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Regulation of quorum sensing and virulence in *Pseudomonas aeruginosa*

Regolazione del quorum sensing e della virulenza in *Pseudomonas aeruginosa*

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"One sometimes finds what one is not looking for."

"I have been trying to point out that in our lives
chance may have an astonishing influence and,
if I may offer advice to the young laboratory workers,
it would be this - never to neglect an extraordinary
appearance or happening."

Alexander Fleming

"It is a capital mistake to theorize before one has data.
Insensibly one begins to twist facts to suit theories,
instead of theories to suit facts."

"How often have I said to you that when you have eliminated the impossible,
whatever remains, however improbable, must be the truth?"

Sherlock Holmes

"Look deep into nature, and then you will understand everything better."

"The true sign of intelligence is not knowledge but imagination."

Albert Einstein

In loving memory of my mother,
Stefania Moscato.
May she always rest in peace
knowing that I finally find my way.

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ABSTRACT

The asocial existence of the bacterial cells has been a major paradigm in microbiology for a long time. However, a huge amount of experimental evidences collected in the last 30 years has revealed that bacteria preferentially live in communities, in which the behavior of individual cells is coordinated by cell-cell communication systems to control phenotypic behaviors at the population level. Bacteria not only form well-organized communities, but they also exploit complex social cooperative and competitive interactions, mimicking multicellular organisms. Bacterial social life mainly relies on their ability to exchange information *via* chemical communication systems. In some cases, these systems allow a group of bacteria to trigger a unified and coordinated response to metabolic and environmental stimuli, so to accomplish tasks which would be difficult, if not impossible, to achieve for individual bacterial cells. One of the most studied bacterial cell-cell communication systems is quorum sensing (QS), by which a bacterial population coordinately reprograms gene expression in response to cell density. The QS-response is achieved when the concentration of a specific signal molecule, produced and secreted by single bacteria cells, reaches a threshold level, corresponding to a certain cell density, at which it is able to trigger a phenotypic and behavioral change in all the members of the bacterial population.

QS in different bacteria is involved in the regulation of a wide variety of physiological processes, including competence, bioluminescence, antibiotic biosynthesis, motility, plasmid conjugal transfer, biofilm formation, and production of bacterial virulence factors in plant, animal and human pathogens. Furthermore, evidence has been accumulated that some bacterial signal molecules are used not only for intra-species communication, but also to exchange information between bacteria of different species or genera occupying the same ecological niche, and also to interact with their eukaryotic hosts.

The QS system of the opportunistic human pathogen *Pseudomonas aeruginosa* is one of the best characterized bacterial communication systems, and it is now considered as a mayor model system for QS studies. In *P. aeruginosa* QS positively controls the expression of virulence related traits, such as virulence factor production and biofilm formation, and it is consequently involved in both acute and chronic infections. Moreover the QS circuit of *P. aeruginosa* is one of the most complex communication systems described up to date, since it is made up of four interconnected QS systems interwoven in an intricate regulatory network, the *las*, *rhl*, *pqs* and IQS systems. The four QS systems of *P. aeruginosa* are hierarchically organized and, in a wide range of cultural conditions, the *las* system is at the top of this hierarchy, being required for full activation of the other three QS circuits. Overall, the *las* QS system positively controls the complex regulative cascade involved in the expression of virulence-related phenotypes in *P. aeruginosa*, and for this reason it is considered one of the most promising target for the development of new anti-virulence drugs. Besides cell density, the *las* QS system is modulated by many regulators and signalling systems in response to metabolic and environmental cues. In particular, the timing and the extent of the QS response are

finely modulated at different levels by a plethora of transcriptional and post-transcriptional regulators. It is believed that this fine-tuning of the *las* QS system might play a major role during *P. aeruginosa* infections, even though this hypothesis has been poorly investigated in mammalian models of infection so far.

Despite in the last 20 years the huge efforts of the scientific community has lead to a refined knowledge of the molecular mechanisms controlling the expression of the *las* QS system in *P. aeruginosa*, our understanding of its regulation and actual role in the infection processes is far from complete. On these bases, the main aim of this PhD project has been to contribute to shed light on some unclear aspects of the *las* QS system regulation. In particular, this PhD work aimed at *(i)* identifying novel transcriptional regulators of the *las* QS system; *(ii)* evaluating the effect of a dysregulation in the timing and the extent of the *las* QS response on the ability of *P. aeruginosa* to establish both acute and chronic infections in a murine model system; *(iii)* investigating new emerging behavioral properties arising from the peculiar regulatory architecture of the *las* QS system.

Briefly, the search for novel direct regulators of LasR, the QS signal molecule receptor that activates the *las* QS system, lead to the identification of a new QS transcriptional regulator PA3699. This protein directly represses *lasR* transcription, and consequent expression of QS-controlled virulence phenotypes in *P. aeruginosa*.

The study of the effect of a dysregulation in the timing and extent of the QS response revealed that an anticipated activation of the *las* QS system does not significantly affect *P. aeruginosa* virulence in a mouse model of infection, while an increased activation of the *las* QS system beyond physiological levels impairs *P. aeruginosa* ability to establish chronic lung infections in mice.

The arrangement of the genes composing the *las* QS system resembles the architecture of a network motif, the type-1 incoherent feedforward loop (IFFL-1), which is known to confer peculiar regulatory properties to the expression of its output genes. In line with this resemblance, we demonstrated that the *las* IFFL-1 confers robustness to the expression of its output genes with respect to possible fluctuations in the levels of the LasR activator.

The main achievements of this PhD work have been published in two international peer-reviewed journals (Longo *et al*., 2013; Bondí *et al*., 2014), and have been collected in an additional manuscript almost ready to be submitted (Bondí *et al*., manuscript in preparation).

- Longo F, Rampioni G, **Bondì R**, Imperi F, Fimia GM, Visca P, Zennaro E, Leoni L (2013) A new transcriptional repressor of the *Pseudomonas aeruginosa* quorum sensing receptor gene *lasR*. *PLoS One* 8:e69554.
- **Bondí R**, Messina M, De Fino I, Bragonzi A, Rampioni G, Leoni L (2014) Affecting *Pseudomonas aeruginosa* phenotypic plasticity by quorum sensing dysregulation hampers pathogenicity in murine chronic lung onfection. *PLoS One*. 9:e112105.
- **Bondì R**, Messina M, Longo F, Leoni L, Rampioni G. Characterization of the incoherent feedforward loop governing quorum sensing in *Pseudomonas aeruginosa.* Manuscript in preparation.

INTRODUCTION

1. Phenotypic plasticity and the evolution of opportunistic pathogens

Standard virulence evolution theory assumes that virulence factors are maintained in a pathogen microorganism to allow the optimal exploitation of the host, or a better transmission among the hosts (Anderson and May, 1982). An increasing number of studies demonstrate that many opportunistic pathogens (OPs) do not conform to these assumptions, with virulence factors being maintained to gain selective advantages in non-parasitic contexts (Woolhouse *et al*., 2001). The classical definition used in medical literature defines an OP as an organism that can become pathogenic following a perturbation to its host, while more recently OPs have been defined as pathogens that are non-obligate and/or non specialist of a focal host (Brown *et al*., 2012). The latter definition is simple and broader than the classical one and, applied to the human host, divides pathogen in four groups depending on the possible combination of the two conditions obligate/non-obligate and specialist/non-specialist (Table 1):

- **Group 1: Specialist on humans and obligate parasite.** This class includes pathogens specifically adapted to the human host (yellow box);
- **Group 2: Specialist on humans and facultative parasite.** This class includes commensal opportunistic pathogens (pink box);
- **Group 3: Non-specialist on humans and obligate parasite.** This class includes zoonotic opportunistic pathogens (green box);
- **Group 4: Non-specialist and facultative parasite.** This class includes environmental opportunistic pathogens (blue box).

Table 1. Classification of pathogens according to Brown definition of OPs.

	Obligate parasite	**Facultative parasite**
Specialist on humans	HIV, influenza virus (A,B,C), *Mycobacterium tubercolosis*	*Staphylococcus aureus, Streptococcus pneumoniae*
Non-specialist on humans	*Borrelia burgdorferi, Salmonella* spp.	*Pseudomonas aeruginosa, Burkholderia cepacia*

Modified from Brown *et al*., 2012.

According to this definition, only the Group 1 includes non-OP for which the standard virulence evolution theory can be still applied to investigate the mechanisms of virulence evolution, maintenance and expression, relating them with the survival and exploitation of the human host. The other groups of pathogens, conversely, all include OPs, and differ from each other for their

ecological niches besides the human host. Indeed, the Groups reported in Table 1 include: Group 2, commensal opportunists, adapted only to the human host but able to coexist peacefully with their hosts in the absence of perturbations; Group 3, zoonotic opportunists, that can colonize other animals besides the human host; Group 4, environmental opportunists, that normally live in the environment exploiting various ecological niches. For these three groups of OPs, the standard virulence evolution theory fails, and cannot explain the evolution, maintenance and regulation of virulence factors only in relation with the survival and colonization of the human host. Two key features seem to drive the evolution of the OPs, and are generalism and phenotypic plasticity. All human OPs are generalists, which means that they are able to grow in more than one environment, and many of them display remarkable phenotypic plasticity, defined as the ability to modify phenotypic expression in response to fluctuations in their environmental context. The study of the mechanisms involved in the plastic response that allows the adaptability of an OP to different environments provides information on why and when an OP expresses its virulence potential, possibly causing a disease. A deep understanding of the environmental conditions and the selective pressures that lead to the evolution, maintenance and expression of virulence traits in OPs, is required to develop new strategies to interfere with the plastic response that controls their pathogenic potential (Brown *et al*., 2012).

2. *Pseudomonas aeruginosa*

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

P. aeruginosa causes severe infections in hospitalised and immunocompromised patients; it is also the main cause of death in people affected by Cystic Fibrosis (CF) (Welsh *et al*., 2001). However, healthy people can also develop mild illnesses with *P. aeruginosa*, commonly after exposure to contaminated water. Ear infections, especially in children, and more generalized skin rashes may occur after exposure to inadequately chlorinated hot tubs or swimming pools. Eye

infections have occasionally been reported in persons using extended-wear contact lenses (Centers for Disease Control and Prevention).

The most common hospital-acquired *P. aeruginosa* infections are related to the use of medical devices, infections of wounds, burns and surgical sites. This infections are very frequent (11-13% of all nosocomial infections and 13.2-22.6% of infections in intensive care units), and associated with high morbidity and mortality rates when compared with infections caused by other bacterial pathogens (Osmon *et al*., 2004). This is mainly due to the fact that *P. aeruginosa* infections are hard to eradicate because this microorganism is intrinsically resistant to many antibacterials, including β-lactams, macrolides, tetracyclines, co-trimoxazole and most fluoroquinolones, and it is particularly prone to acquire new resistances in the hospital environment by horizontal gene transfer (Latifi *et al*., 1995). In CF patients *P. aeruginosa* plays a particularly important role. CF is a genetic disorder affecting approximately 1 in 2,500 newborns in the Caucasian population, and it is caused by a mutation in the gene coding for the transmenbrane conductance regulator, CFTR. The inability to regulate sodium and chloride transport due to aberrant CFTR increases airway secretion viscosity, and within the resulting thick mucus in the lung *P. aeruginosa* finds a favorable niche (Lyczak *et al*., 2000). From 80% to 95% of CF patients die because of respiratory failure due to chronic *P. aeruginosa* infection and concomitant airway inflammation. Up to 97% of CF patients are infected with *P. aeruginosa* by the age of 3 years (Lyczac *et al*., 2002; Murray *et al*., 2007).

The capacity of *P. aeruginosa* to produce such diverse infections, is due to a large number of virulence factors, such LasA and LasB elastases, exotoxin A, phospholipase C, protease IV, PrpL protease, pyocyanin, siderophores, hydrogen cyanide, and rhamnolipids, and also to the production of biofilm, that allows the colonization of host tissues and the protection of bacterial cells from the immune system and antibiotics therapies. These factors are collectively capable of causing extensive tissue damage, bloodstream invasion and dissemination in humans and other mammals (Fig. 1; Smith and Iglewski, 2003).

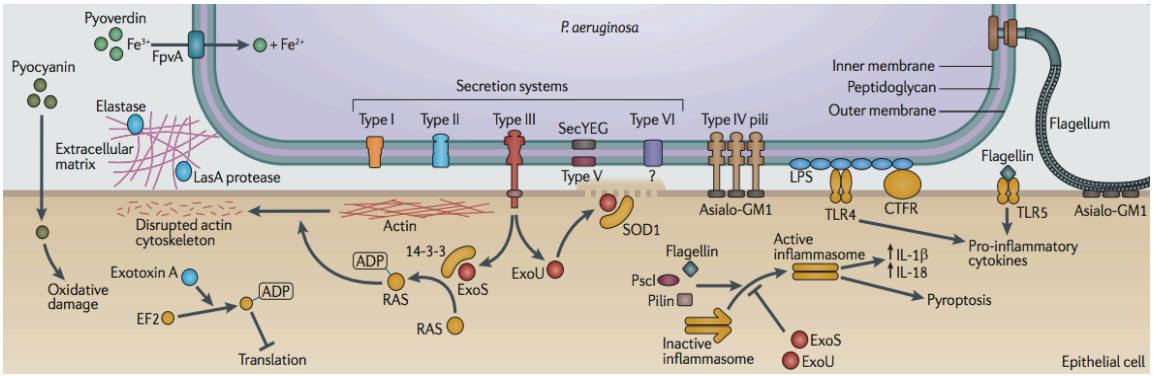

Fig. 1. **Overview of *P. aeruginosa* virulence determinants.** *P. aeruginosa* has both cell-associated (flagellum, pili, lipopolysaccharides, alginate) and extracellular virulence factors. The type III secretion apparatus is used to inject exotoxins S, T, Y, and U in the host cell. Modified from Hauser and Ozer, 2011.

P. aeruginosa can adopt two different lifestyles that reflect two different strategies of infection, the acute and the chronic infection. The acute infection is rapid, systemic, carried out by a planktonic bacterial community expressing high levels of virulence factors and typically has a severe outcome; in patients with damaged airways from mechanical ventilation, trauma, or antecedent viral infection, *P. aeruginosa* colonization of the respiratory tract is often followed by acute pneumonia, sepsis, and death (Sadikot *et al.*, 2005). Conversely, during chronic infections bacterial proliferation is limited to a specific host tissue (*e.g.*, in the CF lung or in association with medical devices), and bacteria can persist in the host for extended periods of time, adopting a slow-growing sessile lifestyle and forming biofilm. In the biofilm mode of growth bacteria are more resistant to the host immune system and prolonged antibiotic therapies, and they produce limited amount of virulence factors (Furukawa *et al.*, 2006; Coggan *et al.*, 2012). In *P. aeruginosa* both the acute and the chronic infections are positively controlled by quorum sensing, a communication system that regulates gene expression in response to cell density (Rutherford and Bassler, 2012).

3. Quorum Sensing

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

The most intensively investigated QS systems in Gram-negative bacteria employ *N*-acylhomoserine lactones (AHLs) as signal molecules. AHL biosynthesis is typically catalysed by LuxI-family synthases, which transfer an acyl group from an acylated acyl carrier protein (acyl-ACP) to the methionyl amine of S-adenosyl-L-methionine (SAM), after which cyclization of the methionyl moiety to homoserine lactone occurs. The length of the acyl side chain (usually from 4 to 18 carbons), saturation and oxidation state at position 3, determine the resulting AHL structure, and thus signal-specificity. Short-chain AHLs generally freely diffuse across membranes, while there is some evidence for active efflux of AHLs with longer acyl side-chains. AHLs generally function by binding to a cognate intracellular receptor protein belonging to the LuxR-family. In most cases, the

LuxR receptor-AHL complex binds to target promoters, activating gene expression (Miller and Bassler, 2001; Atkinson and Williams, 2009).

