspa0084* overexpressing SPA0084 from pGM*spa0084*.

Discussion

We studied the novel *P. aeruginosa* sRNA SPA0084, which appears highly conserved among the sequenced *P. aeruginosa* strains. Overall, our results suggest that SPA0084 is embedded in the *P. aeruginosa* QS playing a role in wiring *las* to *pqs* systems (Fig. 9). The *las*-*pqs* connections described so far [34] base on LasR that, when bound to 3OC12-HSL activates transcription of *rhlR* and *pqsR*. PqsR, bound to the cognate signal molecules PQS or HHQ, positively regulates *pqsABCD* genes, which are required for HHQ and PQS synthesis, the latter in combination with *pqsH* activated by LasR/3OC12-HSL. Conversely, RhlR, bound to C4-HSL, negatively regulates both *pqsR* and *pqsABCD.* PQS and HHQ both function as autoinducers since they each drive the expression of *pqsABCD* in a *pqsR*-dependent manner. PQS and HHQ synthesis is thus positively auto-regulated via PqsR/HHQ/PQS and *las*, and down-regulated by *rhl*. We suggest that, from basal *pqsABCD* transcription, the positive post-transcriptional regulation of SPA0084 on the *pqsABCD*-encoded biosynthetic pathway could assure the achievement of threshold levels of HHQ/PQS required to trigger the *pqsR*-dependent autoinduction. Once auto-induction is triggered, SPA0084 could act further to potentiate the positive feedback loop. These SPA0084 roles would be coupled to *las* by the negative regulation of LasR/3OC12-HSL. We suggest that a fine balancing between the different regulatory effects of LasR on HHQ/PQS synthesis, at both transcriptional (direct positive on *pqsR* and *pqsH*; indirect negative via RhlR on *pqsR* and *pqsABCD*) and post-transcriptional level (indirectly via SPA0084) can influence timing and magnitude of expression of QS-regulated virulence factors.

This view is consistent with the evidence that perturbations of SPA0084 levels affect pyocyanin synthesis, biofilm formation and swarming motility, processes that are known to be influenced by HHQ/PQS synthesis (Fig. 10). In the case of pyocyanin production, we suggest that the SPA0084-mediated modulation of HHQ/PQS levels directly impact the pathways of activation of pyocyanin biosynthetic genes dependent on PqsR/HHQ/PQS, RhlR/C4-HSL and PqsE [48] [32]. The lack of responsiveness in terms of pyocyanin synthesis to SPA0084 overexpression in both Δ*rhlR* and *pqsR*- strains strongly support this hypothesis. Also in case of biofilm formation, the role of SPA0084 in influencing HHQ/PQS levels could be directed to the *pqsR*/*rhlR*/*pqsE* pathway known to induce the

expression of the lectin gene *lecA* [35] that has been shown to play an important role in maintain biofilm architecture [30]. In addition, there could be a direct role of pyocyanin in SPA0084-mediated modulation of biofilm. In fact, pyocyanin was shown to promote the release of extracellular DNA (eDNA) [49]. Furthermore, pyocyanin intercalation with eDNA enhances cell-to-cell interactions by influencing cell surface properties and physico-chemical interactions [50]. The moderate increase of biofilm formation in the *pqsR*$^{-}$ mutant following SPA0084 overexpression suggests also that, at lesser extent, SPA0084 might influence biofilm formation via *pqsR*-independent mechanisms.

Swarming motility and biofilm formation have an inverse relationship in PA14 strain [51]. Inversely regulating biofilm and swarming, SPA0084 overexpression seems to recapitulate the PA14 behavior. However, conversely to biofilm, the repression of swarming motility when SPA0084 is overexpressed appears to be completely *pqsR*-independent. We suggest that, even in the absence of autoinduction (i.e. in a *pqsR*$^{-}$ background), HHQ/PQS levels can reach, under SPA0084 stimulation from basal *pqsABCD* transcription, levels that suffices to repress swarming motility through unknown PqsR-independent mechanisms similar to those described for PAO1 strain [39]. However, the regulation by SPA0084 of functions that inversely regulate biofilm formation and swarming motility, such as the diguanylate cyclase SadC [52] and phosphodiesterase BifA [53] cannot be ruled out.

Besides being negatively regulated by LasR/3OC12-HSL, our results indicate that SPA0084 responds to infection relevant cues that *P. aeruginosa* can experience in mammalian hosts such as temperature and oxygen availability. Furthermore, SPA0084 shows a growth phase-dependent pattern of expression, being up-regulated in stationary phase. The -10 motif in SPA0084 promoter is identical to the heptamer consensus CTATACT recognized by RpoS. This suggests that the growth phase-dependent pattern of SPA0084 expression is due to the activity of this alternative σ factor whose significance as global factor controlling *quorum sensing* gene expression was shown previously [41]. Together, these SPA0084 regulations are expected to contribute to the fine co-modulation of HHQ/PQS synthesis (Fig. 10).

Our results suggest that the stimulation of PQS synthesis is mediated by a positive effect of SPA0084 on translation of *pqsC* mRNA. For this regulation, we propose a model in which *pqsC* translation is self-repressed by a stem-loop which blocks ribosome access and the SPA0084 role is to release this block (Fig. S4). SPA0084 is predicted to bind to mRNA regions RI and RII centered 46 nt upstream and 21 nt

downstream the translation start site of *pqsC*, respectively. As shown in Fig. S4, the region between nt -13 and +32 was predicted to form a large stem-loop structure in which both SD and AUG of *psqC* are trapped by base pairing to RII region. We postulate that SPA0084 binding to RII results in destabilization of stem-loop structure that can promote access of the 30S ribosome subunit on the mRNA, thereby allowing translation initiation. In this context, we suggest that SPA0084 binding to RI plays the role of overall stabilization of SPA0084/mRNA interaction.

Finally, we tested the *in vivo* relevance of SPA0084 with the *G. mellonella* model of systemic (acute) infection. As expected, SPA0084 revealed to be a positive regulator of virulence. Strikingly, its overexpression resulted in a hypervirulent phenotype. This effect can be clearly attributable to enhanced expression of *pqs*-controlled virulence factors.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* strains were routinely grown at 37°C in LB. *P. aeruginosa* strains were grown at 30°C or 37°C in BHI-rich medium or in LB at 120 rpm. Unless otherwise indicated, antibiotics were added at the following concentrations: for *E. coli*, 20 µg/ml gentamicin, 100 µg/ml ampicillin, 50 µg/ml chloramphenicol, 50 µg/ml kanamycin; for *P. aeruginosa* 60 µg/ml gentamicin, 50 µg/ml chloramphenicol, 100 µg/ml km, 100 µg/ml tetracycline, 300 µg/ml carbenicillin. To induce the P_{BAD} promoter in plasmid pGM931, arabinose was added to media at a final concentration of 10 mM.

Anaerobic batch cultivations of *P. aeruginosa* were performed in a Biostat-Q system bioreactor (B-Braun) at 37°C with a stirring speed of 500 rpm in a working volume of 800 mL BHI supplemented with 100 mM KNO_3 to allow anaerobic respiration. Oxygen was excluded from the bioreactor by flushing nitrogen (<3 ppm O_2) with a flow of 0.1–0.31 L/min. Norprene tubes were used to minimize the diffusion of oxygen into the bioreactor. The dissolved oxygen concentration was measured by a Mettler Toledo polarographic oxygen probe. To assess the SPA0084 response to a shift from aerobic to anaerobic conditions, bacterial cells were grown aerobically in BHI supplemented with 100 mM KNO_3 with a stirring speed of 500 rpm until an OD_{600} of 0.8. Then, oxygen was excluded from the bioreactor by flushing nitrogen, and cultivation continued until stationary phase.

Plasmid construction and mutant generation

To construct plasmid pGM-*spa0084*, the *spa0084* wild type gene was amplified by polymerase chain reaction (PCR) from PA14 genomic DNA with primers 6 and 7 (Tab. S2). The PCR products were digested with *NcoI-PstI* and cloned into the shuttle vector pGM931, a pHERD20T [54] derivative carrying a transcriptional terminator downstream of the multiple cloning site [55]. To generate a transcriptional fusion between the *spa0084* promoter ($P_{spa0084}$) and the gene of super-folder variant of the green fluorescent protein (sfGFP), a DNA fragment including the $P_{LtetO-1}$ promoter was removed from plasmid pXG10-SF by digestion with *AatII-NsiI* and replaced with a DNA fragment spanning -156 to + 65 with

respects to the *spa0084* transcription start site amplified using primers 3 and 4 (Tab. S2). This procedure gave rise to pXG-$P_{spa0084}$-*gfp*. A DNA fragment spanning $P_{spa0084}$ and *gfp* gene was amplified from pXG-$P_{spa0084}$-*gfp* using primers 5 and 14 (Tab. S2), digested with *ClaI* and cloned into the shuttle vectors pBBR2-MCS2 and pBBR5-MCS5, giving rise to pBBR2-$P_{spa0084}$-*gfp* and pBBR5-$P_{spa0084}$-*gfp*, respectively. A directional screening was performed using primer 15, 25 and 24 (Tab. S2), to identify plasmids containing the $P_{spa0084}$-gfp sequence cloned in the opposite direction with respect to the pBBR1 and pBBR5 *lac* promoter.

Plasmid pBBR1-$pqsC_{RI\text{-}II}$::*gfp* expressing the $pqsC_{RI\text{-}II}$::*gfp* translational fusion under the control of $P_{LtetO\text{-}1}$ was constructed as follows. A DNA fragment spanning -94 from *pqsC* translation start site and the first +22 codons of *pqsC* mRNA was amplified from PA14 chromosomal DNA with primers 10 and 11 (Tab. S2), digested with *PstI-NheI* and cloned into the sfGFP reporter vector pXG30-SF, giving rise to plasmid pXG30- $pqsC_{RI\text{-}II}$::*gfp*. The DNA fragment spanning $P_{LtetO\text{-}1}$ and the $pqsC_{RI\text{-}II}$::*gfp* fusion was amplified from pXG30-$pqsC_{RI\text{-}II}$::*gfp* with primers 12 and 13 (Tab. S2), digested with *ClaI-XbaI* and cloned into pBBR1-MCS5.