In Gram-positive bacteria QS systems generally rely on genetically encoded peptides as signal molecules, often termed 'autoinducing peptides' (AIPs). AIPs are expressed as inactive pro-peptides via canonical ribosomal synthesis, and later processed and modified to generate the active QS signal. AIPs are not freely diffusible across membranes. AIP perception by the receiver cell is usually mediated by sensor kinases, which transduce the signal from the membrane to cognate response regulators inside the cell *via* a phosphorylation cascade (Miller and Bassler, 2001; Atkinson and Williams, 2009).

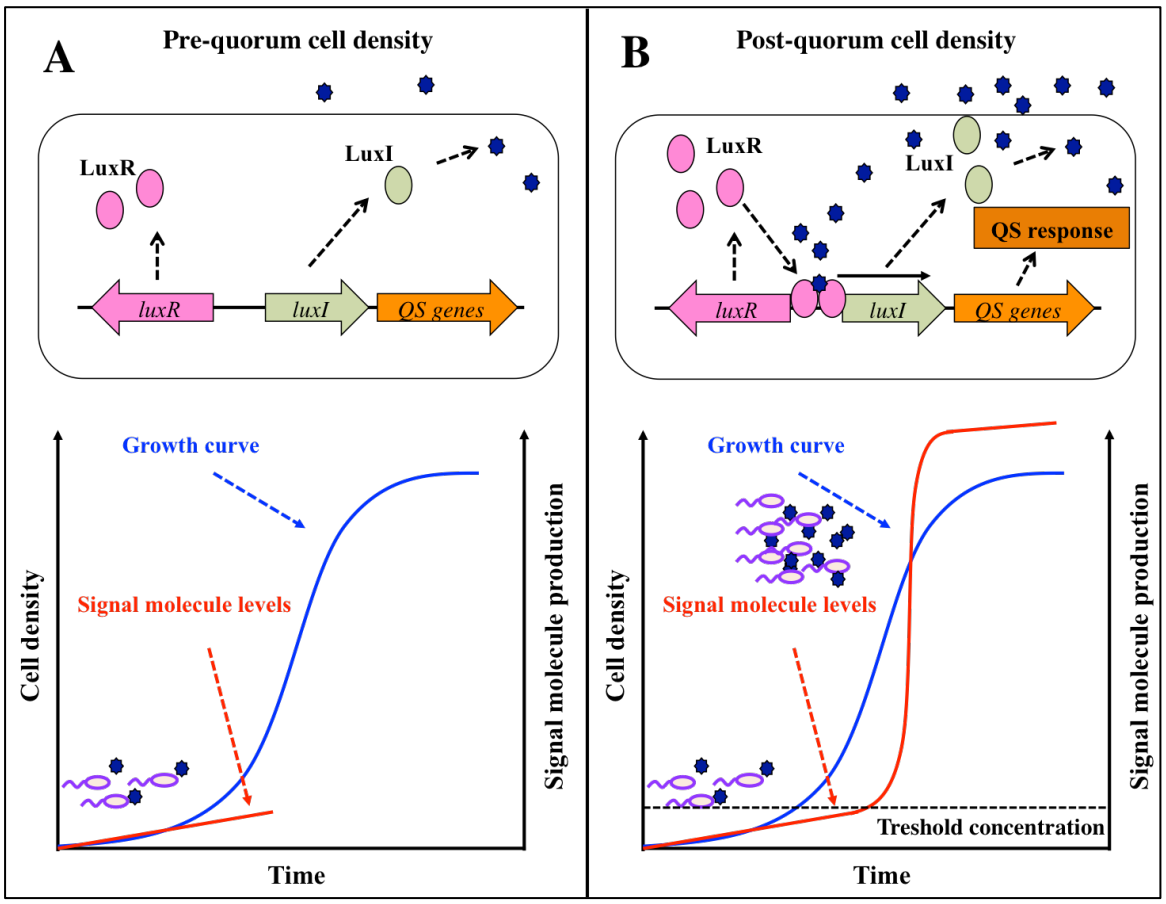

Fig. 2. Quorum sensing. Schematic representation of a general LuxR/LuxI-like QS system. **(A)** At the pre-quorum cell density an acyl-homoserine lactone (AHL) signal molecule is produced by a LuxI-like protein at a basal level (red curve), and diffuses outside the producing cells accumulating in the extracellular environment. **(B)** At the post-quorum cell density the signal molecule reaches a threshold concentration and binds to an intracellular LuxR-like cognate receptor; the LuxR/AHL complex activates QS-regulated genes and triggers the expression of the *luxI-like* gene, generating a positive feedback loop which lead to a rapid increase in signal molecule production (red curve). The blue curve is representative of a general growth curve; solid black arrows indicate positive control; dashed black arrows indicate information flow; dark blue stars represent the QS signal molecules.

The first QS system was described in the early 1970s in the marine bacterium *Vibrio fischeri*, in which it controls bioluminescence emission. This QS system was based on the production of the signal molecule *N*-3-(oxohexanoyl)homoserine lactone ($3OC_6$-HSL). In *V. fischeri* the gene *luxI* encodes the synthase LuxI that, at low cell density, synthesizes $3OC_6$-HSL at a basal level; $3OC_6$-HSL accumulates in the extracellular environment proportionally to cell density of the bacterial culture, and when it reaches a certain threshold concentration, corresponding to the "quorum" cell density, it binds to and activates its cognate intracellular receptor LuxR, encoded by the gene *luxR* (Fig. 2A). The LuxR/$3OC_6$-HSL complex triggers the transcription of genes involved in bioluminescence production, and also of the gene *luxI* gene, generating a positive feedback loop which lead to a rapid increase in the concentration $3OC_6$-HSL (Fig. 2B; Nealson *et al*., 1970; Fuqua *et al*., 1994).

4. QS in *Pseudomonas aeruginosa*

As previously described, in *P. aeruginosa* both acute and chronic infections are positively controlled by QS (Rutherford and Bassler, 2012). *P. aeruginosa* has four QS systems that are interconnected and hierarchically arranged: in rich medium, the *las* QS system is at the top of this hierarchy, because it is required for full activation of the other three QS systems, the *rhl*, the *pqs*, and the recently characterized IQS systems (Latifi *et al*., 1996; Pesci *et al*., 1997; Gallagher *et al*., 2002 Kiratisin *et al*., 2002; Deziel *et al*., 2004; Xiao *et al*., 2006 Lee *et al.,* 2013) (Fig. 3).

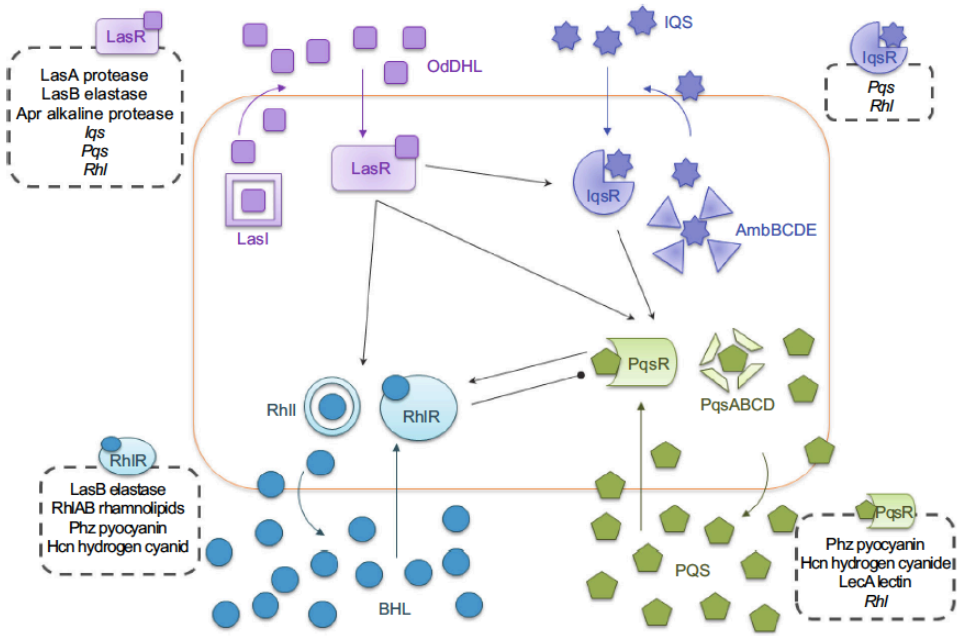

Fig. 3. Schematic representation of the four QS signaling networks in *P. aeruginosa*. Black arrows indicate positive control; circle-headed black lines indicate negative control. Modified from Lee and Zhang, 2015.

The *las* system consists of the LuxR-like transcriptional regulator LasR (encoded by the *lasR* gene), and of the LuxI-like AHL synthase LasI (encoded by the *lasI* gene), that directs the synthesis of the QS signal molecule *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) (Fig. 4; Schuster and Greenberg, 2006). Similarly to the *V. fischeri lux* QS system previously described, at low-cell density the $3OC_{12}$-HSL molecule is synthesized by LasI at a basal level, and is secreted into the surrounding medium; as a consequence, no QS-response occurs (Pearson *et al*., 1999). With increasing cell density, the signal molecule accumulates until its concentration reaches the threshold level; at this critical concentration, $3OC_{12}$-HSL binds its cognate receptor, the QS-activator LasR (Fuqua *et al*., 1996). The LasR/$3OC_{12}$-HSL complex triggers *lasI* transcription, generating a positive feedback loop that leads to the amplification of $3OC_{12}$-HSL production; as a consequence, the QS-system becomes active (Seed *et al*., 1995). Indeed, the LasR/$3OC_{12}$-HSL complex also activates the *rhl*, *pqs* and IQS QS systems, and acts as a global transcriptional regulator, drastically reprogramming *P. aeruginosa* transcriptome (Fig. 4; Schuster *et al*., 2003). As a whole, the QS circuit regulates about 7% of all the *P. aeruginosa* genes, and has a key role in the infection processes being required for the production of many virulence factors and for biofilm formation (Kirisits and Parsek, 2006; Schuster and Greenberg, 2006).

The involvement of QS in *P. aeruginosa* pathogenicity is highlighted by the observation that QS-deficient strains are less virulent than the wild type counterparts in all the animal and plant infection models tested up do date: mice, *Drosophila melanogaster*, *Caenorhabditis elegans, Dictyostelium discoideum*, and *Arabidopsis thaliana* (Smith and Iglewski, 2003, Juhas *et al*., 2005; Diggle *et al*., 2006; Rampioni *et al*., 2010). Moreover, $3OC_{12}$-HSL directly stimulates interferon-γ and interleukin-8 production, inhibits interleukin-12 and tumor necrosis factor α, promotes immunoglobulin-E production, and causes apoptosis in macrophages and neutrophils. In this respect, $3OC_{12}$-HSL can be considered as virulence factors itself (Wagner *et al*., 2006).

Also the *rhl* QS system relies on the production of an acyl-homoserine lactone as signal molecule, the *N*-butanoylhomoserine lactone (C_4-HSL). C_4-HSL is synthesised by the LuxI-like enzyme RhlI, and it is released into the extracellular environment; as the bacterial population grows, C_4-HSL binds to its LuxR-like cognate receptor RhlR, and the RhlR/C_4-HSL complex modulates the expression of target genes. The *pqs* system uses the alkyl-quinolone signals 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) and 2-heptyl-4-hydroxyquinoline (HHQ) as signal molecules. Both PQS and HHQ can activate the LysR-like protein PqsR that, once activate upon signal molecule(s)-binding, acts as a transcriptional regulator. The IQS system has been characterized very recently, and it is based on the production of the signal molecule 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS); the genes involved in IQS synthesis are a non-ribosomal peptide synthase

genes of the *ambBCDE* operon, and the transcriptional regulator IqsR is the IQS receptor. When bacteria are grown in complex media, such as LB, the four QS systems are interconnected and hierarchically organized, with the *las* QS system placed at the top of the QS cascade, since it is required for full activation of the *rhl, pqs* and IQS systems. Therefore the regulative cascade leading to the expression of virulence phenotypes in *P. aeruginosa* mainly relies on the *las* QS system (Lee and Zhang, 2015).

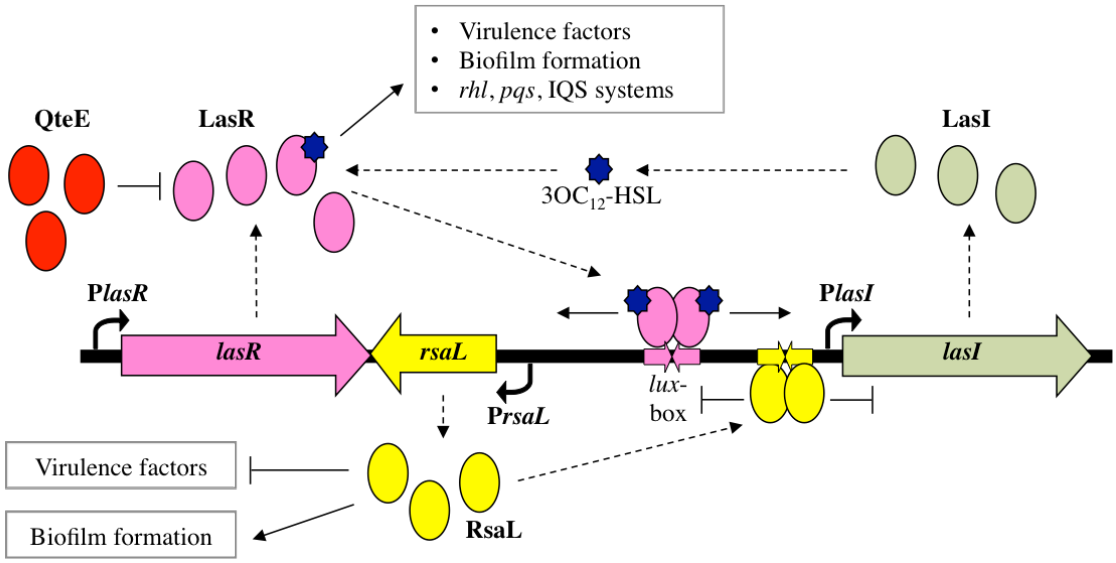

Fig. 4. Schematic representation of *P. aeruginosa las* QS system. The signal molecule $3OC_{12}$-HSL is produced by the synthase LasI at a basal level until the "quorum" cell density is reached; at the "quorum" cell density $3OC_{12}$-HSL binds to and activates its cognate receptor LasR, that in turn promotes the expression of *(i)* genes involved in virulence factors production and in biofilm formation, *(ii)* the *rhl*, *pqs* and IQS systems, *(iii)* the *lasI* gene, generating a positive feedback loop which leads to a rapid increase in $3OC_{12}$-HSL production, *(iv)* the *rsaL* gene, that counteracts the positive feedback loop by repressing *lasI* transcription. The premature activation of the *las* system at low cell density is prevented by the pre-quorum repression exerted by QteE on LasR. Solid arrows indicate positive control; T-lines indicate negative control; dashed arrows indicate information flow; blue stars represent the $3OC_{12}$-HSL signal molecules.