PAO1 and PA14 *Δspa0084* mutants were generated by allelic exchange with a deletion from −23 to +96 with respect to the *spa0084* transcription start site by an enhanced method of marker-less gene replacement [56] with some modifications [17] as follows. The TS1 region spanning the left 533-bp flanking sequence of *spa0084* was amplified with primers 18 and 19 (Tab. S2). The TS2 region spanning the right 500-bp flanking sequence of *spa0084* was amplified with primers 20 and 21 (Tab. S2). PCR was performed from both PAO1 and PA14 genomic DNA. SOE-PCR with oligos 18 and 21 (Tab. S2) was used to join TS1 and TS2. The joined TS1-TS2 DNA fragments were digested with *SacI-BamI* and cloned in CC118 λ*pir* into the poly-linker site of pSEVA612S, giving rise to pSEVApao1-*Δspa0084* and pSEVApa14-*Δspa0084.* These two plasmids were transferred from *E. coli* CC118 λpir to PAO1 and PA14, respectively, with the assistance of the helper E. coli strain HB101(pRK600) in a conjugative triparental mating [57]. Exconjugant *P. aeruginosa* clones were selected on M9-citrate with 60 μg/mL 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. The co-

integration process introduces into the recipient chromosome two sites for the I-SceI endonuclease flanking the multiple cloning site of pSEVA612S. Transient I-SceI endonuclease expression from pSW-I stimulates homologous recombination at the integration site of the pSEVA612S derivative and strongly enhances the resolution leading to allelic exchange [56]. Plasmid pSW-1 was transferred from *E. coli* DH5 to *P. aeruginosa* clones bearing genomic co-integrates of pSEVA612S derivatives by triparental mating as above, and pSW-1-recipient *P. aeruginosa* clones were selected on M9-citrate with 300 µg/mL carbenicillin. Cultures of resulting *P. aeruginosa* clones carrying pSW-I were grown overnight in LB with 300 µg/mL 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 pair 22/23 (Tab. S2). All plasmid constructs and deletion mutants were checked by sequencing with oligos indicated in Tab. S2. The *spa0084* chromosomal deletions and the SPA0084 overexpression from pGM-*spa0084* were validated by Northern blot (Fig. S1).

RNA isolation and analysis

Total RNA extractions and Northern blot analyses were performed as described previously [17, 40]. DNA oligonucleotides 1 and 2 (Tab. S2) were used to probe SPA0084 and 5S RNA, respectively. The SPA0084 transcription start site (+1) was investigated by Northern blot with DNA oligonucleotides val0 and val1 (8 and 9, Tab. S2). Treatment with terminator 5'-phosphate-dependent exonuclease was performed as described previously [17].

In vivo assays for sRNA/mRNA interactions and promoter activity

The *in vivo* analysis of the GFP reporter systems was performed as previously described [17]. *P. aeruginosa* PA14 strains carrying the reporter pBBR1-*pqsC*$_{RI\text{-}II}$*::gfp* or pBBR1-*gfp* combined with pGM931 or pGM-*spa0084* were grown at 37°C in LB with carbenicillin, gentamicin, and 0.2% arabinose. The *E. coli* strains carrying the reporter pXG-P$_{spa0084}$-*gfp* combined with pET28b or pET28b-LasR were grown at 37°C in LB added with chloramphenicol, kanamycin and where indicated 5µM 3OC$_{12}$-HSL. The *P. aeruginosa* PAO1 and PA14 strains carrying the reporter plasmids of $P_{spa0084}$ activity were grown in LB at 37°C with kanamycin (pBBR2-$P_{spa0084}$-*gfp*) or gentamycin (pBBR5-$P_{spa0084}$-*gfp*). All reporter strains

were inoculated in 15 ml tubes at an OD600 of 0.1 in 5 ml of medium. After 24 h, cell samples were collected by centrifugation, washed twice, and resuspended in PBS (10 mM Na_3PO_4, 150 mM NaCl), and 200 µl aliquots were transferred to black polystyrene 96-well microplates with a clear, flat bottom (Corning). The absorbance (Abs_{595}) and fluorescence polarization ($FP_{485/535}$) were measured in a Tecan Infinity PRO 200 reader using Magellan data analysis software (Tecan). GFP activity was expressed in arbitrary units (AU) as $FP_{485/535}/Abs_{595}$. All analyses included three independent experiments.

Pyocyanin production assay

The pyocyanin assay was performed as described previously [58]. *P. aeruginosa* cultures were inoculated from a fresh colony and grown overnight in LB supplemented with appropriate antibiotics at 37°C. Cultures were then diluted at OD_{600} of 0.05 and re-incubated for 18 h at 37°C in 20 ml LB supplemented with antibiotics and arabinose. A 5 ml sample of culture was extracted with 3 ml of chloroform and then re-extracted with 2 ml of 0.2 N HCl. The absorbance of the resulting solution was measured at 520 nm. Concentrations of pyocyanin expressed as µg/ml of culture supernatant were determined using the following formula: $[(A_{520} \times 2)/A_{600} \times 17.072] \times 0.66$.

Biofilm formation assays

Biofilm formation was determined by Crystal Violet staining assay modified from [59]. From overnight cultures grown at 37°C in LB medium supplemented with appropriate antibiotics, bacteria were inoculated in 200µl of BHI at OD_{600} 0.05 in polystyrene microtiter plates (96-Well Cellstar® Cell Culture Plate, U-bottom, Greiner Bio-One) and grown for 48h at 37°C. Cell density of planktonic bacteria was determined by measuring OD_{600}nm. Planktonic cells were removed, and cells attached to microtiter plates were stained with 1% (w/v) Crystal Violet. Crystal Violet staining biofilm cells was solubilized in 0.2 ml of 95% ethanol and measured at OD_{600}. The amount of "adhesion units" was calculated by a ratio between the OD_{600} of Crystal Violet staining biofilm cells and the OD_{600} of planktonic cells. All analyses included three independent experiments.

Motility assays

Swarming assays were performed as described previously [60]. 3µl-aliquots of bacterial cultures diluted at OD_{600} of 3 were spotted onto Swarming Plates (5 g/l Bacto Agar, 8 g/l nutrient broth N°2, 28 mM glucose) and grown for 18 hr at 30 or 37°C. For swimming assays, 1.5 µl-aliquots of bacterial cultures diluted at OD_{600} of 3 were spotted onto 'Swimming Plates' (0.3 % Bacto Agar Difco added to LB) and grown for 18 h at 30°C.

AQs extraction and TLC analysis

The AQs extraction and TLC analysis were performed as described previously [61]. Cell-free supernatants were prepared from cultures grown in 100 ml of LB medium in aerobic conditions until an OD_{600} of 5. Supernatants were obtained by centrifugations at 5000 rpm for 15 min and then 10 ml-aliquots were filtered through sterile Minisart 0.2 µM filters (Sartorius) to remove any un-pelleted cell. AQs were extracted 3 times with an equal volume of acidified ethyl acetate. The collected organic phases were dried over Na_2SO_4 and the solvent was removed under vacuum; the residue was suspended in 100 µl of methanol. 5 µl-samples were spotted onto normal-phase silica 60F254 TLC plates (Merck) (pretreated with 5% K_2HPO_4 for 30 min and activated at 100°C for 1 h) and eluted using a dichloromethane/methanol (95:5, v/v) until the solvent front reached the top of the plate. PQS and HHQ were visualized under UV light at 254nm, PQS only under UV light at 366 nm. 2 µl of 20 mM stocks of synthetic PQS and HHQ were spotted as references.

Galleria mellonella killing assay

Bacterial overnight cultures grown in LB were pelleted by centrifugation at 5000 rpm for 10 min at 4°C, washed twice with 10 mM $MgSO_4$, resuspended at OD of 1 ($1x10^9$ cell/ml) in 10 mM $MgSO_4$ and adjusted to $2x10^3$ cells/ml of by serial dilutions in 10 mM $MgSO_4$. 10 µl-aliquots (about 20 bacterial cells) were used to infect ten *G. mellonella* larvae for each bacterial strain. Following infection, larvae were incubated at 37°C and scored dead/live from 16 to 24 hours post-infection. A larva was considered death when it displayed no movement in response to touch. A mock inoculation with 10 µl of 10 mM $MgSO_4$ was performed in each experiment to monitor the killing due to physical injury or infection by

pathogenic contaminants. To assess the number of infecting bacterial cells, CFU/ml were determined for the bacterial suspensions used for infection.

Statistical analysis

Statistical significance was determined by calculating the *p*-values using the two-tailed Student-t test for unpaired data sets; differences with a *p*-value ≤ 0.05 were considered statistically significant.

Supporting Information

Table S1. Strains and plasmids

Table S2. Oligonucleotides

Table S3. Bioinformatics analysis of the interaction of SPA0084 with *pqsC* mRNA

Fig. S1. Alignment of the intergenic region containing the *spa0084* gene of thirteen *P. aeruginosa* sequenced strains.

Fig. S2. Validation of overexpression levels of SPA0084 in PAO1 and PA14 strains.

Fig. S3. *P. aeruginosa* growth is not affected by SPA0084 overexpression or deletion.

Fig. S4. Model of SPA0084 mediated activation of *pqsC* translation.

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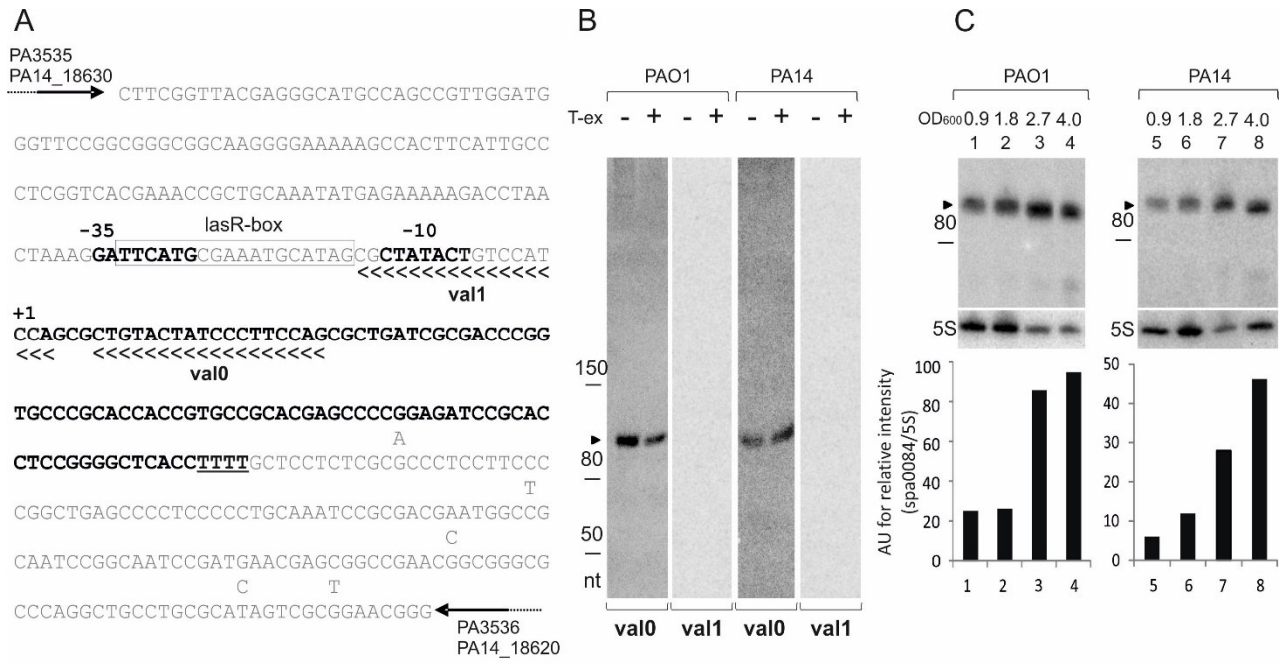

Fig. 1. SPA0084 genomic context and expression analysis.