5. Regulation of QS in *P. aeruginosa*

The *las* QS system is more complex than the minimal *lux*-like QS system exemplified by *V. fischeri* and described in Fig. 2, because it undergoes a fine modulation of the timing and the extent of $3OC_{12}$-HSL production. As an example, in *V. fischeri* exogenous provision of $3OC_6$-HSL to a low cell-density culture activates the *lux* QS system (Fuqua *et al.*, 1994). Conversely, the exogenous provision of $3OC_{12}$-HSL to a *P. aeruginosa* low cell-density culture is not sufficient to activate the *las* QS system (Witheley *et al.*, 1999). This difference is due to a strong pre-quorum repression exerted on the *P. aeruginosa las* system by multiple repressors, such as the LasR post-translational repressors QscR, QslA and QteE (Chugani *et al.*, 2001; Ledgham *et al.*, 2003; Sienhel

et al., 2010; Seet *et al*., 2011). QscR has been characterized by Chugani *et al*. in 2001 for its ability to repress some QS regulated genes, and a mutant defective in *qscR* shows an anticipation of $3OC_{12}$-HSL production (Fig. 5). Its mechanism of action was investigated by Ledgham *et al*. in 2013, and revealed that QscR, in the absence of $3OC_{12}$-HSL, forms heterodimers with LasR unable to bind DNA; however, since QscR has a functional DNA binding site, the reason why the heterodimers with LasR are impaired in DNA binding is not clear. Exogenous provision of high levels of $3OC_{12}$-HSL disrupt QscR inhibition of LasR (Chugani *et al*., 2001; Ledgham *et al*., 2003; Coggan *et al.*, 2012). Similarly to QscR, also QslA represses LasR *via* protein-protein interaction; for QslA however, Seet *et al.* in 2011 demonstrated that its interaction with LasR prevents LasR binding to the promoter of the *lasI* gene (P*lasI*); QslA is also able to disrupt a pre-formed LasR-P*lasI* complex, and the exogenous provision of $3OC_{12}$-HSL does not affected QslA repression on LasR, suggesting that QslA may control the overall QS threshold (Seet *et al.*, 2011; Coggan *et al.,* 2012). QteE was first characterized by Sienhel *et al*. in 2010 for its role in modulating the QS threshold level; it has been supposed that QteE affects LasR stability at low cell density. Further characterization of QteE is discussed in the Chapter II of this thesis; the work of Bondì *et al.* of 2014 demonstrates that a *P. aeruginosa* mutant strain defective in *qteE* shows an anticipation of $3OC_{12}$-HSL production and of QS-related virulence phenotypes expression with respect to the wild type strain (Sienhel *et al*., 2010; Coggan *et al.*, 2012; Bondì *et al.,* 2014) (Fig. 5).

The timing of the QS response seems to be very important in *P. aeruginosa* considering that its regulation is committed to three distinct regulators; it is possible, however, that they are not simply redundant, but respond to different external cues to control the exact timing of QS activation in different environmental conditions (Coggan *et al.,* 2012).

Besides the pre-quorum repression, another difference between the traditional *lux*-like QS systems and the *P. aeruginosa las* system is the post-quorum repression exerted by the repressor of *lasI*, RsaL. As previously described, the traditional *lux*-like QS systems consist of the *luxI*-like gene, encoding the synthase of the signal molecule, and of the *luxR*-like gene, encoding the receptor of the signal molecule; conversely, in the *las* system the gene *rsaL* is part of the *las* genetic locus together with *lasI* and *lasR*, and its transcription is activated by the LasR/$3OC_{12}$-HSL complex (Fig. 4). RsaL is the only transcriptional regulator, besides LasR, known to bind the *lasI* promoter region (Rampioni *et al*., 2006). RsaL represses *lasI* transcription in a post-quorum phase of growth, and therefore it counteracts the positive feedback loop generated by the LasR/$3OC_{12}$-HSL complex (Rampioni *et al*., 2007). In the traditional QS systems, when the threshold concentration of the signal molecule is reached, the positive feedback loop leads to the accumulation of the signal molecule during the all growth curve; conversely, in the *las* system, $3OC_{12}$-HSL production reaches

a stationary level before the end of exponential phase of growth due to the RsaL-dependent homeostatic regulation of *lasI* transcription (Fig. 4 and Fig. 5; Chugani *et al*., 2001; Ward *et al*., 2004; Rampioni *et al*., 2007). RsaL is also a global regulator that in *P. aeruginosa* regulates more than 300 genes, including genes involved in virulence factors production (pyocyanin and hydrogen cyanide), in the efflux of antibiotic outside the cell, and in biofilm formation (Rampioni *et al*., 2007; Rampioni *et al*., 2009).

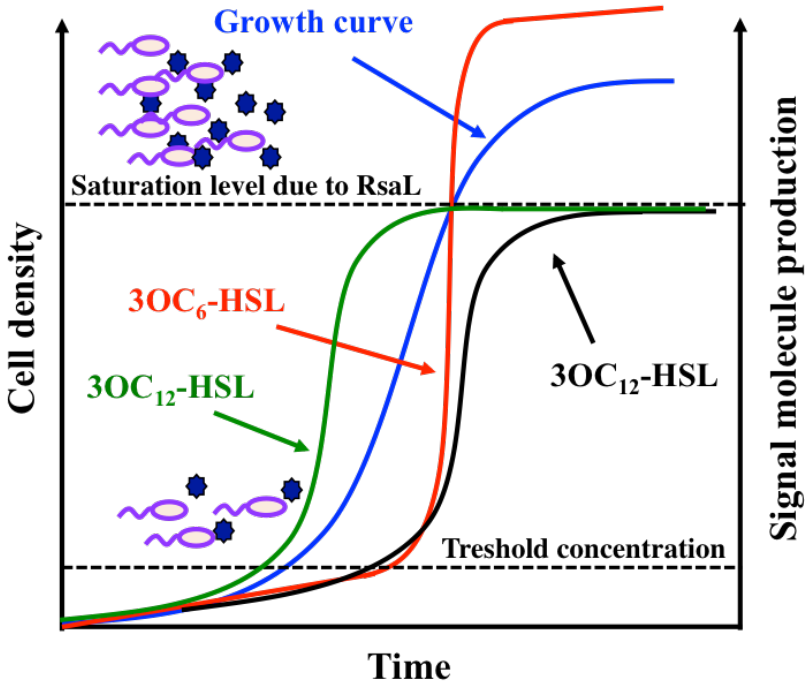

Fig. 5. Schematic representation of $3OC_6$-HSL production in *V. fischeri* and of $3OC_{12}$-HSL production in *P. aeruginosa*. In *V. fischeri*, the positive feedback loop generated by the LuxR/$3OC_6$-HSL complex leads to the accumulation of the signal molecule during the all growth curve (red curve); in *P. aeruginosa*, the levels of $3OC_{12}$-HSL reach a saturation level before the end of exponential phase of growth because the positive feedback loop generated by the LasR/$3OC_{12}$-HSL complex is counteracted by the RsaL repressive effect on *lasI* transcription (black curve). The mutation of the *qscR* or *qteE* genes anticipates $3OC_{12}$-HSL production (green curve). Dark blue stars represent the QS signal molecules.

P. aeruginosa QS circuit is integrated into a wide regulatory network, and is modulated by environmental and metabolic signals beside cell density (Soberón-Chávez *et al*., 2005; Dunn and Stabb, 2006; Duan and Surette, 2007; Coggan *et al.*, 2012). If considering only the regulators acting on the *las* QS system, a number of transcriptional and post-transcriptional regulators have been described that exert a direct or an indirect regulation on its activity. However, in most cases, their physiological role and molecular mechanism of action remain unclear (Coggan *et al.*, 2012). Some

of these regulators and their effect on the *las* system are summarized in Fig. 6, and the best characterized ones are also described below.

Besides QscR, QslA and QteE, that have been previously described, MvaT and AlgQ negatively regulate the expression of LasR, while Vfr and GacA positively regulate its expression. MvaT belongs to family of the *histone-like nucleoid structuring* (H-NS), that are involved in compacting chromosomal DNA (Dorman, 2004). In *P. aeruginosa* MvaT regulates the expression of genes involved in virulence, in arginine metabolism, in antibiotic resistance and in biofilm formation, through direct binding to the promoters or through indirect regulation (Diggle *et al.*, 2002; Vallet *et al.*, 2004; Westfall *et al.*, 2004 and 2006; Li *et al.*, 2009). In an *mvaT* mutant the production of $3OC_{12}$-HSL is anticipated with respect to the wild type strain, suggesting its role as repressor of the *las* system (Diggle *et al.*, 2002).

AlgQ (also named AlgR2) was originally identified as a regulatory protein involved in alginate production. It also regulates the synthesis of secreted virulence factors, up-regulating neuraminidase and siderophore synthesis and down-regulating rhamnolipids and extracellular proteases synthesis. AlgQ negatively modulates the expression of *lasR* by the directly binding to its promoter region (Ledgham *et al.*, 2003).

Vfr was first identified in *P. aeruginosa* as a *virulence factor regulator*, due to its positive effect on the production of several virulence factors, such as proteases and exotoxin A. It is a member of the cAMP receptor protein (CRP) family, and links the QS circuit and the cyclic adenosine monophosphate (cAMP) signaling system by directly activating *lasR* transcription (Albus *et al*., 1997). Differently from *Escherichia coli*, cAMP levels in *P. aeruginosa* are not related to glucose uptake and utilization (West *et al.,* 1994), and the role played by cAMP in *P. aeruginosa* physiology, as well as the environmental stimuli to which Vfr responds, are still unknown (Lazdunkski *et al.*, 2007).

GacA is the response protein of the two-component system GacS/GacA, and it activates the transcription of LasR and also of its repressor QscR. Moreover, GacA is essential for the expression of the two non-coding regulatory RNAs RsmZ and RsmY, that work in tandem with the regulator RsmA. RsmA is a pleiotropic post-transcriptional regulator that together with RsmZ and RsmY controls the production of secondary metabolites both directly and indirectly. RsmA represses *lasI* transcription and exerts a negative effect on $3OC_{12}$-HSL production. In a *rsmA* mutant strain the expression of *lasI* is prematurely induced and reaches higher levels with respect to the wild type strain (Lazdunkski *et al.*, 2007).

VqsR is a LuxR-like protein with no signal molecule-binding domain. Its synthesis is dependent on the LasR/3OC$_{12}$-HSL complex, and in turn VqsR represses *lasR* transcription. A mutant defective in *vqsR* is impaired in 3OC$_{12}$-HSL production (Lazdunkski *et al.*, 2007).

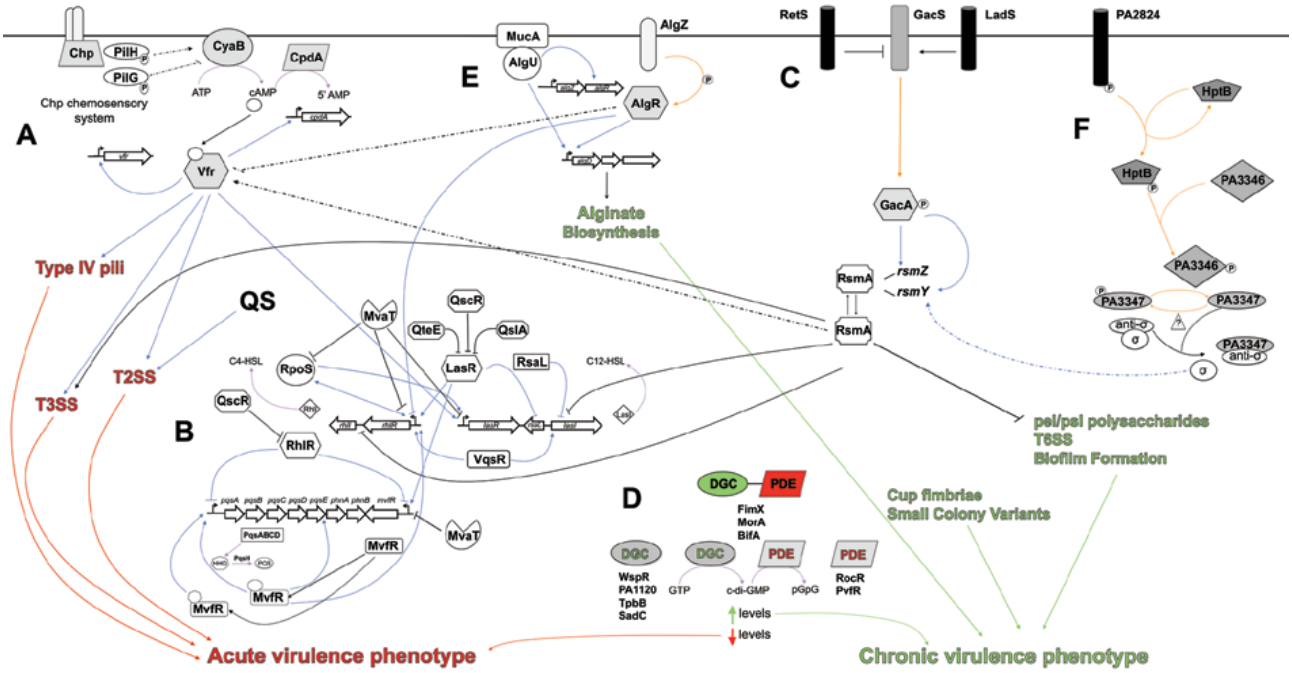

Fig. 6. Schematic representation of the regulatory pathways that modulate *P. aeruginosa* lifestyle. Lines depict direct regulatory mechanisms within given signaling pathways, as well as cross-talk between regulatory systems. Arrows represent positive regulation; T-lines indicate negative regulation; blue lines represent transcriptional regulation while purple lines represent enzymatic reactions; orange lines depict post-translational modification events; black lines illustrate post-translational regulation events; dashed lines indicate unknown mechanisms of regulation. **(A)** cAMP/Vfr signaling. **(B)** Quorum Sensing. **(C)** Gac/Rsm pathway. **(D)** c-di-GMP signaling. **(E)** MucA signaling. **(F)** HptB signaling. Modified by Coggan *et al.*, 2012.

AIMS AND RATIONALE

P. aeruginosa resistance to antibiotics demand the discovery of new therapeutic approaches. Targeting bacterial virulence instead of bacterial growth is an alternative approach to antimicrobial therapy that offers promising opportunities to inhibit pathogenesis and its consequences without placing immediate life-or-death pressure on the target bacterium (Cegelski *et al.*, 2008). However, as described above, virulence in *P. aeruginosa* is due to the production of an arsenal of virulence factors: for this reason the appropriate anti-virulence drug should target the regulatory networks controlling virulence instead of a single virulence factor (Rasko and Sperandio, 2010). In this context, QS is a good target for anti-virulence drugs because it controls genes involved in multiple virulence factors production and in biofilm formation, playing a positive role in the instauration of both the acute and the chronic infection in humans (Rutherford and Bassler, 2012). Moreover, QS is interwoven in an intricate regulatory network (Fig. 6; Venturi, 2006; Coggan *et al.*, 2012) and it has been suggested that also the regulators of QS play an important role during the infections; therefore, QS regulators could be good candidates for the development of anti-virulence drugs.

The general aim of this PhD project has been the study of the regulation of the *P. aeruginosa las* QS system, an. This project is structured in three main objectives: *(i)* the identification of novel transcriptional regulators controlling LasR expression (Longo *et al.*, 2013; Chapter I); *(ii)* the study of the effect of a dysregulation in timing and magnitude of the *las* QS response on *P. aeruginosa* ability to establish infections; (Bondì *et al.*, 2014; Chapter II); *(iii)* the investigation of new possible regulative properties arising from the peculiar architecture of the *las* QS system (Bondì *et al.*, manuscript in preparation; Chapter III).

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Chapter I

A new transcriptional repressor of the *Pseudomonas aeruginosa* Quorum Sensing receptor gene *lasR*

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A New Transcriptional Repressor of the *Pseudomonas aeruginosa* Quorum Sensing Receptor Gene *lasR*

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Abstract

Pseudomonas aeruginosa pathogenic potential is controlled *via* multiple regulatory pathways, including three quorum sensing (QS) systems. LasR is a key QS signal receptor since it acts as a global transcriptional regulator required for optimal expression of main virulence factors. *P. aeruginosa* modulates the QS response by integrating this cell density-dependent circuit to environmental and metabolic cues. Hence, QS also controls the adaptation to challenging environmental niches, such as infection sites. However, little is known about the molecular mechanisms connecting QS and other signalling pathways. In this work, DNA-affinity chromatography was used to identify new *lasR* transcriptional regulators. This approach led to the identification and functional characterization of the TetR-like transcriptional repressor PA3699. This protein was purified and shown to directly bind to the *lasR* promoter region *in vitro*. The induction of PA3699 expression in *P. aeruginosa* PAO1 cultures repressed *lasR* promoter activity and the production of LasR-dependent virulence factors, such as elastase, pyocyanin, and proteases. These findings suggest a role for PA3699 in *P. aeruginosa* pathogenicity. *P. aeruginosa* genome encodes at least 38 TetR-family proteins, and PA3699 is the eighth member of this group functionally characterized so far and the first one shown to bind the *lasR* promoter *in vitro.*

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Introduction

The definition of quorum sensing (QS) was coined about 20 years ago to describe a communication system based on the production, detection, and response to signal molecules, allowing bacterial populations to trigger a coordinated response at a threshold cell density [1]. Today it is well known that QS regulation plays a key role in a number of relevant bacterial processes, including colonization of plants and animal tissues, and production of antibiotics [2,3].