A. Comparison of the *P. aeruginosa* PAO1 and PA14 intergenic region between PA3535 and PA3536 PAO1 ORFs and PA14_18630 and PA14_18620 PA14 ORFs. At positions with different bases between the two strains, the PAO1 alternative is reported above. The *spa0084* sequence is in bold. The predicted transcription start site of *spa0084* is indicated with +1, while the transcription terminator poly-T tail is underlined. The DNA sequence identity shared by PAO1 and PA14 extends upstream of the +1 position and includes the −35 sequence GATTCATG and the −10 sequence CTATACT that strictly matches the eptamer consensus sequence recognized by RNA polymerase containing the σ factor RpoS. A *las-rhl* consensus box overlapping the -35 site and the downstream -35/-10 spacer is indicated. Two oligo probes, Val1 (from nt -15 to +3) and Val0 (from nt +7 to + 24) used for Northern blot analysis in (B) are indicated by arrowheads. **B**. Northern blot analysis of SPA0084 on 10 µg of total RNA extracted from PAO1 and PA14 at the end of the exponential growth phase, treated (+) or untreated (−) with terminator 5'-phosphate-dependent exonuclease (T-ex). The arrowhead indicates the primary transcript of ≈100 nt. **C**. Levels of SPA0084 in PAO1 and PA14 LB medium at 37°C. Samples were taken at the indicated OD_{600} and processed for total RNA extraction and analysis by Northern blot. Intensities of the bands of SPA0084 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.

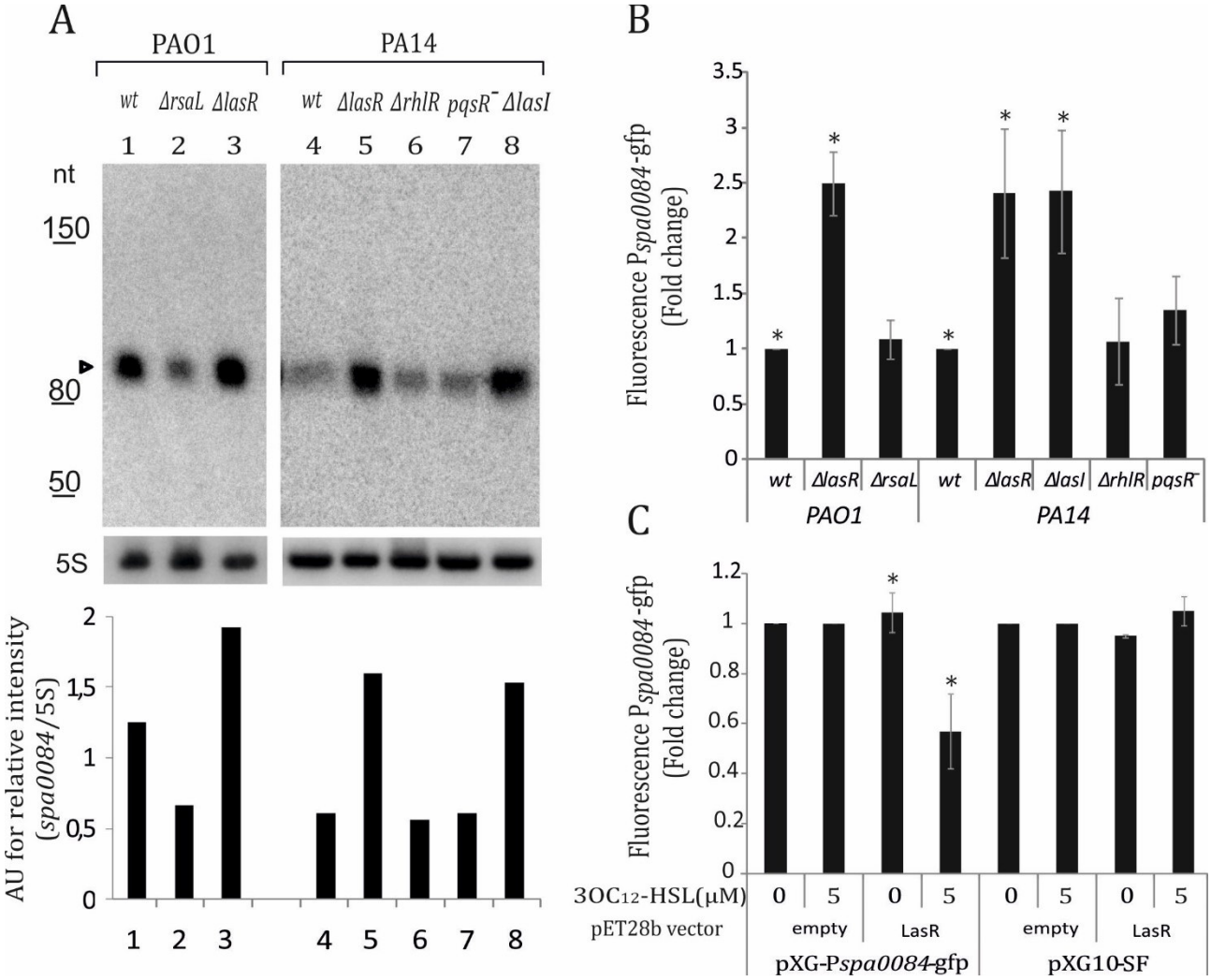

Fig. 2. SPA0084 expression is negatively regulated by LasR-3OC12-HSL complex.
A. Levels of SPA0084 in PAO1 wild-type, PAO1 *ΔrsaL*, PAO1 *ΔlasR*, and PA14 wild-type, PA14 *ΔlasR*, PA14 *ΔrhlR*, PA14 *pqsR*-, PA14 *lasI*. These strains were inoculated in BHI at OD_{600} of 0.1 and grown at 37°C with agitation. At late exponential phase, culture samples were taken and processed for total RNA extraction and analysis by Northern blot. Intensities of the bands of SPA0084 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. **B.** Comparison of the fluorescence polarization resulting from PAO1 wild-type, PAO1 *ΔrsaL*, PAO1 *ΔlasR*, and PA14 wild-type, PA14 *ΔlasR*, PA14 *ΔrhlR*, PA14 *pqsR*-, PA14 *ΔlasI* carrying the $P_{spa0084}$ promoter transcriptional fusions with sfGFP cloned into pBBR2-$P_{spa0084}$-*gfp* or pBBR5-$P_{spa0084}$-*gfp*. The results are expressed in fold changes with respect to *wt* strains. Asterisks indicate a p-value<0.05. **C.** Comparison of the fluorescence polarization from *Escherichia coli Top10* strain, carrying the $P_{spa0084}$ promoter transcriptional fusions with sfGFP cloned into the pXG-$P_{spa0084}$-*gfp* combined with the control vector pET28b (empty) or pET28b-LasR (LasR), in absence (0) or presence (5) of 5μM $3OC_{12}$-HSL. The pXG10-SF containing only the sfGFP gene was used as negative control. The results are expressed in fold changes with respect to strains carrying the pET28b empty vector in the absence of $3OC_{12}$-HSL. Asterisks indicate a p-value<0.05.

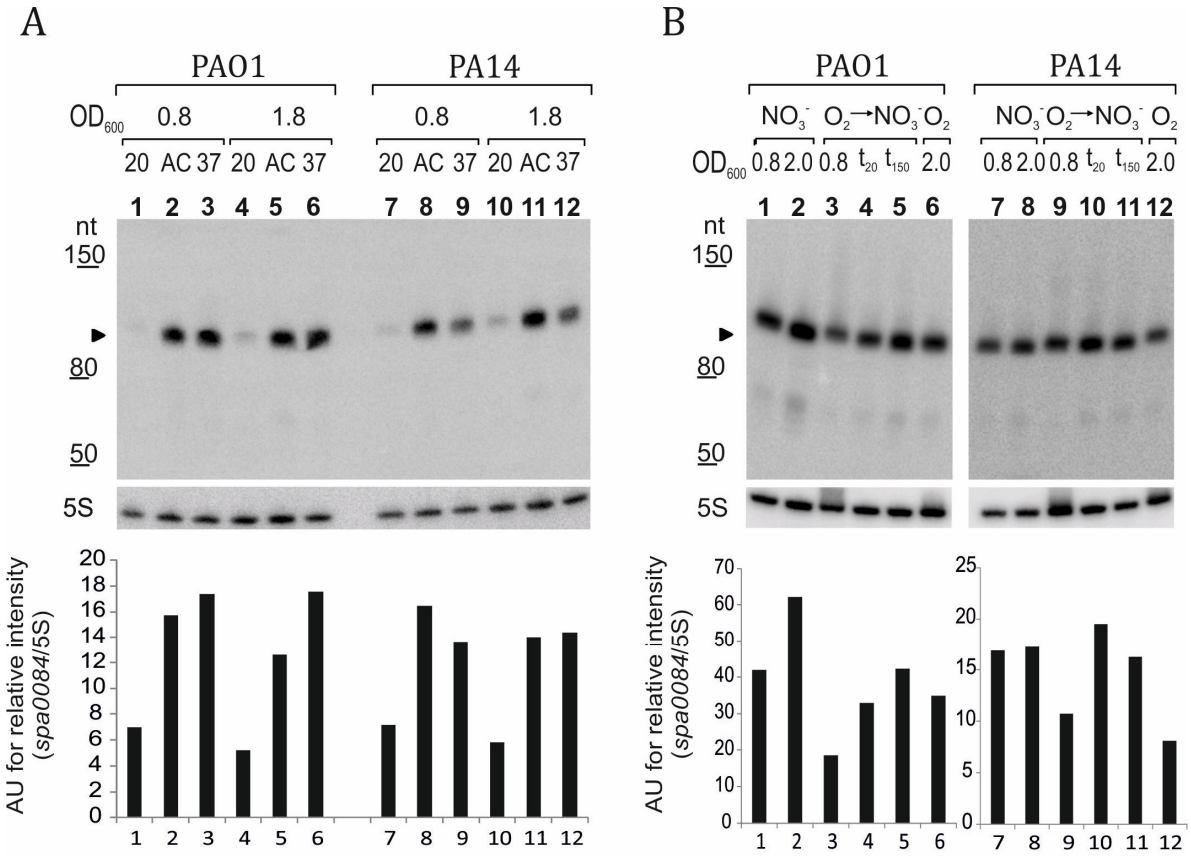

Fig. 3. SPA0084 expression is induced by temperature shift and low availability of oxygen.

A. Levels of SPA0084 in PAO1 and PA14 grown in BHI at 20°C (lanes 1, 4, 7 and 10), 37°C (lanes 3, 6, 9 and 12) or following 20 min of acclimation (AC) from 20 to 37°C (lanes 2, 5, 8 and 11). Culture samples were taken at middle (OD_{600} of 0.8) and late (OD_{600} of 1.8) exponential growth phase. **B.** Levels of SPA0084 in PAO1 and PA14 grown in BHI anaerobically (NO_3^-; lanes 1, 2, 7 and 8), aerobically (O_2) (lanes 6 and 12) and aerobically until an OD_{600} of 0.8 and then shifted from aerobic to anaerobic conditions ($O_2 \rightarrow NO_3^-$; lanes 3, 4, 5, 9, 10 and 11). 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 SPA0084 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.

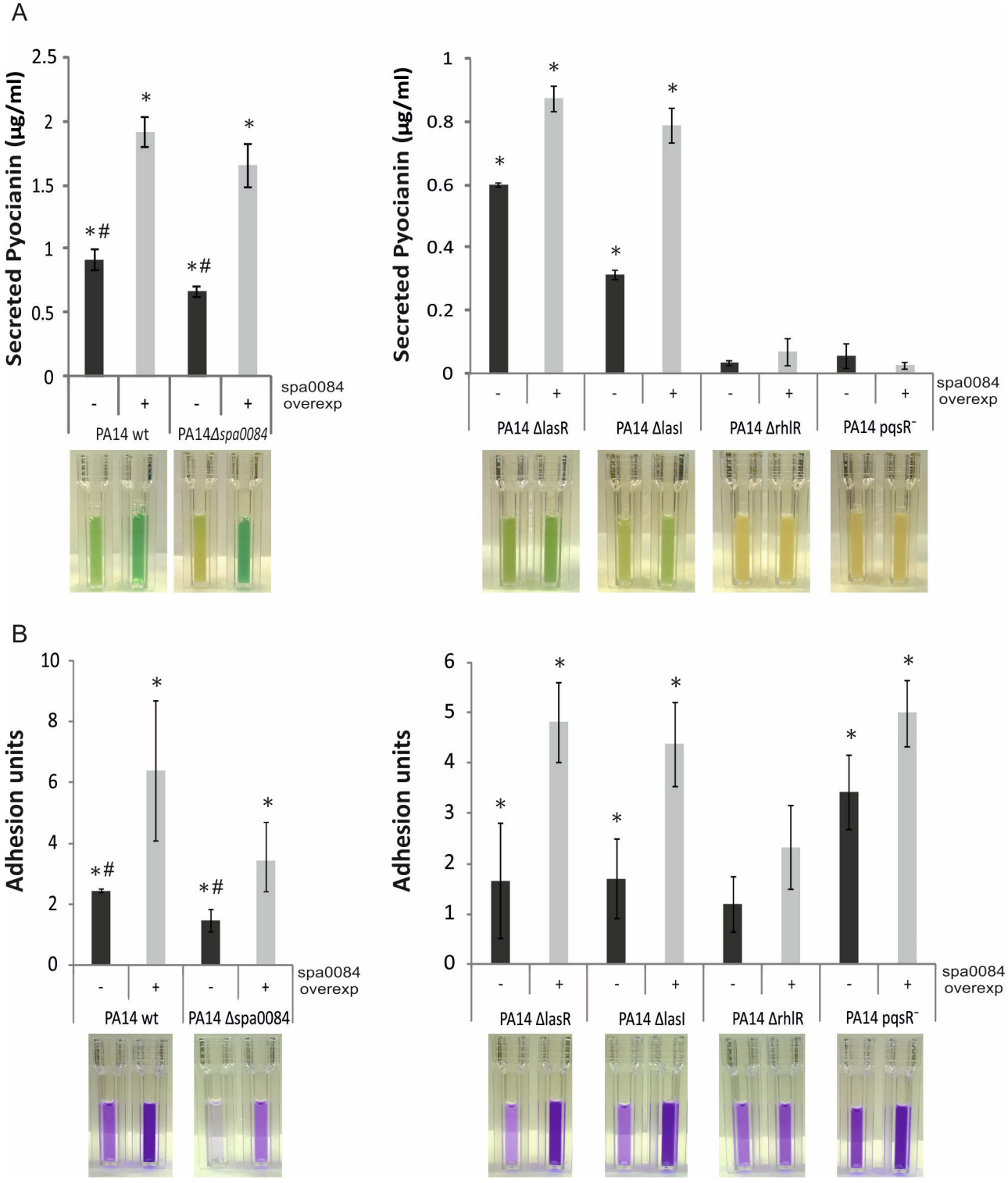

Fig. 4. SPA0084 positively regulates pyocyanin production and biofilm formation.
A. Pyocyanin production was assessed after 18 h of growth ($OD_{600} \simeq 4.5$) in LB supplemented with carbenicillin and arabinose starting from 5 ml of culture supernatant of the indicated strains carrying pGM931 empty vector (-) or pGM*spa0084* (+). The amount of pyocyanin was calculated using the following formula: $[(A_{520} \times 2)/(A_{600} \times 17.072)] \times 0.66$ = µg of pyocyanin per ml modified from Essar, 1990. Images of culture samples before pyocyanin extraction are reported below charts. **B**. Biofilm formation assays of the indicated strains carrying pGM931 empty vector (-) or pGM*spa0084* (+) grown in BHI supplemented with carbenicillin and arabinose for 48 h at 37°C in polystyrene 96-well microtiter plates. Adhesion units were calculated using the following formula: (OD_{600} of Crystal Violet staining biofilm cells)/(OD_{600} planktonic cells). Images of samples of the ethanol volumes used to solubilize Crystal Violet bound to biofilm cells are reported below charts. Asterisks and hashes indicate a p-value<0.05.

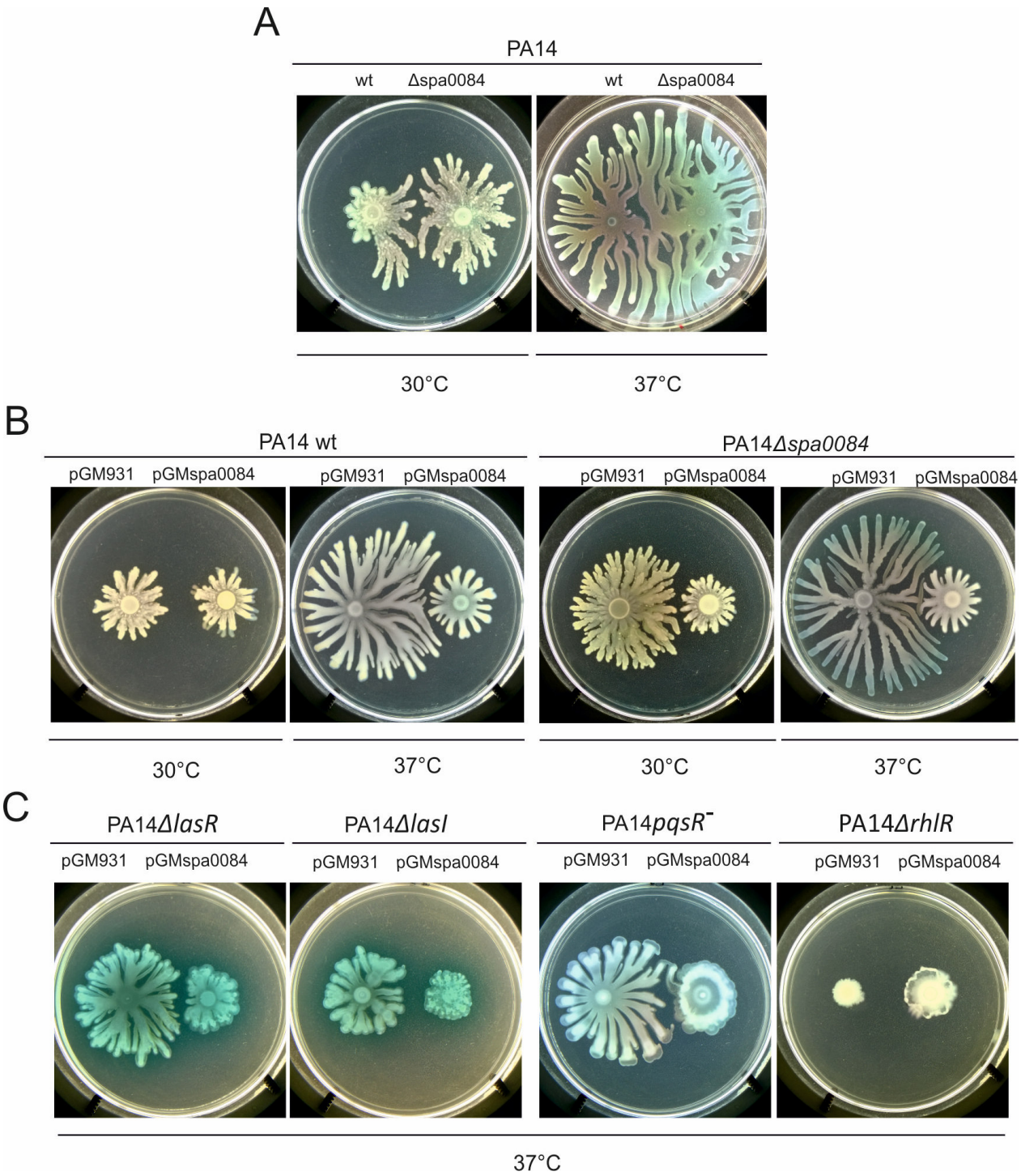

Fig. 5. SPA0084 negatively regulates swarming motility.
Swarming motility assays of: **A.** PA14 *wt* and PA14 *Δspa0084*. **B.** PA14 *wt* and PA14 *Δspa0084* carrying either pGM931 or pGM-*spa0084*. **C.** PA14 *ΔlasR,* PA14 *ΔlasI, PA14pqsR*-and PA14*ΔrhlR* quorum sensing mutants carrying either pGM931 or pGM-*spa0084*. For all strains, 3µl of an overnight culture diluted at OD_{600} of 3 were spotted onto swarming plates and incubated at the indicated temperature. For strains carrying either pGM931 or pGM-*spa0084* swarming plates were supplemented with carbenicillin and arabinose. Pictures were taken after 18 h of incubation.