In many bacteria, the QS regulatory device is interwoven with other global regulatory networks responsive to different environmental cues (e.g., temperature, pH, osmolarity, oxidative stress, nutrient starvation). These networks cross-talk in the bacterial cell in order to determine the optimal survival strategy [4]. The advantage of integrating QS with other regulatory pathways becomes a compelling necessity when the QS response controls multiple functions and its activation commits bacteria to a strong reorganization of the whole cellular metabolism [5].

One of the most studied model organisms in QS research is *Pseudomonas aeruginosa*. This versatile bacterium is able to thrive in a wide range of environmental niches, including the human body. The high adaptability of *P. aeruginosa* is reflected by its behaviour as a pathogen. In humans, *P. aeruginosa* causes community- and hospital-acquired infections, colonizing different body districts like lungs, eyes, ears, urinary tract, injured skin (burns and wounds). Such infections are often difficult to eradicate as a consequence of antibiotic resistance and biofilm formation [6]. In particular, *P. aeruginosa* chronic lung infection is the major cause of death in cystic fibrosis patients, a genetic disease affecting about 1/3,000 newborns in the Caucasian population [6].

P. aeruginosa pathogenicity strongly depends on the fine and coordinated regulation of a wide array of virulence factors [7,8]. This is achieved thanks to a complex network of regulatory and signalling pathways controlling virulence-related phenotypes, in response to environmental cues and bacterial population structure [9]. Among these pathways, a preeminent role in *P. aeruginosa* virulence is played by three

interconnected QS systems, based on the production of different signal molecules. The QS signal molecule *N*-3-oxo-dodecanoyl-homoserine lactone ($3OC_{12}$-HSL) is synthesized by LasI, encoded by the *lasI* gene, and its cognate receptor is the cytoplasmic transcriptional regulator LasR, encoded by the *lasR* gene. $3OC_{12}$-HSL progressively accumulates in the bacterial culture and at a threshold concentration it binds LasR. The LasR/$3OC_{12}$-HSL complex, in turn, activates transcription of hundreds of genes, including the genes coding for the receptors of the other two QS systems, based on *N*-butanoylhomoserine lactone (C_4-HSL) and 2-heptyl-3-hydroxy-4-quinolone (PQS) signal molecules. As a whole, the *P. aeruginosa* QS circuit has a key role in pathogenesis, regulating the production of virulence factors, the formation of biofilm and the expression of antibiotic efflux pumps [10].

The three QS systems of *P. aeruginosa* are interwoven and connected to other regulatory pathways [10–12]. Indeed the expression of genes involved in the synthesis and perception of QS signal molecules is finely regulated at the transcriptional and post-transcriptional level in response to various metabolic and environmental stimuli [12–15]. However, the proteins involved in this regulation and their mechanisms of action are largely unknown [11,12]. To date only few transcriptional regulators have been shown to directly bind the promoters of genes involved in *P. aeruginosa* QS signal synthesis and perception. In particular, RsaL directly represses *lasI* transcription [16,17], while Vfr directly activates the *lasR* promoter region [18]. Moreover, AlgR2 (AlgQ), besides acting as an anti-sigma factor for the vegetative sigma RpoD [19], can also bind to and downregulate the *lasR* promoter [20]. However, the stimuli controlling the transcriptional activity of Vfr, AlgR2 (AlgQ) and RsaL are still unknown.

The objective of this work has been the identification of novel transcriptional regulators of LasR expression. To this aim, *P. aeruginosa* cytoplasmic proteins able to bind the *lasR* promoter region have been picked-up by DNA-affinity chromatography and identified by mass spectrometry. This led to the functional characterization of the TetR-like protein PA3699, which acts as a novel repressor of *lasR* transcription and of *P. aeruginosa* virulence factors production.

Results and Discussion

Identification of *P. aeruginosa* proteins interacting with the *lasR* promoter

To increase the probability of fishing proteins interacting with the *lasR* promoter (P*lasR*), the activity of P*lasR* was preliminarily monitored along the *P. aeruginosa* PAO1 growth curve by means of a P*lasR*::*lacZ* transcriptional fusion (Figure 1A Table S2). P*lasR* activity increased steadily during the exponential phase and reached a plateau at the onset of the stationary phase. The maximal promoter activity was maintained for about four hours, and then declined (Figure 1A), suggesting that at the end of the exponential phase and during the stationary phase of growth, one or more transcriptional regulator(s) bind to P*lasR* and affect its promoter activity. Hence, crude protein extracts were prepared from *P. aeruginosa* PAO1 cultures grown to A_{600} = 2.0 and A_{600}= 5.0, corresponding to late exponential and stationary phase of growth, respectively (Figure 1A).

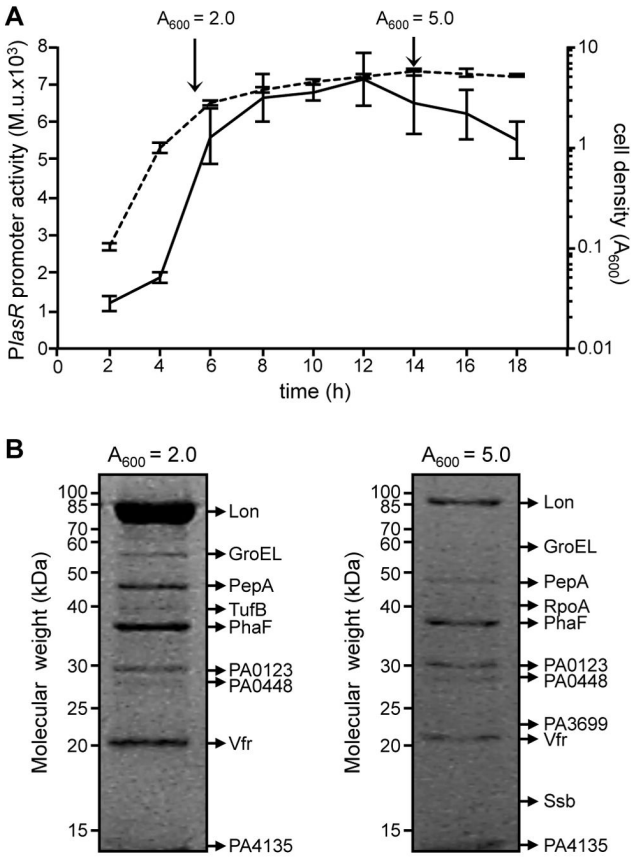

Figure 1. P*lasR* activity and P*lasR*-affinity chromatography. (A) Growth curve of the *P. aeruginosa* PAO1 wild type strain carrying the pMP*lasR*::*lacZ* plasmid (dashed line) and corresponding P*lasR* promoter activity (solid line). The points of the growth curve at which the protein crude extracts for DNA-affinity chromatography were prepared are indicated by arrows. (B) SDS-PAGE analysis of proteins bound to the P*lasR* promoter region. Protein crude extracts were prepared from *P. aeruginosa* PAO1 cultures grown in LB broth to the indicated cell densities (A_{600}). Bands analysed by MALDI-TOF mass spectrometry are indicated by arrows, and the corresponding protein name is reported.

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To prepare the DNA-affinity chromatography matrix, a biotinylated double-stranded DNA fragment, corresponding to the *lasR* promoter region cloned in the P*lasR*::*lacZ* transcriptional fusion, was immobilized on a streptavidin-conjugated chromatography resin. Protein crude extracts were independently incubated with the matrix and, after extensive washing, proteins specifically bound to P*lasR* were eluted, separated by SDS-PAGE, and identified by MALDI-TOF mass spectrometry (Table 1). The experiment was performed in duplicate for each crude extract. Examples of SDS-PAGE gels are shown in Figure 1B. Overall, twelve protein bands were reproducibly detected on the SDS-PAGE gels (Table 1), while

Table 1. List of P*lasR*-binding proteins identified by MALDI-TOF analysis from the SDS-PAGE gels shown in Figure 1B.

PA number [a b]	Gene name[a]	Functional class[a]	Predicted molecular mass (kDa)	Protein score[c] – No. of peptides matched	
				A_{600} = 2.0	A_{600} = 5.0
PA0123		probable transcriptional regulator	33.5	447-10	708-21
PA0448		probable transcriptional regulator	34.1	652-18	777-19
PA0652	*vfr*	Transcriptional regulator Vfr	24.5	564-14	621-16
PA0779	*lon*	probable ATP-dependent protease	88.6	276-11	709-34
PA3699		probable transcriptional regulator	26.3	-	279-13
PA3831	*pepA*	leucine aminopeptidase	52.6	891-24	412-20
PA4135		probable transcriptional regulator	16.5	293-10	258-9
PA4232	*ssb*	single-stranded DNA-binding protein	18.5	-	134-6
PA4238	*rpoA*	DNA-directed RNA polymerase alpha chain	39.7	-	390-14
PA4277	*tufB*	elongation factor Tu	43.7	709-21	-
PA4385	*groEL*	GroEL protein	57.1	1210-22	723-21
PA5060	*phaF*	polyhydroxyalkanoate synthesis protein PhaF	30.6	439-11	456-20

a. PA number, gene name and functional class refer to the *Pseudomonas* Genome Database annotation [22].

[b] Proteins identified only when using P*lasR* as DNA bait are in bold characters.

c. The protein score is according to the Mascot programme (scores ≥ 76 correspond to an error probability $p < 0.05$ in our data set) [37].

no protein bands were visible in control experiments with beads uncoupled to DNA (data not shown). Among these, RpoA, Ssb and TufB are involved in general DNA processing, while the PhaF phasin is involved in polyhydroxyalkanoate segregation during cell division [21]. Moreover three proteins not involved in DNA processing (i.e., Lon, PepA and GroEL) were also retrieved (Table 1).

Of the two transcription factors previously known to bind P*lasR* (i.e., Vfr and AlgR2) [18,20], only Vfr was picked-up (Table 1), indicating that the DNA-affinity chromatography approach here described allows identification of some, but not all, the transcription factors specific for P*lasR*. Therefore, besides AlgR2, other regulators of this promoter may have escaped our analysis.

Last, but of primary importance with respect to our aims, among the picked-up proteins PA0123, PA0448, PA3699 and PA4135 were annotated in the *P. aeruginosa* genome as putative transcription factors with unknown function (Table 1). On the basis of their sequence, PA0123 and PA0448 are annotated as putative LysR-like family transcriprional regulators [22]. Interestingly, the three-dimensional structure of PA3699 and PA4135 has been recently solved (Protein Data Bank accession numbers, 3KKD and 2FBI, respectively). On the basis of primary sequence and structural features, PA3699 and PA4135 can be assigned to the TetR-like and MarR-like family of transcriptional regulators, respectively [22]. In this view, the above results provide the first experimental evidence that PA0123, PA0448, PA3699 and PA4135 are expressed in *P. aeruginosa* cultures and able to bind DNA *in vitro*. On the basis of the above considerations, these four factors were selected for further analysis as novel putative *lasR* regulators (Table 1).

In vivo characterization of putative *lasR* transcriptional regulators

In order to study the *in vivo* effect of PA0123, PA0448, PA3699 and PA4135 on P*lasR* activity, a set of *P. aeruginosa* PAO1 in frame deletion mutants in the corresponding genes was generated. As a control, also a mutation in the *vfr* gene was introduced in *P. aeruginosa* PAO1. A genetic cassette carrying the P*lasR*::*lux* transcriptional fusion was introduced in a chromosomal neutral site of the five mutant strains and of *P. aeruginosa* wild type to conveniently measure P*lasR* activity as a function of light emission along the growth curve. The DNA region cloned in this genetic cassette was identical to that used as a bait in the DNA-affinity chromatography experiment.

In accordance with literature data, P*lasR* activity was strongly decreased as a consequence of *vfr* mutation (Figure S1) [18], while no significant differences with respect to the wild type activity were observed in the PA0123, PA0448, PA3699 and PA4135 mutant strains (Figure S1). Similar results were obtained by using the P*lasR*::*lacZ* transcriptional fusion as reporter system (data not shown).

It was surprising that inactivation of the four new putative regulators able to bind P*lasR in vitro* did not affect the activity of this promoter *in vivo*. However, the DNA-affinity chromatography allows the purification of proteins present in a synthetic binding buffer, a condition that is unlikely to mimic the intracellular milieu. Thus, some of the factors picked-up *in vitro* using P*lasR* as bait could be not sufficiently expressed and/or active *in vivo* under the experimental conditions used for the promoter activity assay. To overcome this problem, the PA0123, PA0448, PA3699, and PA4135 genes were cloned in the expression vector pHERD30T, under the control of an L-arabinose-inducible promoter (Table S2) [23]. The resulting plasmids were independently introduced in the wild type *P. aeruginosa* PAO1 strain carrying the P*lasR*::*lux* transcriptional fusion, and promoter activity was measured along the growth curve in the presence of L-arabinose; the pHERD30T empty vector was used as control. With the only exception of PA0448, L-arabinose-induced expression of the tested proteins reduced the activity of the P*lasR*::*lux* fusion. However, induction of PA0123 and PA4135 also resulted in a strong inhibition of *P. aeruginosa* growth (Figure 2A). Since bioluminescence emission is an energy demanding process, we hypothesized that PA0123 and PA4135 induction might affect growth only in a *lux* proficient background. However, PA0123 and PA4135 induction had a negative effect on *P. aeruginosa* growth also in the PAO1 wild type strain lacking the P*lasR*::*lux* fusion in the chromosome (data not shown). Overall, these results indicate that PA0123 and PA4135 are toxic for *P. aeruginosa* when their expression is induced, at least in the growth conditions used in our experimental setting.

Since an effect on bacterial growth is particularly meaningful when studying a cell density-dependent pathway like QS, further studies were focused on PA3699, the only protein that, once induced, strongly inhibited P*lasR* activity without affecting *P. aeruginosa* growth (Figure 2A).

It could be reasonably argued that the procedure described in the above paragraphs could isolate DNA binding proteins that do not play a role *in vivo* on the regulation of the target promoter and that the overexpression of transcriptional regulators could lead to pleiotropic effects/phenotypes. These causalities likely occurred in the case of PA0448, PA0123 and PA4135, since the first protein has no effect on P*lasR* activity and the other two are toxic to the cell when overexpressed. Conversely, PA3699 induction did not affect growth, while it repressed P*lasR* activity during the whole growth curve and proportionally to the amount of L-arabinose present in the growth medium (Figure 2B and 2C). All these features argue for a *bona fide* repressor activity of PA3699 on P*lasR*.

PA3699 binds the *lasR* promoter region in EMSA assays

In order to confirm DNA-affinity chromatography results, electrophoretic mobility shift assay (EMSA) of a DNA probe (P*lasRp*) encompassing the promoter region of *lasR* were conducted using the purified PA3699 protein. To this purpose, PA3699 was fused to a six-histidine tag (6xHis) at the N-terminal domain and purified by nickel-nitrilotriacetic acid (Ni-NTA)-affinity chromatography. The 6×His tag was subsequently

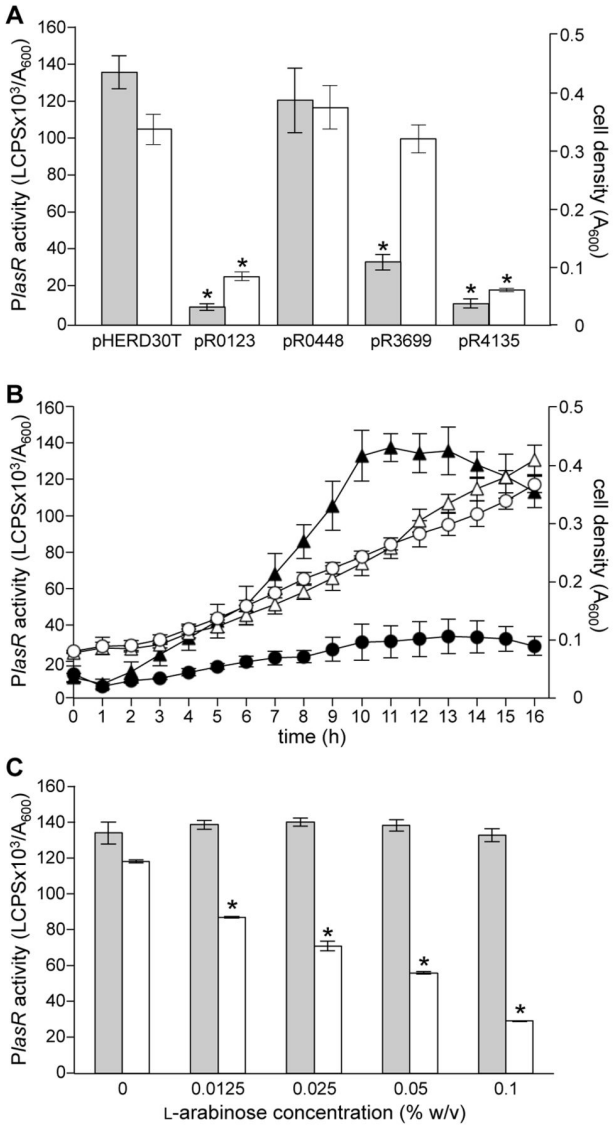

Figure 2. Effect of the induction of P*lasR*-bound proteins on P*lasR* promoter activity. . (A) Histogram reporting P*lasR* maximal promoter activity (grey bars) and the corresponding cell density (white bars) measured in *P. aeruginosa* PAO1 P*lasR::lux* strains carrying the plasmids indicated below the graph, grown in LB supplemented with 0.1% (w/v) L-arabinose. (B) Graph reporting P*lasR* promoter activity (filled symbols) and cell density (open symbols) measured during the growth curve in *P. aeruginosa* PAO1 P*lasR::lux* carrying pHERD30T (triangles) or pR3699 (circles), grown in LB supplemented with 0.1% (w/v) L-arabinose. (C) Histogram reporting P*lasR* maximal promoter activity measured in *P. aeruginosa* PAO1 P*lasR::lux* strains carrying pHERD30T (grey bars) or pR3699 (white bars) grown in LB supplemented with different L-arabinose concentrations (%, w/v), indicated below the graph. In (A), (B) and (C) the average of three independent experiments is reported with standard deviations; in (A) and (C) statistical significance with respect to *P. aeruginosa* PAO1 P*lasR::lux* (pHERD30T) is indicated with one asterisk ($p < 0.01$).

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cleaved by thrombin digestion, yielding the native PA3699 protein in soluble and highly pure form (Figure 3A and 3B).

As shown in Figure 3C, incubation of the P*lasRp* probe with purified PA3699 led to the formation of a complex endowed with lower electrophoretic motility with respect to the free probe, and the amount of the shifted complex was proportional to the concentration of purified PA3699. Since the electrophoretic motility of an unspecific DNA probe did not change in the presence of PA3699, the binding of this protein to P*lasRp* is specific (Figure 3C).

Altogether, these data provide an unequivocal proof of the direct molecular interaction between PA3699 and the *lasR* promoter region.

PA3699 affects the production of QS-controlled virulence factors

The LasR/3OC$_{12}$-HSL complex is required for full expression of most *P. aeruginosa* virulence factors, including elastase, pyocyanin, and proteases [24].

In order to assess whether the repression exerted by PA3699 on LasR expression may affect *P. aeruginosa* pathogenic potential, the expression of LasR-dependent virulence phenotypes was compared in *P. aeruginosa* carrying either the pHERD30T empty vector or its derivative plasmid for L-arabinose-dependent induction of PA3699 (pR3699). Consistent with the strong repressive effect exerted by PA3699 induction on P*lasR* activity, results showed that also the production of LasR-dependent phenotypes such as elastase, pyocyanin, and proteases was strongly decreased in *P. aeruginosa* upon PA3699 induction (Figure 4). However, pyocyanin, elastase and protease production remained unaffected in the *P. aeruginosa* PA3699 mutant strain with respect to the wild type, consistently with what observed for the P*lasR* promoter activity (data not shown).

CONCLUDING REMARKS

The masterly management of an enormous metabolic potential *via* multiple regulative networks and signalling systems allows *P. aeruginosa* adaptation to the most challenging environments, including the human host [9,11,12].

The *P. aeruginosa las* QS system is among the most studied and best characterized bacterial regulatory pathways, also because it is considered a promising target for anti-virulence therapies [25–27]. Nevertheless, its interconnections with other *P. aeruginosa* regulative networks and signalling systems are still poorly understood [9].

So far, *in vivo* (genetic) approaches led to the identification of a number of genes involved in the modulation of the *las* system activity/response, highlighting the multiplicity of pathways connecting cell-density and environmental/metabolic signalling. However, the transcriptional factors directly controlling the expression of the *las* genes in response to environmental/metabolic stimuli are mostly unknown [11,12,15]. In this work, DNA-affinity chromatography has been used for the first time for the identification of transcriptional regulators of the *lasR* gene, encoding the 3OC$_{12}$-HSL receptor.

Unexpectedly, the genetic inactivation of the four putative P*lasR* transcription factors identified by *in vitro* DNA-affinity chromatography (i.e., PA0123, PA0448, PA3699, and PA4135) did not affect the activity of P*lasR in vivo*. A possible explanation for this result is that at least some of these factors are not sufficiently expressed and/or active *in vivo* under the conditions used in the promoter activity assay. This hypothesis

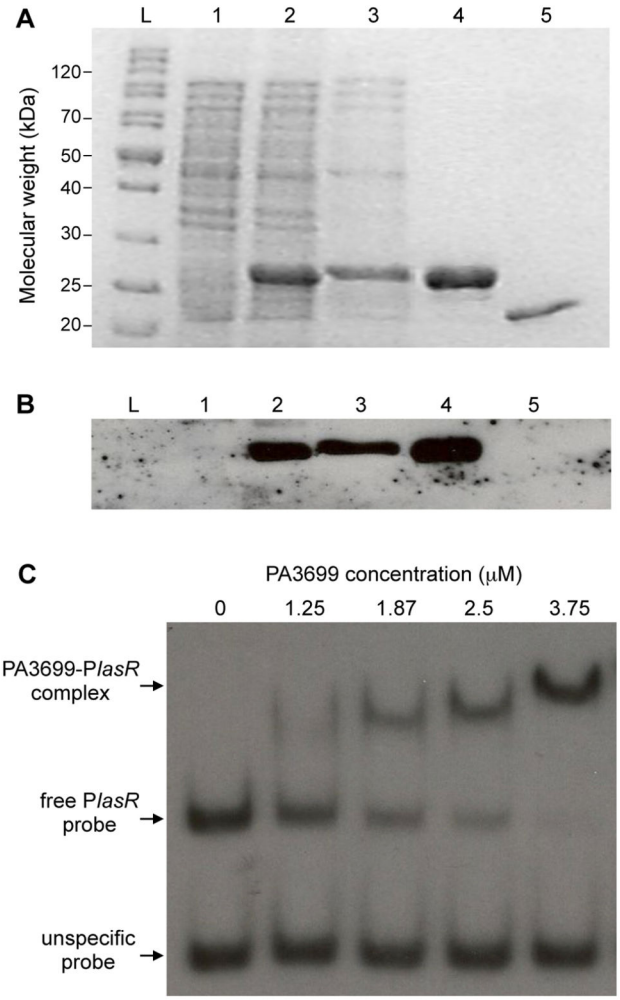

Figure 3. PA3699 purification and P*lasR*-binding assay. . (A) SDS-PAGE analysis of samples withdrawn at different steps of PA3699 purification. Lane L, PageRuler Unstained Protein Ladder (Fermentas); lane 1, non-induced protein crude extract; lane 2, induced protein crude extract; lane 3, soluble fraction of the induced protein crude extract; lane 4, purified protein; line 5, purified protein after thrombin cleavage. (B) Western blot analysis performed with mouse anti-6xHis primary antibody and anti-mouse peroxidase-conjugated secondary antibody on a gel identical to the one shown in (A). (C) Autoradiography of an EMSA showing direct interaction between a DNA probe encompassing the *lasR* promoter region and purified PA3699. PA3699 concentration (μM) is indicated above each lane. An unspecific probe was added in the reaction mixture as control. The PA3699-P*lasR* complex and the free DNA probes are indicated.

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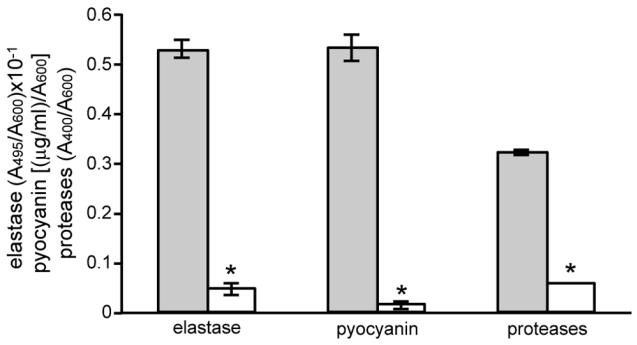

Figure 4. Effect of PA3699 induction on *P. aeruginosa* virulence factors production. . Histogram reporting elastase, pyocyanin and proteases production measured in *P. aeruginosa* PAO1 carrying pHERD30T (grey bars) or pR3699 (white bars), grown in LB supplemented with 0.1% (w/v) L-arabinose. The average of three independent experiments is reported with standard deviations; statistical significance with respect to *P. aeruginosa* PAO1 (pHERD30T) is indicated with one asterisk ($p < 0.01$).

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is supported by the observation that a progressive increase of PA3699 induction caused a parallel decrease in P*lasR* activity, indicating this factor as a novel transcriptional repressor of P*lasR*.

According to its structural features, PA3699 belongs to the TetR-family, a preeminent group of transcriptional regulators in bacteria [28]. Transcriptional regulators of the TetR-family typically activate upon binding of a specific ligand to an allosteric site [28]. Thus, it is likely that PA3699 regulates *lasR* transcription only in the presence of a specific ligand when expressed at a physiological level. Since the interaction of a transcriptional regulator with its DNA target sequence is governed by a thermodynamic equilibrium between the bound and unbound form, the induction of PA3699 expression could shift this equilibrium to the bound state, resulting in P*lasR* regulation also in the absence of the actual stimulus/ligand. Phenotypic microarray experiments are in progress to identify the growth conditions leading to PA3699 full activity and/or expression.

P. aeruginosa genome encodes at least 38 TetR-family proteins, and PA3699 is the eighth member of this group functionally characterized so far and the first one shown to bind the *lasR* promoter *in vitro* [28–33].

Overall, DNA-affinity chromatography combined with induction of selected factors has been proven as a useful approach for the identification of regulators that are not highly expressed or active under standard laboratory conditions, but that might be relevant in specific environmental niches, like for instance infection sites.

Although further studies are required to assess the involvement of PA3699 in *P. aeruginosa* physiology and pathogenesis, this work represents a further step toward the disclosure of the complex network controlling cell-cell communication, social behaviours and host adaptability of *P. aeruginosa*, and hopefully toward the identification of new promising targets for future drug-research programmes.

Materials and Methods

Bacterial strains and media

The bacterial strains used in this study are listed in Table S1 (Supporting Information). All *E. coli* and *P. aeruginosa* strains were routinely grown at 37°C in Luria-Bertani broth (LB), LB supplemented with 1.5% (w/v) agar, or Pseudomonas Isolation Agar (PIA) [34]. Unless otherwise stated, antibiotics were added at the following concentrations: *E. coli*, 20 µg/ml gentamicin (Gm), 100 µg/ml ampicillin (Ap), 25 µg/ml kanamycin (Km), 30 µg/ml chloramphenicol (Cm), 10 µg/ml tetracycline (Tc), 10 µg/ml nalidixic acid (Nal); *P. aeruginosa*, 100 µg/ml Gm, 400 µg/ml Cm, 100 µg/ml Tc, 300 µg/ml carbenicillin (Cb). When required, media were supplemented with glucose, isopropyl β-D-1-thiogalactopyranoside (IPTG) or L-arabinose at the concentration reported in the text.

Recombinant DNA techniques

Plasmids used or generated in this study and details on their construction are reported in Table S2 (Supporting Information). Preparation of plasmid DNA, purification of DNA fragments, restrictions, ligations, and transformations of *E. coli* were carried out by standard procedures [34]. PCR amplifications were performed using Bio Red-Taq DNA polymerase (Bioline) or Accuzyme DNA polymerase (Bioline). The oligonucleotides used in this study are listed in Table S3 (Supporting Information). Automated sequencing was performed by Genechron sequence service (Genechron).

When required, plasmids were transferred from *E. coli* S17.1 λ*pir* to *P. aeruginosa* PAO1 by bi-parental conjugation, and from *E. coli* DH5α to *P. aeruginosa* PAO1 by tri-parental conjugation with the helper strain *E. coli* HB101 pRK2013 [35].

DNA-affinity chromatography

DNA-affinity purification was performed as previously described [36]. A biotinylated DNA fragment encompassing the *lasR* promoter region (from nucleotide -359 to +13 relative to the *lasR* start codon) was PCR amplified with primers FW381 and RV334 (Table S3); primer FW381 was biotinylated.

P. aeruginosa PAO1 protein crude extracts were obtained from cells grown in 300 ml of LB at 37°C to an absorbance at 600 nm wavelength (A_{600}) of 2.0 or 5.0, with 200 r.p.m. shaking. Bacterial cells were harvested by centrifugation and suspended in 2 ml of Sonication Buffer [10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.05% (v/v) Triton X-100] supplemented with 1 mg/ml lysozyme. After 30 min of incubation at 37°C, cells were disrupted by sonication. Cellular debris were removed by centrifugation and subsequent filtration. Protein concentration in the supernatant was determined with the Bradford Protein Assay Kit (Bio-Rad) according to manufacturer's instruction.

One mg of paramagnetic streptavidin-conjugated resin (Dynabeads M-280, Invitrogen) was equilibrated in 200 µl of Wash Buffer 2X [10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl, 0.05% (v/v) Triton X-100], and then incubated with 20 µg

of the biotin-labelled PCR product for 25 min at 22°C. Unbound DNA was removed by magnetic separation in a magnet particle concentrator (Dynal, Invitrogen). Dynabeads were washed three times with 1 ml Wash Buffer 1X, and then incubated with 50 mg of *P. aeruginosa* protein crude extract for 2 hours at 22°C. Unbound proteins were removed by magnetic separation in a magnet particle concentrator, and the Dynabeads were washed six times with Sonication Buffer. Proteins specifically bound to the DNA-bead complexes were eluted in 20 µl of Elution Buffer [1.2 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.05% (v/v) Triton X100]. Eluted proteins were separated by Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) on a 12% (v/v) polyacrylamide gel and stained with Coomassie Brilliant Blue [34]. Protein identity was determined by Matrix-Assisted Laser Desorption/ Ionization-Time Of Flight (MALDI-TOF) mass spectrometry. MALDI-TOF analyses were performed as previously described [37].