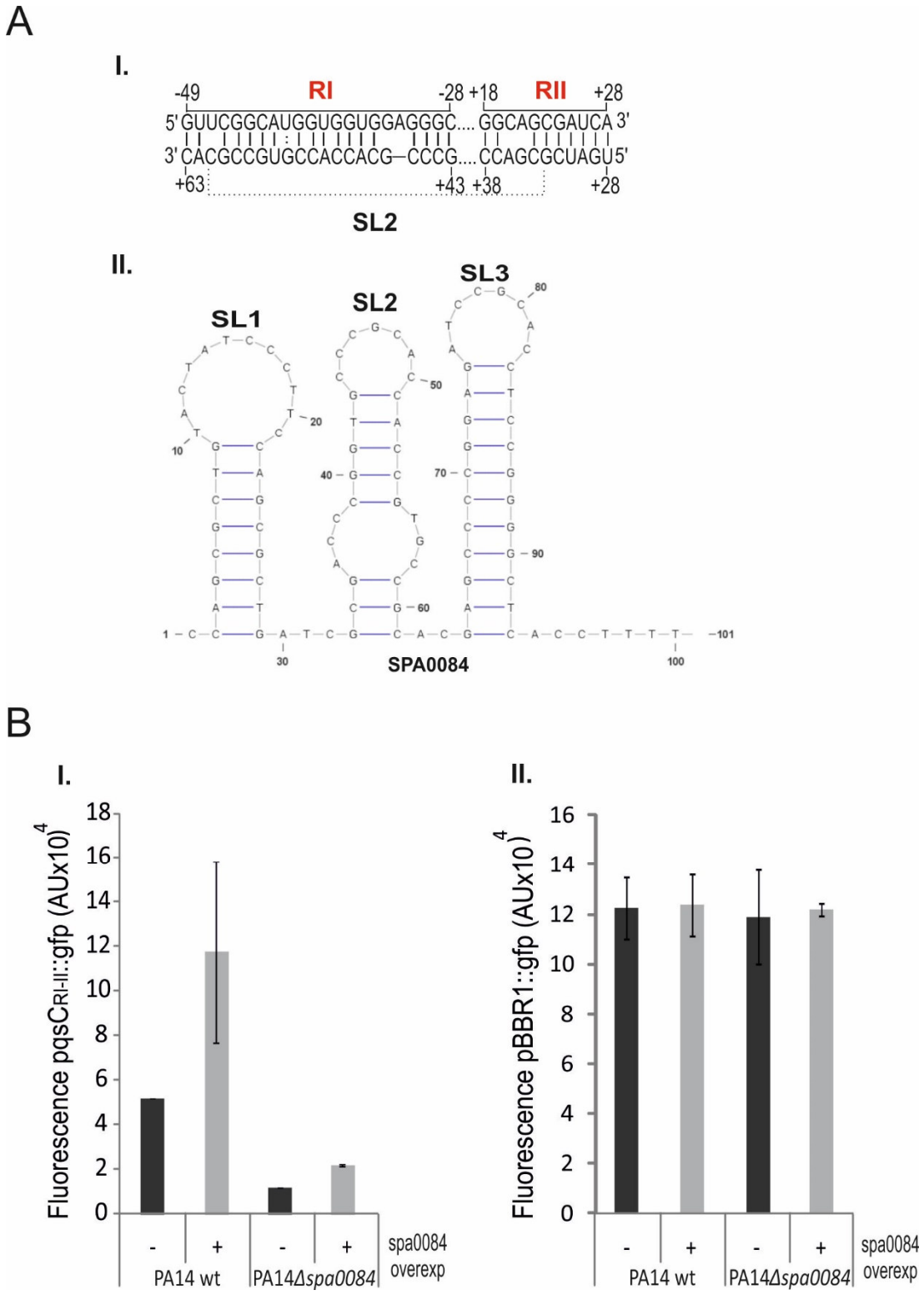

Fig. 6. SPA0084 positively regulates *pqsC* expression.
A. (I) Sequence alignment between regions RI and RII of *pqsC* mRNA and SPA0084. The SPA0084 region implicated in the interaction with the *pqsC* mRNA encompassing stem-loop SL2 (see below) is indicated. (II) The SPA0084 RNA secondary structure predicted by Mfold tool that contains three stable stem-loops (SLs), SL1 (nt 2-29), SL2 (nt 33-61) and SL3 (nt 64-93), the latter having a strong resemblance to a Rho-independent transcription terminator. **B.** (I) Comparison of the fluorescence polarization expressed in arbitrary units (AU) resulting from translational fusion *pqsC*$_{RI-II}$*::gfp* (I) or the *sfGFP* control alone (II) combined with the control vector pGM931 (-) or the plasmid pGM-*spa0084* (+) in PA14 *wt* and PA14 *Δspa0084* strains.

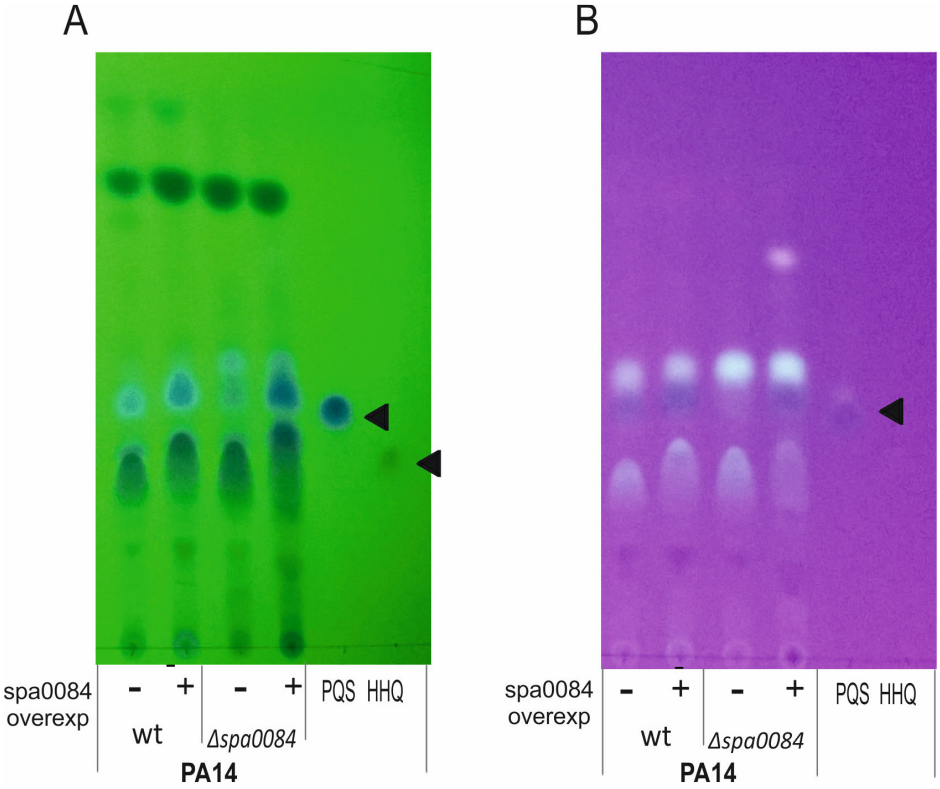

Fig. 7. SPA0084 stimulates the synthesis of PQS.

TLC analysis of AQs extracted from cell-free supernatants resulting from cultures of PA14 *wt* and PA14 *Δspa0084* strains carrying pGM931 empty vector (-) or pGM*spa0084* (+) grown in 100ml of LB medium under aerobic conditions until an OD_{600} of 5. Five µl of extracted AQs were spotted onto normal-phase silica 60F254 TLC plates (Merck), developed with dichloromethane/methanol (95:5, v/v) and visualized under 254nm (A) or 366nm (B) UV light. Synthetic PQS and HHQ standards loaded as control are indicated with arrowheads.

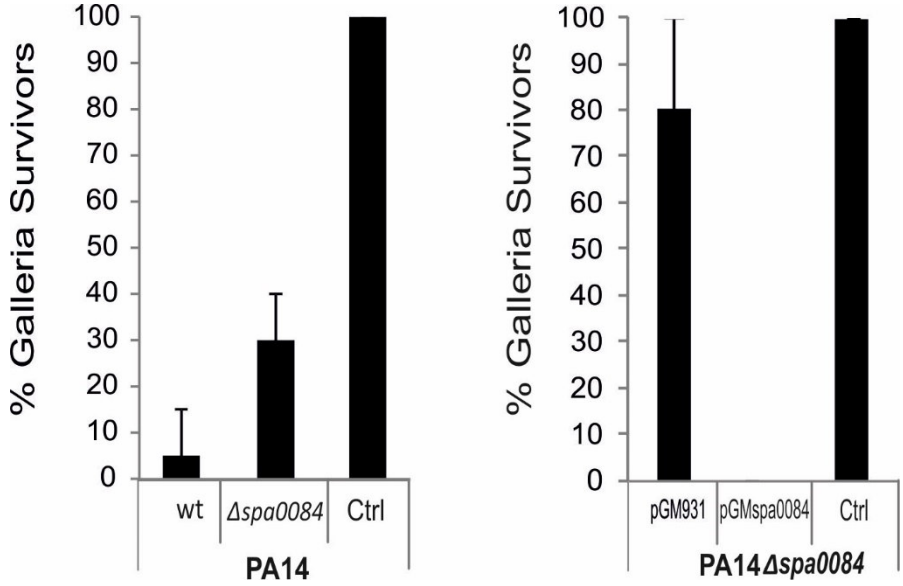

Fig. 8. SPA0084 influence virulence of *P. aeruginosa* PA14 strain.
Comparison of killing activity in *G. mellonella* larvae between PA14 *wt* and PA14 *Δspa0084* (left) and between PA14 *Δspa0084* strains carrying pGM931 or pGM-*spa0084* (right). For each *P. aeruginosa* strain, ten *G. mellonella* larvae were inoculated with about twenty cells and incubated for 24 h at 37°C. Bars represent the means along with standard deviations of *G. mellonella* survivors at 24 h from two independent experiments.