Construction of *P. aeruginosa* mutants

P. aeruginosa in frame deletion mutants were generated using the pDM4 plasmid as previously described [38]. Briefly, the flanking DNA regions (about 650 bp each) of the selected genes were PCR amplified with primers listed in Table S3 and sequentially cloned in pDM4. The resulting pDM4-derivative plasmids, listed in Table S2, were independently introduced in *P. aeruginosa* PAO1 by conjugation. Clones with a chromosomal insertion of the pDM4-derivative plasmids were selected on LB agar plates supplemented with 400 µg/ml Cm e 10 µg/ml Nal. Plasmid excision from the chromosome was subsequently selected on LB agar plates supplemented with 10% (w/v) sucrose. Mutant strains were checked by PCR analysis.

Construction of *PlasR* transcriptional fusions

The DNA region encompassing the *lasR* promoter (spanning from nucleotide -359 to +13 relative to the *lasR* start codon) was PCR amplified with the primers FW333 and RV334 (Table S3) and cloned by EcoRI-PstI restriction in the pMP220 and mini-CTX-*lux* plasmids [39,40]. The resulting pMP*lasR*::*lacZ* and mini-CTX-P*lasR*::*lux* plasmids (Table S2) were conjugated in selected *P. aeruginosa* strains. The mini-CTX plasmid backbone was removed from the *P. aeruginosa* strains carrying the P*lasR::lux* cassette by using the pFLP2 plasmid (Table S2). pFLP2 was subsequently cured by sucrose counterselection as previously described [41].

Over-expression of putative *lasR* regulators

The PA0123, PA0448, PA3699 and PA4135 genes were PCR amplified using the primers listed in Table S3 and cloned in the pHERD30T vector [23], generating the plasmids pR0123, pR0448, pR3699 and pR4135, respectively (Table S2). These plasmids were introduced in *P. aeruginosa* PAO1 carrying the P*lasR::lux* transcriptional fusion by conjugation. Positive clones were selected on PIA plates supplemented with 100 µg/ml Gm.

Promoter activity assay

P*lasR* activity in *P. aeruginosa* PAO1 carrying the pMP*lasR*::*lacZ* plasmid was measured by standard Miller assay [42]. An over-night culture was diluted to an A_{600} of 0.05 in fresh LB, and incubated for 18 hrs at 37°C. Promoter activity (M.u.) and cell density (A_{600}) were determined every 2 hr for 18 hrs.

P*lasR* activity in *P. aeruginosa* strains carrying the P*lasR*::*lux* chromosomally-inserted transcriptional fusion was determined as bioluminescence emission per cell by using the automated luminometer-spectrometer Wallac 1420 VICTOR 3V (PerkinElmer). Over-night cultures were diluted to an A_{600} of 0.05 in fresh LB, and 0.2 ml of these cultures were grown at 37°C in microtiter plates. Luminescence and cell density (A_{600}) were determined every 1 hr for 16 hrs. Promoter activity is given as Light Counts Per Second (LCPS) divided by A_{600}. The strains carrying the pHERD30T-derivative plasmids were grown in LB supplemented with Gm 100 µg/ml and 0.1% L-arabinose. For both the *lacZ*- and *lux*-based promoter assays average values and standard deviations were calculated from three independent experiments.

PA3699 purification

The PA3699 gene was cloned in the pET-28b(+) vector (Novagen), generating the pE3699-N6 plasmid (for more details see Table S2). In this plasmid the PA3699 gene is transcriptionally-coupled in frame with a sequence coding for a 6 histidine tag (6xHis) at the N-terminus.

The pE3699-N6 plasmid was transformed in *E. coli* BL21 (DE3, pLysS) (Novagen). The resulting strain was grown over-night at 37°C with 200 r.p.m. shaking in LB supplemented with 25 µg/ml Km, 30 µg/ml Cm and 0.2% (w/v) glucose. The over-night culture was diluted 1:100 in 250 ml of the same medium and after 1 hr growth at 37°C with 200 r.p.m. shaking it was induced for 4 hrs with 0.5 mM IPTG.

PA3699-N6 purification was performed with the QIAexpress® Ni-NTA Fast Start Kit (Qiagen), according to manufacturer's instructions. The PA3699-N6 recombinant protein was eluted from the Ni-NTA resin with 1 ml of Elution Buffer Native, dialyzed 1:1000 in Dialysis Buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl), and concentrated with Centricon YM-10 centrifugal filter device (Amicon). PA3699-N6 identity was verified by SDS-PAGE and Western blot analysis performed with anti-6xHis primary antibody (Qiagen) and peroxidase-conjugated anti-mouse IgG secondary antiboby (Sigma-Aldrich) on aliquots withdrawn at different steps of the purification process [34]. The 6×His tag was subsequently cleaved from 1 mg of purified PA3699-N6 by digestion with 100 µg thrombin in Thrombin Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 3.3 mM $CaCl_2$). The PA3699 protein was further purified by gel permeation chromatography on a Superdex 200 HR column (Amersham Pharmacia Biotech), dialyzed 1:1000 in Storage buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 50% glycerol) and stored at -20°C.

Protein concentration was determined with the Bradford protein assay kit (Bio-Rad) according to manufacturer's instruction.

Electrophoretic Mobility Shift Assay (EMSA)

A 210 bp DNA fragment encompassing the *lasR* promoter region (from nucleotides -359 to -150 relative to the *lasR* start codon) was PCR amplified with primers FW 333 and RV 535

(Table S3) from *P. aeruginosa* PAO1 genome and T/A cloned in the pDRIVE-T-EASY vector (Qiagen), generating pDP*lasR* (for more details see Table S2). pDP*lasR* was digested with EcoRI, and the resulting 218 bp DNA fragment was labeled with [α-^{32}P] dATP by fill-in with Klenow enzyme [43]. The radio-labelled DNA probe was purified with SigmaSpin post-reaction purification columns (Sigma-Aldrich) followed by phenol-chloroform (1:1) extraction and ethanol precipitation.

In the EMSA experiments, the labelled DNA probe (0.2 nM) was incubated with different protein concentrations, in Binding Buffer [20 mM Tris-HCl, 2 mM EDTA, 5 mM $MgCl_2$, 30 mM KCl, 5% (v/v) glycerol, 0.025% (v/v) nonidet P-40, 30 µg/ml poly(dI): (dC); pH 8.0]. A labelled aspecific probe (0.2 nM) was added to the binding mix. After 15 min of incubation at 22°C the reaction mixtures were loaded onto a 30 min pre-run 6% (w/v) polyacrylamide gel under non-denaturing conditions. The ratio acrylamide: bis-acrylamide was 37.5:1. The electrophoresis was carried out at 22°C in TBE 0.5X [34] at 5 V/cm for 4 hrs. Gel was then dried and autoradiographed.

Phenotypic assays

P. aeruginosa PAO1 wild type carrying the pHERD30T empty vector or the pR3699 plasmid (see Table S2) were grown over night at 37°C in LB supplemented with 100 µg/ml Gm. Cultures were diluted to an A_{600} of 0.02 in LB supplemented with 0.1% L-arabinose, and supernatants were collected for elastolytic, proteolytic and pyocyanin assays after 6 hrs incubation at 37°C with 200 r.p.m shaking.

Elastolytic and proteolytic activities were determined by elastin-Congo red and azocasein assays, as previously described [44–46]. Pyocyanin quantification was performed as previously described [47]. The amount of pyocyanin, in µg/ml, was calculated using the following formula: A_{520}/A_{600} X 17.072 = µg of pyocyanin per ml [48].

The average data and standard deviations of the phenotypic assays were calculated from three independent experiments.

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 ≤ 0.01 are considered as statistically significant.

Supporting Information

Figure S1. Effect of the mutations in the genes coding for the new putative *lasR* transcriptional regulators on P*lasR* activity. Graph reporting P*lasR* promoter activity measured in *P. aeruginosa* PAO1 wild type (black line) and in isogenic clear deletion mutants in the PA0123 (green line), PA0448 (red line), PA3699 (blue line), PA4135 (yellow line) or *vfr* (grey line) gene. Each strain contained the P*lasR*::*lux* transcriptional fusion in the chromosomal *attB* neutral site, and was grown for 16 hrs in LB at 37°C with 200 r.p.m. shaking. P*lasR* activity is given as Light Counts Per Second (LCPS) divided by cell density (A_{600}). The average values and standard deviations were calculated from three independent experiments. Statistical significance with respect to the *P. aeruginosa* PAO1 wild type strain is indicated with one asterisk ($p < 0.01$).
(PDF)

Table S1. Bacterial strains used in this study.
(PDF)

Table S2. Plasmids used in this study.
(PDF)

Table S3. Oligonucleotides used in this study.
(PDF)

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Author Contributions

Conceived and designed the experiments: FL GR EZ PV LL. Performed the experiments: FL GR RB FI GMF. Analyzed the data: FL GR RB FI GMF PV EZ LL. Wrote the manuscript: LL.

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SUPPORTING INFORMATION

Table S1. Bacterial strains used in this study.

Strain	Relevant characteristics	Reference/Source
E. coli		
DH5α	Cloning strain; Nal^R.	Grant *et al*., 1990
S17.1 λ*pir*	Conjugative strain for suicide plasmids.	Simon *et al*., 1983
BL21 (DE3, pLysS)	High stringency expression host; Cm^R.	Novagen
HB101 (pRK2013)	Helper strain for tri-parental conjugations; Km^R.	Figursky and Helinski, 1979
SM10 (pFLP2)	Strain carrying the pFLP2 plasmid (FLP; Ap^R/Cb^R) for Flp-mediated recombination.	Hoang *et al*., 1998
P. aeruginosa		
PAO1 (ATCC15692)	wild type strain.	American Type Culture Collection
PAO1 P*lasR*::*lux*	wild type strain carrying the P*lasR*::*lux* transcriptional fusion integrated in the chromosome.	This study
ΔPA0123	*P. aeruginosa* PAO1 mutant strain with a complete deletion of the PA0123 gene.	This study
ΔPA0123 P*lasR*::*lux*	ΔPA0123 carrying the P*lasR*::*lux* transcriptional fusion integrated in the chromosome.	This study
ΔPA0448	*P. aeruginosa* PAO1 mutant strain with a complete deletion of the PA0448 gene.	This study
ΔPA0448 P*lasR*::*lux*	ΔPA0448 carrying the P*lasR*::*lux* transcriptional fusion integrated in the chromosome.	This study
ΔPA3699	*P. aeruginosa* PAO1 mutant strain with a complete deletion of the PA3699 gene.	This study
ΔPA3699 P*lasR*::*lux*	ΔPA3699 carrying the P*lasR*::*lux* transcriptional fusion integrated in the chromosome.	This study
ΔPA4135	*P. aeruginosa* PAO1 mutant strain with a complete deletion of the PA4135 gene.	This study
ΔPA4135 P*lasR*::*lux*	ΔPA4135 carrying the P*lasR*::*lux* transcriptional fusion integrated in the chromosome.	This study
Δ*vfr*	*P. aeruginosa* PAO1 mutant strain with a complete deletion of the *vfr* gene.	This study
Δ*vfr* P*lasR*::*lux*	Δ*vfr* carrying the P*lasR*::*lux* transcriptional fusion integrated in the chromosome.	This study

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SUPPORTING INFORMATION

Table S2. Plasmids used in this study.

Plasmid	Relevant characteristics and plasmid construction	Reference Source
pDM4	Suicide vector for construction of deletion mutants; *sacBR*; *oriR6K*; Cm^R.	Milton *et al*., 1996
mini-CTX-*lux*	Promoter-probe vector containing the *luxCDABE* operon; Tc^R.	Becher and Schweizer, 2000
pHERD30T	Plasmid for L-arabinose-inducible protein expression in *P. aeruginosa*; Gm^R.	Qiu *et al*., 2008
pMP220	*lacZ*-based promoter probe vector; Tc^R.	Spaink *et al*., 1987
pET-28b(+)	Plasmid for recombinant protein expression in *E. coli*; Km^R.	Novagen
pDRIVE-T-EASY	*E. coli* T/A based cloning vector; Ap^R.	Qiagen
pDM4 ΔPA0123	The upstream DNA region and downstream DNA region of the PA0123 gene were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW416/RV417 and FW418/RV419 (Tab. S3), and cloned in the pDM4 plasmid by XbaI-XhoI restriction.	This study
pDM4 ΔPA0448	The upstream DNA region and downstream DNA region of the PA0448 gene were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW428/RV429 and FW430/RV431 (Tab. S3), and cloned in the pDM4 plasmid by XbaI-XhoI restriction.	This study
pDM4 ΔPA3699	The upstream DNA region and downstream DNA region of the PA3699 gene were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW448/RV449 and FW450/RV451 (Tab. S3), and cloned in the pDM4 plasmid by XbaI-XhoI restriction.	This study
pDM4 ΔPA4135	The upstream DNA region and downstream DNA region of the PA4135 gene were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW424/RV425 and FW426/RV427 (Tab. S3), and cloned in the pDM4 plasmid by XbaI-XhoI restriction.	This study
pDM4 Δ*vfr*	The upstream DNA region and downstream DNA region of the *vfr* gene were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW456/RV457 and FW458/RV459 (Tab. S3), and cloned in the pDM4 plasmid by XbaI-SalI restriction.	This study
pMP*lasR*::*lacZ*	pMP220 derivative plasmid containing a 372 bp DNA fragment encompassing the *lasR* promoter region (P*lasR*). P*lasR* was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW333 and RV334 (Tab. S3) and cloned in the pMP220 plasmid by EcoRI-PstI restriction.	This study
mini-CTX-P*lasR::lux*	mini-CTX-*lux* derivative plasmid containing a 372 bp DNA fragment encompassing the *lasR* promoter region (P*lasR*). P*lasR* was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW333 and RV334 (Tab. S3) and cloned in the mini-CTX-*lux* plasmid by EcoRI-PstI restriction.	This study
pR0123	The PA0123 gene was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW479 and RV480 (Tab. S3) and cloned in pHERD by NcoI-XbaI restriction. This plasmid allows the over-expression of PA0123 in *P. aeruginosa*.	This study

pR0448	The PA0448 gene was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW485 and RV486 (Tab. S3) and cloned in pHERD by NcoI-PstI restriction. This plasmid allows the over-expression of PA0448 in *P. aeruginosa*.	This study
pR3699	The PA3699 gene was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW495 and RV496 (Tab. S3) and cloned in pHERD by NcoI-XbaI restriction. This plasmid allows the over-expression of PA3699 in *P. aeruginosa*.	This study
pR4135	The PA4135 gene was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW483 and RV484 (Tab. S3) and cloned in pHERD by NcoI-PstI restriction. This plasmid allows the over-expression of PA4135 in *P. aeruginosa*.	This study
pE3699-N6	The PA3699 gene was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW514 and RV515 (Tab. S3) and cloned in pET-28b(+) by NdeI-XhoI restriction. This plasmid allows the over-expression of PA3699 fused with a 6xHis at the *N*-terminus.	This study
pDP*lasR*	The P*lasR* promoter region was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW333 and RV535 (Tab. S3) and cloned in pDRIVE-T-EASY by T/A cloning. This plasmid was generated to label the probe encompassing the P*lasR* promoter.	This study

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SUPPORTING INFORMATION

Table S3. Oligonucleotides used in this study.

Name	Sequence (5'-3') [a]	Position [b]	Restriction site
FW333	NNNCCGGAATTCGGTCGCACGCGTGGCG	1557809	EcoRI
RV334	NNAAAACTGCAGCAACCAAGGCCATAGCGCT	1558182	PstI
FW381	Biotin-GGTCGCACGCGTGGCG	1557809	-
FW416	NNNGCTCTAGAATCGATGAAGGCTTGGCCG	143925	XbaI
RV417	NNCCGGAATTCCGCTGGAGCTGACCGAC	143256	EcoRI
FW418	NNCCGGAATTCCGCTGGAGCTGACCGAC	142370	EcoRI
RV419	NNCCGCTCGAGAACGCCGGAGGGCCTGA	141721	XhoI
FW424	NNNGCTCTAGATTCTCGCCTATGGGCGGC	4625678	XbaI
RV425	NNNAACTGCAGTGGCGTTGACATAGGGTCC	4624992	PstI
FW426	NNNAACTGCAGATAAAGCCCTGAAGCGAC	4624591	PstI
RV427	NNCCGCTCGAGGTCGAGAAGATGCACAGC	4623928	XhoI
FW428	NNNGCTCTAGAAGGCCGAAGCAGCCGATG	503485	XbaI
RV429	NNNAACTGCAGGATCTTGCGTCGCATGGA	504135	PstI
FW430	NNNAACTGCAGGGGCTGGGCTGAACGCG	505018	PstI
RV431	NNCCGCTCGAGTCGCTCTACTGGCTGCTCA	505661	XhoI
FW448	NNNGCTCTAGACCCAAGGTCCTCGGCCACG	4141323	XbaI
RV449	NNNCGGAATTCTACTCGGGACACGGTGTTT	4140661	EcoRI
FW450	NNNCGGAATTCATGGGCCTGTGACCTGA	4139970	EcoRI
RV451	NNCCGCTCGAGATCTCATTGGGTGACCCGC	4139308	XhoI
FW456	NNNGCTCTAGATCTCCACCCCGCCGCG	707316	XbaI
RV457	NNNCGGAATTCAATAGCTACCATGCCCGAGT	706661	EcoRI
FW458	NNNCGGAATTCGGCACCCGCTGAACAG	706024	EcoRI
RV459	NACGCGTCGACCGGAGATCGCCCGCAGCT	705365	SalI
FW479	NCATGCCATGGATTCGCTGAAAGGC	143246	NcoI
RV480	NNNGCTCTAGATCGGTCAGCTCCAGCG	142352	XbaI
FW483	NCATGCCATGGCAACGCCAAGACCCTCCC	4624980	NcoI
RV484	NAAAACTGCAGGTCGCTTCAGGGCTTTATCT	4624575	PstI
FW485	NCATGCCATGGGACGCAAGATCCCTTCCAC	504124	NcoI
RV486	NAAAACTGCAGGCGTTCAGCCCAGCCCG	505015	PstI
FW495	NCATGCCATGGCCCGAGTAGCTCCCAGAG	4140649	NcoI
RV496	NNNGCTCTAGATCAGGTCACAGGCCCATGA	4139953	XbaI
FW514	GGAATTCCATATGTCCCGAGTAGCTCCCA	4140651	NdeI
RV515	NNCCGCTCGAGTCACAGGCCCATGACCA	4139957	XhoI
FW535	CCCACTCTATAGAGTTGGCG	1558001	-

a. Introduced restriction sites are underlined.
b. Position with respect to the *Pseudomonas* Genome Database (www.pseudomonas.com).

Chapter II

Affecting *Pseudomonas aeruginosa* phenotypic plasticity by quorum sensing dysregulation hampers pathogenicity in murine chronic lung lnfection

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Affecting *Pseudomonas aeruginosa* Phenotypic Plasticity by Quorum Sensing Dysregulation Hampers Pathogenicity in Murine Chronic Lung Infection

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Abstract

In *Pseudomonas aeruginosa* quorum sensing (QS) activates the production of virulence factors, playing a critical role in pathogenesis. Multiple negative regulators modulate the timing and the extent of the QS response either in the pre-quorum or post-quorum phases of growth. This regulation likely increases *P. aeruginosa* phenotypic plasticity and population fitness, facilitating colonization of challenging environments such as higher organisms. Accordingly, in addition to the factors required for QS signals synthesis and response, also QS regulators have been proposed as targets for anti-virulence therapies. However, while it is known that *P. aeruginosa* mutants impaired in QS are attenuated in their pathogenic potential, the effect of mutations causing a dysregulated timing and/or magnitude of the QS response has been poorly investigated so far in animal models of infection. In order to investigate the impact of QS dysregulation on *P. aeruginosa* pathogenesis in a murine model of lung infection, the QteE and RsaL proteins have been selected as representatives of negative regulators controlling *P. aeruginosa* QS in the pre- and post-quorum periods, respectively. Results showed that the *qteE* mutation does not affect *P. aeruginosa* lethality and ability to establish chronic infection in mice, despite causing a premature QS response and enhanced virulence factors production in test tube cultures compared to the wild type. Conversely, the post-quorum dysregulation caused by the *rsaL* mutation hampers the establishment of *P. aeruginosa* chronic lung infection in mice without affecting the mortality rate. On the whole, this study contributes to a better understanding of the impact of QS regulation on *P. aeruginosa* phenotypic plasticity during the infection process. Possible fallouts of these findings in the anti-virulence therapy field are also discussed.

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Introduction

Quorum sensing (QS) is an intercellular communication process based on the synthesis and secretion of signal molecules that bind to cognate receptors. The signal-activated receptors trigger the expression of target genes. Since the concentration of signal molecules is proportional to cell density, QS coordinates gene expression when the bacterial population reaches a critical threshold level. The population density at which gene expression is triggered is called the "quorum", while the phase before expression is called the "pre-quorum" period [1–3].

QS processes are widespread in the bacterial world and they are studied with particular intensity in *Pseudomonas aeruginosa*. This bacterium is one of the most dreaded Gram-negative pathogens in developed countries, being responsible for both community- and hospital-acquired infections. In addition, *P. aeruginosa* chronic lung infection is the major cause of death in patients with cystic fibrosis (CF), a genetic disease affecting about 1/3,000 newborns in the Caucasian population. *P. aeruginosa* infections are difficult to eradicate as a consequence of intrinsic antibiotic resistance and growth in bacterial communities referred to as biofilms [4,5]. Since in *P. aeruginosa* QS plays a critical role in the production of virulence factors and in biofilm formation, it is considered a very promising target for the development of anti-virulence drugs [6–9].

P. aeruginosa has at least three QS systems based on the production, secretion and perception of distinct signals: *N*-3-oxo-dodecanoyl-homoserine lactone ($3OC_{12}$-HSL), *N*-butyryl-homoserine lactone (C_4-HSL), and molecules belonging to the 2-alkyl-4-quinolones (AQs) family. The signal molecule $3OC_{12}$-HSL is required for optimal production of the other QS signals, though this hierarchy is dependent upon growth conditions [3,10–13]. $3OC_{12}$-HSL is produced by the synthase LasI, encoded by the *lasI* gene, and perceived by the signal receptor LasR, encoded by *lasR* (Fig. 1). The LasR/$3OC_{12}$-HSL complex activates the transcription of hundreds of genes, including: *i*) the *lasI* gene, thus

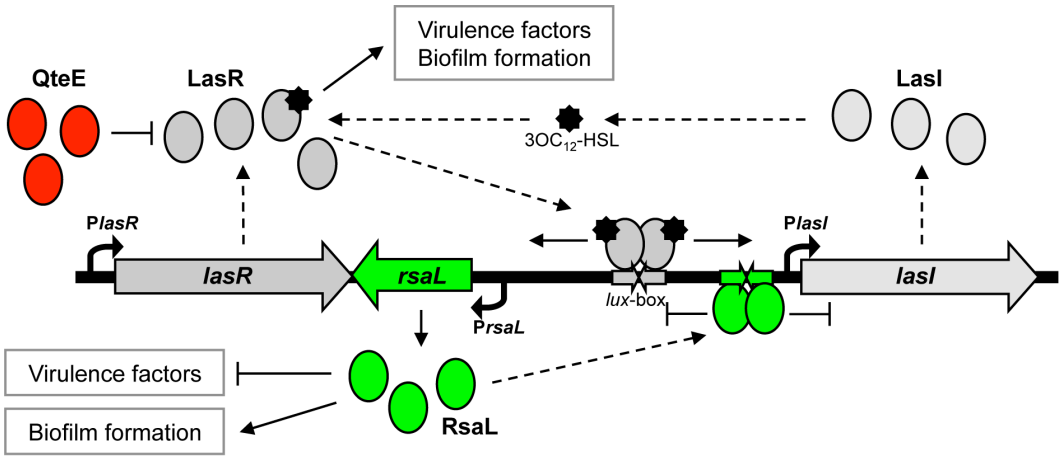

Figure 1. Schematic representation of QteE- and RsaL-dependent regulation of the *P. aeruginosa las* QS system. In the pre-quorum period, QteE binds to the LasR receptor and prevents the binding of the LasR-3OC$_{12}$-HSL complex to the *rsaL-lasI* bidirectional promoter [20], hence delaying the onset of the QS response. Once the quorum has been reached, the LasR/3OC$_{12}$-HSL complex triggers the transcription of both *rsaL* and *lasI* genes. The consequent increase of 3OC$_{12}$-HSL levels, and thus of activated LasR, generates a positive feedback loop also responsible for the increase of RsaL levels. RsaL binding to the *rsaL-lasI* bidirectional promoter represses the expression of both *rsaL* and *lasI* genes, thus counteracting the positive feedback loop. This circuit provides 3OC$_{12}$-HSL homeostasis [24]. Solid arrows represent positive control; T-shaped lines represent negative control; dashed arrows indicate information flow; curved arrows represent the transcription start points of the indicated genes.
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generating the autoinduction circuit typical of most QS systems; *ii*) the genes required for C$_4$-HSL and AQs synthesis and reception; *iii*) many genes involved in virulence factors production and biofilm formation [3,10,11].

In *P. aeruginosa* the autoinduction circuit is coupled to multiple negative regulatory mechanisms that modulate the timing and the extent of the QS response, hence limiting the metabolic burden for virulence factors production when unnecessary. Overall, this fine-tuning is believed to increase *P. aeruginosa* phenotypic plasticity and population fitness, ultimately facilitating colonization of challenging environments such as higher organisms' tissues [3,14–17].

Multiple negative modulators delaying the onset of the QS response by restraining the expression of the *lasI* gene in the pre-quorum period have been identified so far [18–21]. As an example, QteE decreases LasR stability at low cell density. Accordingly, in a *qteE* mutant the transcription of both *lasI* and the 3OC$_{12}$-HSL-dependent gene *lasB*, encoding the secreted virulence factor elastase, is prematurely activated (Fig. 1) [17,20]. Moreover, the overexpression of *qteE* causes a strong reduction of virulence in potato and fruit fly models of infection [22], suggesting that QteE might limit *P. aeruginosa* virulence also in mammals. However, artificial gene overexpression could produce aberrant effects, and the impact of *qteE* mutation on 3OC$_{12}$-HSL and virulence factor production, and on the overall pathogenic potential of *P. aeruginosa* in either test tube cultures or animal models of infections is still unexplored.

After the QS threshold has been reached, in the post-quorum growth phase, additional regulatory factors may intervene, modulating the overall levels of signal molecule produced by the bacterial population. The RsaL protein, a repressor of *lasI* transcription encoded by the *rsaL* gene, is the best characterized post-quorum regulator in *P. aeruginosa*. Since the LasR/3OC$_{12}$-HSL complex activates both *rsaL* and *lasI* transcription, it generates a homeostatic regulatory loop that allows a population of *P. aeruginosa* cells to avoid 3OC$_{12}$-HSL accumulation in the post-quorum period (Fig. 1) [23–25]. The effect of the *rsaL* mutation on 3OC$_{12}$-HSL and virulence factor production in test tube cultures and in an insect infection model has been thoroughly investigated in a previous study [23,26]. Briefly, it has been shown that *rsaL* mutation leads to enhanced motility and secreted virulence factors production such as pyocyanin and elastase, resulting in a hypervirulent phenotype in the *Galleria mellonella* model of systemic (acute) infection. On the other hand, the *rsaL* mutant strain displays increased sensitivity to different antibiotics and reduced ability to form biofilm [26].

P. aeruginosa mutants impaired in QS are less pathogenic in various animal models of infection, especially in those developed to investigate the acute infection process [27]. However, despite *P. aeruginosa* biofilm-based chronic infections have higher social and economic costs in developed countries [4,28], studies on mammalian models of chronic infection have received less attention so far, likely due to the complexity to establish long-term chronic infection in animal models [27,29,30].

Given the importance of QS and phenotypic plasticity in *P. aeruginosa* pathogenicity, it is believed that pre- and post-quorum control of QS response could play a key role in the establishment and persistence of the infection [15,16]. Accordingly, ancillary regulators controlling the timing and extent of the QS response have been proposed as targets for the development of anti-virulence therapies against *P. aeruginosa* [31]. However, to the best of our knowledge, the effect of mutations causing a dysregulated timing and/or magnitude of the QS response on *P. aeruginosa* virulence has never been investigated in mammalian models of chronic infection.

The general objective of this study has been to investigate the impact of QS dysregulation in *P. aeruginosa* pathogenesis, by selecting QteE and RsaL as representatives of two negative regulators that control the *las* QS system in the pre- and post-quorum periods, respectively. As summarized above, the effect of the *rsaL* mutation in test tube cultures has been thoroughly characterized by our group in previous studies, while the effect of this mutation in animal infection models has not been investigated so far [23,24,26]. On the other hand, very little is known about the effect of the *qteE* mutation on *P. aeruginosa* 3OC$_{12}$-HSL production and virulence, both *in vitro* and *in vivo*. For this

reason, the first part of this study has been focused on the characterization of the *qteE* mutant virulence-related phenotypes in test-tube cultures. Next, the effect of both *qteE* and *rsaL* mutations on *P. aeruginosa* pathogenesis has been investigated in a murine model of chronic infection. Results showed that a mutation in *qteE* causes a premature QS response and hyperproduction of virulence factors in *P. aeruginosa* cultures. However, the anticipation of the QS response in the pre-quorum period due to the *qteE* mutation does not affect *P. aeruginosa* pathogenicity, while the post-quorum dysregulation caused by the *rsaL* mutation hampers the establishment of chronic lung infection. Overall these findings contribute to fill-in the current gap of knowledge about the relevance of QS modulation in *P. aeruginosa* pathogenesis, and stimulate a re-discussion of the overall role played by QS during the infection process.

Results and Discussion

Phenotypic characterization of the *P. aeruginosa qteE* mutant

The effect of *qteE* mutation on *P. aeruginosa* $3OC_{12}$-HSL-dependent response was determined along growth by comparing the levels of this signal molecule and of selected QS-dependent virulence factors in wild type and in *qteE* cultures carrying either the empty vector pBBR1MCS-5 or its derivative plasmid (named pQteE) expressing the *qteE* gene.

As shown in Figure 2A, the *qteE* mutant produced detectable levels of $3OC_{12}$-HSL earlier than the wild type strain, reaching a $3OC_{12}$-HSL concentration about 6-fold higher at $A_{600}\approx1$. Interestingly, $3OC_{12}$-HSL levels measured in the *qteE* and in the wild type strains plateaued at the same level in the post-quorum phase of growth ($A_{600}\approx2$). This trend of $3OC_{12}$-HSL production in the *qteE* mutant is also consistent with previous western hybridization experiments showing that the positive effect of the *qteE* mutation on LasR protein stability is restricted to the pre-quorum period [20]. Conversely, as previously shown [24], the *rsaL* mutant disclosed normal $3OC_{12}$-HSL production in the pre-quorum period, while this mutant produced higher $3OC_{12}$-HSL levels than the wild type strain after the QS threshold has been reached ($A_{600}>1.8$; Fig. 2A).

In agreement with the precocious synthesis of $3OC_{12}$-HSL, the *qteE* mutant also anticipated the production of elastase (Fig. 2B) and protease (Fig. 2C). Differently from $3OC_{12}$-HSL levels, it seems that the anticipated expression of proteases and elastase levels in the *qteE* mutant causes accumulation of these secreted factors also in the post-quorum period (compare panels A, B and C of Fig. 2). The homeostatic control of $3OC_{12}$-HSL levels in the post-quorum period is likely due to specific mechanisms that do not affect proteases and elastase production, including the transcriptional repression exerted by RsaL on *lasI*, and the activity of the acyl-HSL degrading enzymes produced by *P. aeruginosa* [15,23–25,32–34]. Also the biosynthesis of the cytotoxic secondary metabolite pyocyanin is activated by the LasR/$3OC_{12}$-HSL complex, though it starts later during the growth with respect to proteases and elastase biosynthesis [35]. Interestingly, when the wild type and *qteE* mutant cultures reached an $A_{600}\approx3.5$, the supernatants of the *qteE* mutant contained high pyocyanin levels, while this virulence factor was almost undetectable in the wild type strain (Fig. 3).