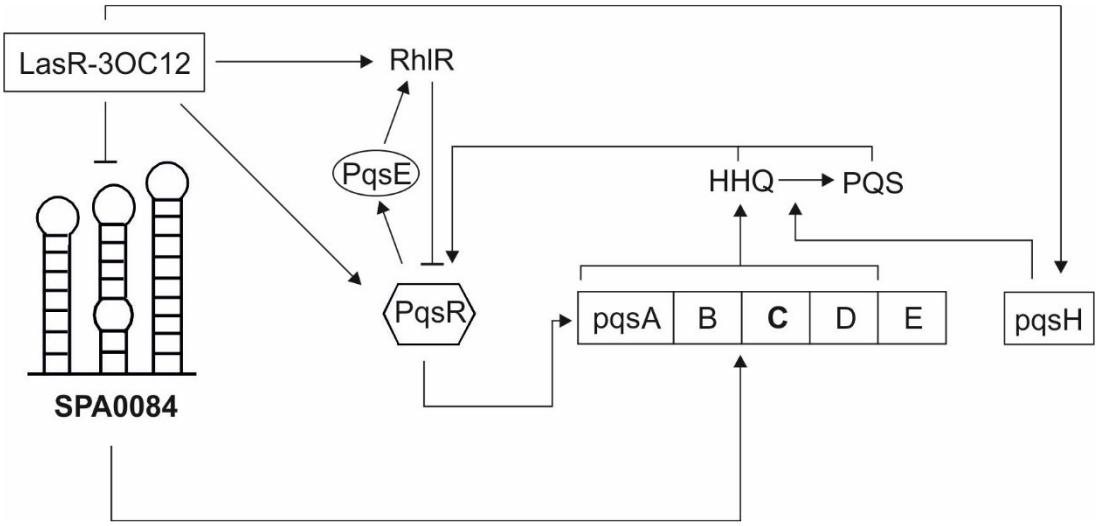

Fig. 9. Schematic representation of the SPA0084 role of wiring *las* to pqs QS systems.
The *las-pqs* connections described so far by Williams, 2009, are depicted along with the suggested role of SPA0084. LasR, when bound to 3OC12-HSL activates transcription of *rhlR* and *pqsR*. PqsR, bound to the cognate signal molecules PQS or HHQ, positively regulates *pqsABCD* genes, which are required for HHQ and PQS synthesis, the latter in combination with *pqsH* activated by LasR/3OC12-HSL. Conversely, RhlR, bound to C4-HSL, negatively regulates both *pqsR* and *pqsABCD*. PQS and HHQ both function as autoinducers since they each drive the expression of *pqsABCD* in a *pqsR*-dependent manner. PQS and HHQ synthesis is thus positively auto-regulated via PqsR/HHQ/PQS and *las*, and downregulated by *rhl*. From basal *pqsABCD* transcription, the positive post-transcriptional regulation of SPA0084 on the *pqsABCD*-encoded biosynthetic pathway is supposed to assure the achievement of threshold levels of HHQ/PQS required to trigger the *pqsR*-dependent autoinduction. Once auto-induction is triggered, SPA0084 could act further to potentiate the positive feedback loop. These SPA0084 roles are coupled to *las* by the negative regulation of LasR/3OC12-HSL.

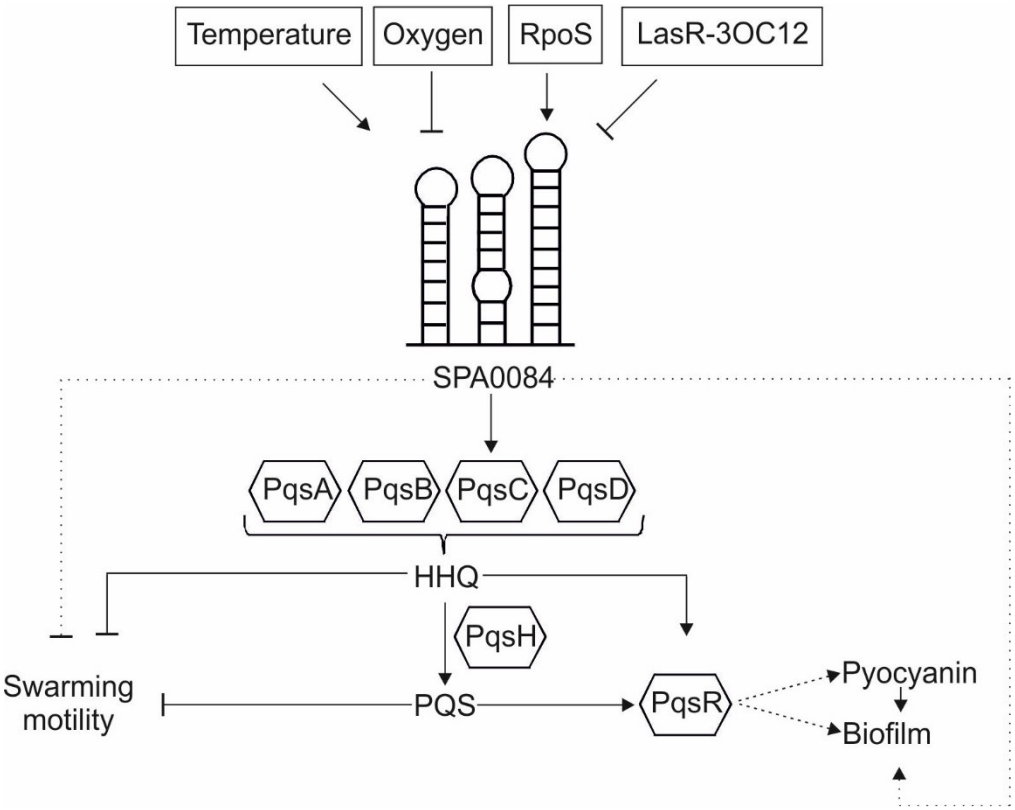

Fig. 10 Schematic representation of the regulatory influence of SPA0084 on the expression of virulence traits.
SPA0084 is depicted as responding to temperature, oxygen availability, stationary phase (RpoS) and to LasR bound to $3OC_{12}$-HSL (LasR-3OC12), and positively influencing HHQ and PQS levels via a post-transcriptional regulation on *pqsC*. SPA0084 levels are thus expected to affect pyocyanin synthesis, biofilm formation and swarming motility, which are processes known to be influenced by HHQ/PQS synthesis. In the case of pyocyanin production and biofilm formation, the SPA0084-mediated modulation of HHQ/PQS levels directly impact the pathways of activation of pyocyanin biosynthetic genes and biofilm formation dependent on PqsR/HHQ/PQS, RhlR/C4-HSL and PqsE. In addition, there could be, in the case of biofilm, a direct role of pyocyanin in promoting the release of extracellular DNA and cell-to-cell interactions. Swarming motility is supposed to be negatively regulated by HHQ/PQS levels through unknown PqsR-independent mechanisms. Possible HHQ/PQS-independent regulations by SPA0084 of functions that inversely regulate biofilm formation and swarming motility are also depicted.

Table S1. Strains and plasmids

Strains or plasmids	Genotype or description	Reference
Pseudomonas aeruginosa		
PAO1	Wild type	(1)
PAO1 Δ*spa0084*	markerless Δspa0084	This work
PAO1 Δ*lasR*	Derivative from (ATCC 15692)	
PAO1 Δ*rsaL*	Derivative from (ATCC 15692)	
PA14	Wild type	(2)
PA14 Δ*spa0084*	markerless Δspa0084	This work
PA14 Δ*lasR*		(3) Deziel et al., 2004
PA14 Δ*lasI*		(4) Liberati et al., 2006
PA14 Δ*rhlR*		(5) Deziel et al., 2005
PA14 *pqsR*⁻ *(mvfr*⁻*)*		(6) Cao et al., 2001
Escherichia coli		
TOP10		Invitrogen
CC118λpir		(7)
Plasmids		
pGM931	pHERD20T derivative,araC/$P_{BAD,}$ Ap^r	(8) Delvillani et al., 2014
pGM-*spa0084*	pGM931 derivative*spa0084* under P_{BAD} , Ap^r	This work
pXG10-SF	sfGFP reporter plasmid; lacZ::gfp, $P_{LtetO-1}$, translational fusion of dicistronic targets, Cm^r	(9)
pXG30-SF	sfGFP reporter plasmid; FLAGLacZ'::gfp$P_{LtetO-1}$, translational fusion of dicistronic targets, Cm^r	(9)
pXG-$P_{spa0084}$-*gfp*	pXG10-SF derivative, lacking $P_{LtetO-1}$ promoter and containing transcriptional phusion of $P_{spa0084}$ -->*gfp*	This work
pXG30-*pqsCRI-II*::*gfp*	pXG30-SF derivative; $P_{LtetO-1}$-->FLAGLacZ':*pqsB-pqsCRI-II*::gfp, Cm^r	This work
pBBR1-MCS5	lacZ ,Gm^r	(10)
pBBR1-MCS2	lacZ, Km^r	(10)
pBBR1-gfp	pBBR1-MCS5 derivative, $P_{LtetO-1}$-->gfp, Gm^r	(11)
pBBR5-$P_{spa0084}$-*gfp*	pBBR1-MCS5 derivative, $P_{spa0084}$ -->gfp, Gm^r	This work
pBBR2-$P_{spa0084}$-gfp	pBBR1-MCS2 derivative; $P_{spa0084}$ -->gfp, Km^r	This work
pBBR1-*pqsCRI-II*::gfp	pBBR1-MCS5 derivative; pqsC::gfp, Gm^r	This work
pSEVA612S	oriR6K, *lacZα;* MCS flanked by two I-SceI sites, Gm^r, Ap^r	(12)
pSW-1	oriRK2, *xylS; Pm*--> I-SceI, Ap^r	(13)
pSEVApao1-Δ*spa0084*	pSEVA612S derivative, Ap^r	This work
pSEVApa14-Δ*spa0084*	pSEVA612S derivative, Ap^r	This work
pET28b	bacteria protein expression, high number of copy, Km^r	Novagen
pET28b-LasR	pET28b derivative, lasR expression	

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Table S2. Oligonucleotides

Oligo	Oligo Name	Sequence (5'-3')	Utilization
1	SPA0084 AS29SR_1-SR7_14	AAGGTGAGCCCCGGAGGT	Northern-blot probe for *spa0084*
2	PA5SRNA02	GGAGACCCCACACTACCATCGGCGATG	Northern-blot probes for 5S
3	SPA0084 promregion AatII FW	GTTTTgacgtcCTTCGGTTACGAGGGCATGCCA	Amplification of $P_{spa0084}$ with AatII/PstI ends for cloning in frame with gfp in pXG30-SF vector
4	SPA84PR+63 rev pstI	AAAActgcagTCGTGCGGCACGGTGGTG	
5	SPA0084 promregion ClaI fw	GTTTTatcgatCTTCGGTTACGAGGGCATGCCA	Amplification of $P_{spa0084 \text{ -->}}$ gfp with ClaI/ClaI end for cloning in pBBR1-MCS5 and pBBR1-MCS2
6	NcoI_5'SPA0084_f	GAccatggAGCGCTGTACTATCCCTTCCA	Amplification of *spa0084* with NcoI/PstI ends for cloning in pGM931 vector
7	PstI 3'SPA0084_r	GAActgcagAGGGTGCGAGAGGAGCAA	
8	Val0 SPA0084 39-19	CTGGAAGGGATAGTACAG	Northern-blot probe for *spa0084*
9	Val1 SPA0084 19-0	TGGATGGACAGTATAGCG	
10	PqsCleader_pXG30_PstI_fw	AAAActgcagCAGCGCGACCGTCTGGAT	Amplification of the -94nt to the first 21 codons of the *pqsC* open reading frame with PstI/NheI ends for cloning in frame with gfp in pXG30-SF vector
11	PqsC+21aa_pXG30_NheI_rev	GTTTTgctagcATCGTCGTTTTCGTAGCTG	
12	ptet-O1_ClaInw_f	GTTTTatcgatCGTCTTCACCTCGAGTC	Amplification from the $P_{LtetO-1}$ to the stop codon of gfp of pXG30-*pqsC*::gfp/pXG1-SF with ClaI/XbaI end for cloning in pBBR1_MCS5
13	sfGFP_TAA_XbaI_r	ATGCCtctagaTTATTTGTAGAGC	
14	sfGFP_TAA_ClaI_r	ATGCCatcgatTTATTTGTAGAGCTC	Reverse primer for the amplification from pXG10-$P_{spa0084}$ --> gfp/pXG1-SF with ClaI ends for cloning in pBBR1_MCS5
15	sfGFP_+96_r	TGTGCCCATTAACATCACCAT	Reverse primer used to verify the pXG30-pqsC::gfp/pXG1-SF, pXG10-pqsC ORF::gfp and pXG10-$P_{spa0084}$ --> gfp
16	pSEVA_fw	TAAAACGACGGCCAGTATAG	Primers on pSEVA vector to verify the constructs/sequencing of TS1 and TS2
17	pSEVA_rw	AGCTATGACCATGATTACG	
18	TS1SPA84 EcoRIF	GCgaattcATGACTTCCGCGAACGCGGTAGCG	Amplification of the genomic region TS1 using genomic DNA of either PAO1 or PA14
19	TS1SPA84 Rev	AGGGTGCGAGAGGAGCAAAACTCGAGTTTCGCATGAATCCTTTAGTTAG	
20	TS2SPA84 For	ACTCGAGTTTTGCTCCTCTCGCACCCT	Amplification of the genomic region TS2 using genomic DNA of either PAO1 or PA14
21	TS2SPA84 Pst1R	GAActgcagCTGTCGCTGTTCCTCGCCCTG	
22	SPA0084_300up	AGCATCGACAGCTACCACCT	Primers 300 bp downstream/upstream of the spa0084 intergenic region to verify the deletion
23	SPA0084_300down	CGCTCATCGCTGACTACCTT	
24	pHERD_rev	GCAAGGCGATTAAGTTGG	Reverse primer used to verify the cloning direction of Pspa0084 in pBBR1-MCS2 and pBBR1-MCS5
25	M13_rev	CAG GAA ACA GCT ATG ACC	Reverse primer on pGM931 backbone to verify the constructs

Table S3. Bioinformatics analysis of the interaction of SPA0084 with *pqsC* mRNA

Webtool	Gene	locus tag	Energy (Kcal/mol)	Confidence*	mRNA start	mRNA end	sRNA
TargeRNA2	pqsC	PA14_51410	-11,36	0,011 [a]	-48	-28	40-59
RNAPredator	pqsC	PA14_51410	-21,86	-3,51 [b]	-40	-19	41-61
StarPicker	pqsC	PA14_51410	-14,5	1 [c]	-48	-28	40-59
IntaRNA	pqsC	PA14_51410	-15,6293		-48	-28	40-62
IntaRNA	pqsC	PA14_51410	-6		18	28	25-37

[a] p-value
[b] zeta score
[c] probability

							5' flanking gene		3' flanking gene	
	Strains	**Percent identity**	**Mismatches**	**Gaps**	**Subject start**	**Subject stop**	**gene name**	**gene function**	**gene name**	**gene function**
1	*Pseudomonas aeruginosa PAO1-UW*	100.0	0	0	3957895	3958150	PA3535	Putative serine protease	PA3536	Hypothetical protein
2	*Pseudomonas aeruginosa UCBPP-PA14*	100.0	0	0	1600240	1599985	PA14_18630	Putative serine protease	PA14_18620	Hypothetical protein
3	*Pseudomonas aeruginosa RP73*	100.0	0	0	4014634	4014889	M062_18730	Putative membrane protein	M062_18735	Hypothetical protein
4	*Pseudomonas aeruginosa B136-33*	100.0	0	0	1540218	1539963	G655_07125	Putative Serine protease	G655_07120	Hypothetical protein
5	*Pseudomonas aeruginosa DK2*	100.0	0	0	1454305	1454050	PADK2_06690	Serine protease	PADK2_06685	Hypothetical protein
6	*Pseudomonas aeruginosa NCGM2.S1*	100.0	0	0	5022050	5022305	NCGM2_4660 EprS	Putative Serine protease	NCGM2_4661	Hypothetical protein
7	*Pseudomonas aeruginosa 39016*	100.0	0	0	4368654	4368909	PA39016_001560025	Putative Serine protease	PA39016_001560026	Hypothetical protein
8	*Pseudomonas aeruginosa LESB58*	100.0	0	0	1624047	1623792	PALES_14981	Putative Serine protease	PALES_14971	Hypothetical protein
9	*Pseudomonas aeruginosa PACS2*	100.0	0	0	4639012	4639267	PaerPA_01004116	Hypothetical protein	PaerPA_01004117	Hypothetical protein
10	*Pseudomonas aeruginosa 2192*	100.0	0	0	3266633	3266888	PA2G_02929	Putative Serine protease	PA2G_02930	Hypothetical protein
11	*Pseudomonas aeruginosa C3719*	100.0	0	0	2739259	2739514	PACG_02446	Putative Serine protease	PACG_02447	Hypothetical protein
12	*Pseudomonas aeruginosa M18*	99.61	1	0	1594487	1594232	PAM18_1451	Putative serine protease	PAM18_1450	Hypothetical protein
13	*Pseudomonas aeruginosa PA7*	96.46	8	1	1644808	1644555	PSPA7_1611	Putative Serine protease	PSPA7_1610	Hypothetical protein

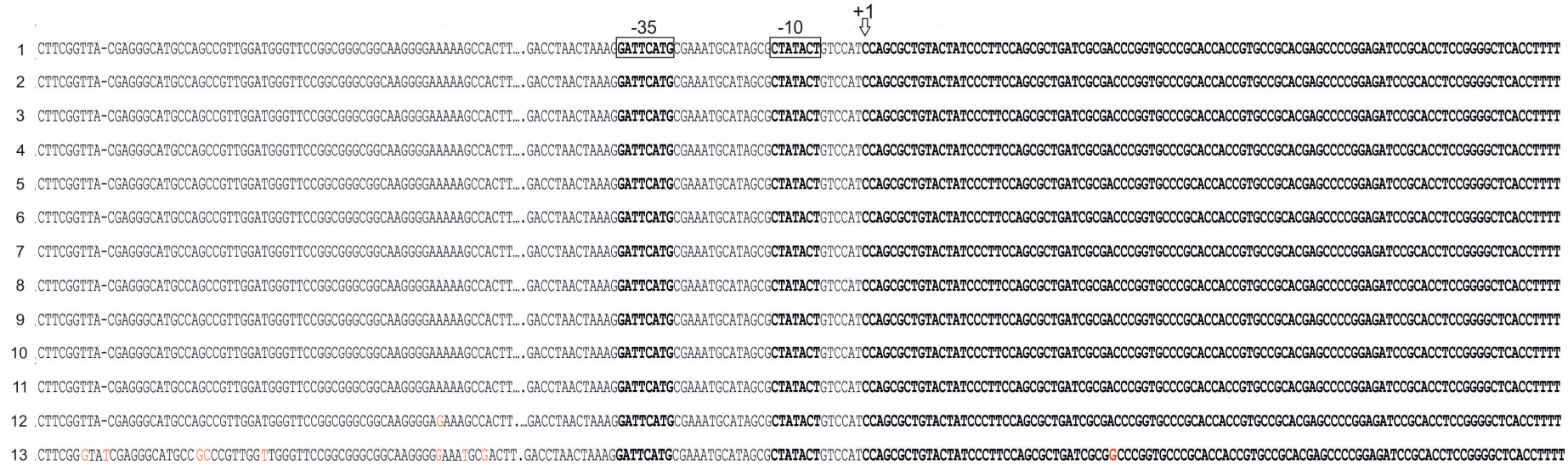

Fig. S1. Alignment of the intergenic region containing the *spa0084* gene of thirteen *P. aeruginosa* sequenced strains.
The sequences of the intergenic regions containing *spa0084* gene from thirteen sequenced *P. aeruginosa* strains were compared by reciprocal-best-BLAST. The sequence regions corresponding to *spa0084* gene and to -35 and -10 motifs are in bold. Non-strictly conserved positions are in red.

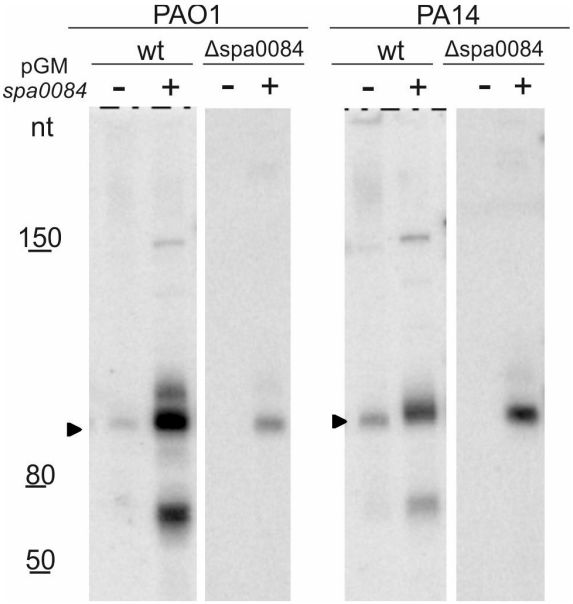

Fig. S2. Validation of overexpression levels of SPA0084 in PAO1 and PA14 strains.
PAO1 wt, PA14 wt, PAO1 *Δspa0084*, and PA14 *Δspa0084* strains harbouring pGM931 empty vector (-) or pGM-*spa0084* (+) were grown in BHI medium with carbenicillin and 10 mM arabinose until and OD_{600} of 1.8. Culture samples were then processed for total RNA extraction and analysis by Northern blot probing SPA0084 RNA.

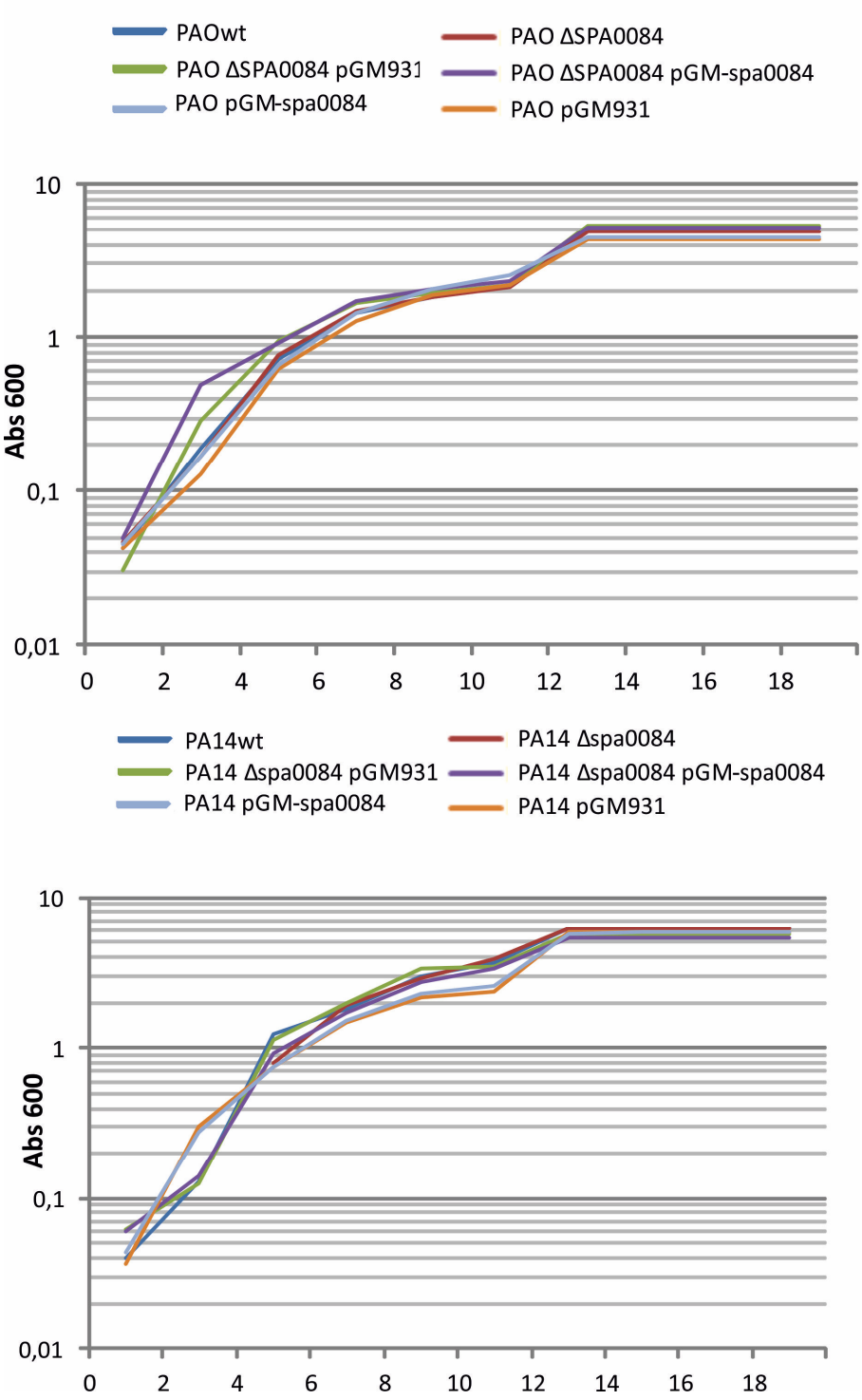

Fig. S3. *P. aeruginosa* growth is not affected by SPA0084 overexpression or deletion. Growth curves in LB medium of the indicated strains. For strains carrying pGM931 or pGM-*spa0084*, 10 mM arabinose was supplemented. Plotted OD_{600} values reflect the average of three independent experiments.

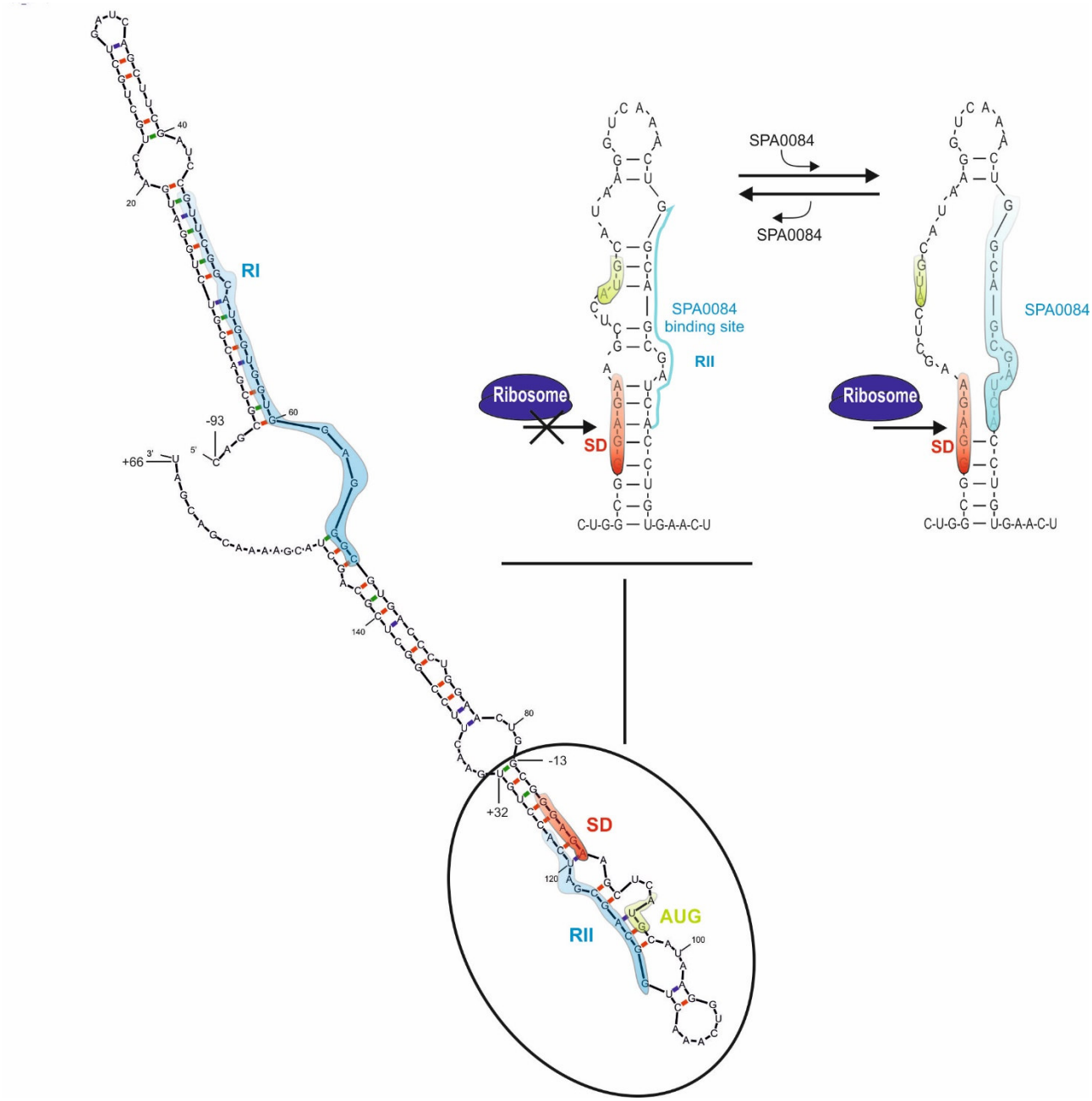

Fig. S4. Model of SPA0084 mediated activation of *pqsC* translation.
The secondary structure of the mRNA region spanning -93 to +66 from the *pqsC* translation start site is represented on the left. Regions RI and RII predicted to be targeted by SPA0084 are highlighted in light blue. Shine-Dalgarno (SD) and start AUG codon of *pqsC* are highlighted in red and green, respectively. The region between -13 and +32 is predicted to form a large stem-loop structure in which both SD and AUG are trapped by base pairing to RII region, which would act as anti-SD/AUG. In this configuration, translation of *pqsC* is repressed. SPA0084 binding to RII results in destabilization of stem-loop structure that can promote access of the 30S ribosome subunit on the mRNA, thereby allowing translation initiation. SPA0084 binding to RI is suggested to play the role of overall stabilization of SPA0084/mRNA interaction.