The growth curve of the *qteE* mutant was similar to those of the *rsaL* mutant and wild type strains and was not affected by the presence of the pBBR1MCS-5 vector, ruling out the possibility that differences in the growth rates could account for the diverse phenotypes described above (Fig. S1 in File S1). Moreover,

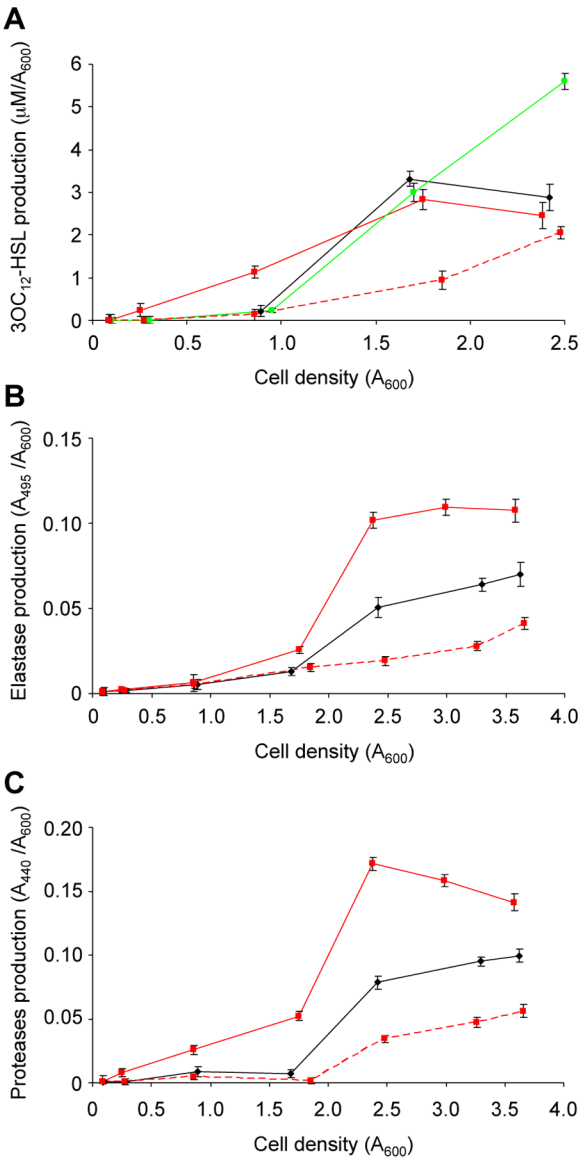

Figure 2. Effect of QS dysregulation caused by *qteE* mutation on *P. aeruginosa* virulence-related phenotypes. Levels of (A) $3OC_{12}$-HSL, (B) elastase, (C) proteases produced along growth by *P. aeruginosa* wild type (black lines), *qteE* (red lines) and *rsaL* strains (green line) carrying the pBBR1MCS-5 empty vector, or by the *qteE* strain carrying the pQteE plasmid (pBBR1MCS-5-derived) for the expression of *qteE* (dashed red line). Values are the means (± standard deviations) of at least three independent experiments.
doi:10.1371/journal.pone.0112105.g002

complementation of the *qteE* mutant strain with the pQteE plasmid caused a repression of both $3OC_{12}$-HSL, proteases, elastase, and pyocyanin production, ruling out any involvement of possible artefacts caused by *qteE* mutagenesis on the tested phenotypes (Figs. 2 and 3). We also verified that the pBBR1MCS-5 empty vector did not affect the levels of $3OC_{12}$-HSL, proteases, elastase and pyocyanin either in the wild type or in the *qteE* mutant *per se* (Fig. S2 in File S1 and Fig. 3).

Overall, the above results are in agreement with previous studies showing that the *lasI* and *lasB* promoters are prematurely activated in the *qteE* mutant with respect to the wild type [17,20], and that QteE overexpression causes repression of elastase

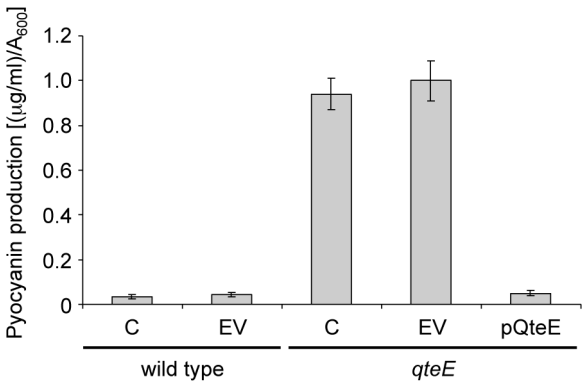

Figure 3. Effect of QS dysregulation caused by *qteE* mutation on pyocyanin production in *P. aeruginosa*. Levels of pyocyanin measured in cell-free supernatants from cultures of the indicated strains grown till $A_{600}\approx3.5$. C, no plasmid; EV, pBBR1MCS-5 empty vector; pQteE, pBBR1MCS-5 derivative plasmid for *qteE* expression.
doi:10.1371/journal.pone.0112105.g003

and pyocyanin production in *P. aeruginosa* [20,22]. Notably, our results demonstrate that in the *qteE* mutant the production of $3OC_{12}$-HSL and of main QS-controlled virulence factors is anticipated, and that these exo-products accumulate along growth at higher levels with respect to the wild type strain, supporting the hypothesis that *qteE* may play a role in *P. aeruginosa* pathogenesis.

Effect of QS dysregulation on *P. aeruginosa* pathogenicity in a murine model of infection

A bacterial population can adopt distinct behaviours in acute and chronic infections, and distinctive phenotypes characterizing these two processes are often inversely regulated. An acute infection is rapid, systemic and carried out by a planktonic bacterial community expressing high levels of virulence factors. Conversely, in a chronic infection bacterial proliferation is limited to a specific host tissue (*e.g.*, in the CF lung or in association with medical devices), and bacteria can persist in the host for extended periods of time, adopting a slow-growing sessile lifestyle (biofilm). In the biofilm mode of growth bacteria are more resistant to the host immune system and prolonged antibiotic therapies, and they produce limited amount of virulence factors despite high cell density [29,36].

In order to test the effect of pre-quorum and post-quorum dysregulation of the *lasI* gene *in vivo*, the virulence of *P. aeruginosa* PAO1 wild type and of its isogenic *qteE* and *rsaL* mutant strains was compared by using the "agar beads" murine model of lung infection [37–39]. In this model, *P. aeruginosa* cells embedded in agar beads are inoculated into the murine lungs, where replicate in microaerobic/anaerobic conditions in the form of microcolonies, similarly to the growth in the mucus of CF patients [40]. Mice dying within three days from the challenge are killed by a systemic (acute) infection. The surviving mice are sacrificed after 14 days from the challenge and bacterial load is evaluated in their lungs: the subset of survived mice containing *P. aeruginosa* (CFU $\geq$1000) in their lungs are considered chronically infected, while the others are considered cleared from the infection [37–39]. This murine model of infection has been previously used to test the virulence of a *P. aeruginosa* PAO1 QS mutant impaired in both $3OC_{12}$-HSL and C_4-HSL production, showing that the QS mutant had strongly attenuated ability to cause chronic infection with respect to the wild type, while the two strains showed similar ability to kill mice within three days from the challenge [41]. In addition, this infection model has been used to validate the anti-virulence activity of anti-*Pseudomonas* compounds, including QS inhibitors [7,42].

When mice were challenged with the *P. aeruginosa* PAO1 wild type strain used in this study, 54% of mortality (20/37 mice) was observed in the first three days, while 76.5% of the survived mice (13/17 mice) were chronically infected after 14 days from the challenge (Fig. 4). Surprisingly, the ability of *qteE* mutant and of the wild type strains to cause both mice mortality and chronic infection, in the survived mice, did not show statistically significant differences (Fig. 4). Hence, despite the production of virulence factors regulated by QS is anticipated and increased in the *qteE* mutant *in vitro*, inactivation of this gene has no effect on *P. aeruginosa* ability to cause infection, at least in this model system.

Concerning the *rsaL* mutant, the ability of this strain to kill the mice by systemic infection (64.3%; 18/28 mice) did not show significant difference with respect to the wild type. This result was surprising, because the LD_{50} of the *rsaL* mutant is seven fold lower with respect to the wild type strain in the *G. mellonella* systemic infection model [26]. Although previous studies testing the virulence of QS defective or attenuated mutants showed a good correlation of the results between *G. mellonella* and murine infection models [43], it seems that this correlation is missing for mutants with dysregulated QS response.

Interestingly, only 30% (3/10 mice) of the survived mice developed a chronic infection after 14 days from the challenge

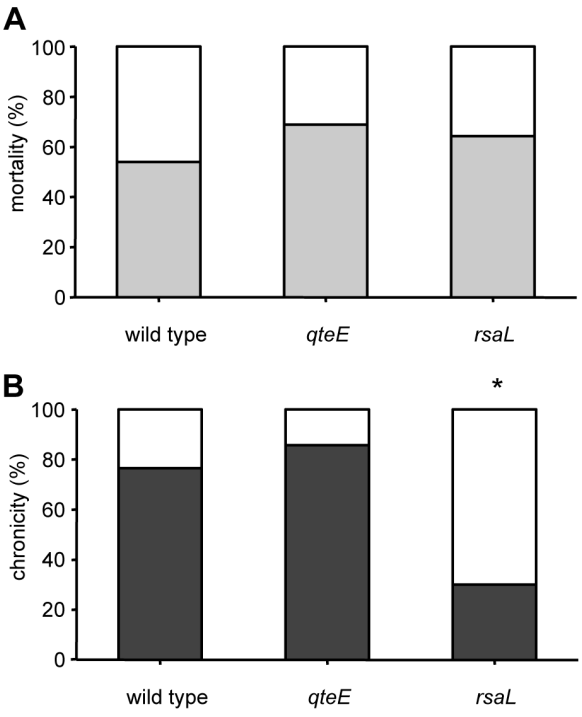

Figure 4. Effect of QS dysregulation caused by *qteE* and *rsaL* mutations on *P. aeruginosa* pathogenesis in mice. C57Bl/6 mice were infected with the indicated strains embedded in agar beads. (A) Mice mortality induced by bacteremia (light grey) and survival (white) were evaluated on challenged mice. (B) Clearance (white) and capacity to establish chronic airways infection (dark grey) were determined on surviving mice after 14 days from challenge. The results are averages of three independent experiments. Statistical significance is indicated by an asterisk comparing *P. aeruginosa* wild type versus *qteE* or *rsaL* strains ($p<0.05$).
doi:10.1371/journal.pone.0112105.g004

with the *rsaL* strain, a percentage significantly lower than that observed with the wild type strain (76.5%; Fig. 4). Hence, the *rsaL* mutation decreased *P. aeruginosa* ability to cause chronic infection. This result strongly supports the hypothesis that the RsaL-mediated post-quorum homeostatic regulation of QS plays a positive role in the establishment of chronic lung infection in mice.

The surviving mice sacrificed 14 days from the challenge were either totally cleared (no *P. aeruginosa* cells in the lungs) or contained similar bacterial loads (>1000 CFU/lungs), independent from the *P. aeruginosa* strain used for the challenge (Fig. S3 in File S1). This result is overall in line with previous studies using this infection model [38].

Conclusions

Since the discovery that *P. aeruginosa* virulence genes expression is QS-dependent and that QS mutants have attenuated pathogenicity in animal models of infection, researchers have tried to explain why QS favours the infection. An early and still in vogue theory is that the QS-control of virulence factors avoids the stimulation of the host immune response at early infection stages, when the size of the bacterial population is small [1]. Another hypothesis, not excluding the former one, is that QS could be important to save energy from unprofitable exoproducts production in environments with high mass transfer, allowing their synthesis only if bacteria are within a low diffusion rate environment, such as an infected tissue [44]. However, considering the key role played by the QS circuitry in *P. aeruginosa* central and secondary metabolism, and its poorly understood links with other cellular regulatory networks, it seems quite an hard task to find an univocal and simple explanation for the role played by QS in the infection.

P. aeruginosa has evolved as a tough versatile organism, able to thrive in a wide range of environmental niches rather than as a specialized pathogen. In accordance with its phenotypic plasticity, *P. aeruginosa* can cause a range of different acute and chronic infections in almost all areas of the human body [4,16,36], implying that the relevance of the different factors affecting the timing and extent of the QS response *in vivo* could be dependent upon the kind of infection.

In the lung infection model used in this study, the similar pathogenic behaviour of the *P. aeruginosa* wild type and *qteE* mutant indicates that the restrained production of $3OC_{12}$-HSL and expression of virulence genes in the pre-quorum period does not favour *P. aeruginosa* ability in either mice killing or in establishing a chronic lung infection. This finding argues against the hypothesis that delaying virulence factors production until cells amass to a certain density could favour the establishment of the infection [1,20].

A relevant finding of this study is that the post-quorum homeostatic regulation of QS exerted by RsaL favours the establishment of the chronic *P. aeruginosa* infection. The importance of this factor also in the human chronic infection is supported by studies on *P. aeruginosa* strains isolated from the chronically infected lungs of CF patients. During the course of this infection, that can last for decades, the *P. aeruginosa* population that initially settles in the lung is subject to a microevolution process leading to the emergence of mutants with phenotypic traits unusual in the environmental strains, including loss of motility, increased ability to form biofilm, increased antibiotic resistance, reduced production of secreted virulence factors [45]. It is striking how these phenotypes inversely correlate with those disclosed by the *rsaL* mutant [26]. Accordingly, in the CF chronic lung infection there is a positive selection for cells expressing high levels of RsaL [46,47].

Since *rsaL* transcription is strongly dependent upon LasR, this factor should not be expressed in a *lasR* mutant. Hence it is interesting to discuss our results by considering that *lasR* mutants are frequently isolated from the lungs of CF patients. It is still under debate whether these mutants arise because they are social cheaters gaining a growth advantage by utilizing "public goods" (*i.e.*, virulence factors) produced by neighbour wild type cells, rather than producing their own [45,48,49], or whether they are better adapted than the wild type to the peculiar environment of the CF lung [50,51]. Overall, it is still unclear whether and how the emergence of *lasR* mutants could contribute to the CF lung decline. However, a recent work showed that *lasR* mutants were able to produce very high levels of pyocyanin under the slow-growing conditions typical of the chronic infection, while wild type cells did not [13]. Moreover, in co-cultivation experiments, the *lasR* mutant was able to cooperate with the wild type for pyocyanin production [13]. Pyocyanin overproduction in the *lasR* mutant is due to the loss of repression normally exerted by RsaL on phenazine biosynthetic genes, because RsaL itself is not expressed as a consequence of *lasR* mutation [13]. However, mutations in *rsaL* are not commonly isolated in CF clinical samples, suggesting that the constitutive expression of QS regulated factors caused by this mutation is unfavourable in the CF lung environment, and that a mutation in the *rsaL* gene can be tolerated only when associated to the lack of expression of the entire LasR regulon.

It has been proposed that targeting the function and the cellular levels of the regulatory factors that modulate the QS pre-quorum and post-quorum response could be a strategy to inhibit *P. aeruginosa* virulence [31]. Though the hypervirulent phenotype disclosed by the *rsaL* mutant *in vitro* and in the *G. mellonella* infection model might cause some concern, our results indicate that a compound targeting RsaL could reduce the ability of *P. aeruginosa* to establish a chronic infection. Moreover, since the *rsaL* mutant is also less resistant to antibiotics, with respect to the wild type [26], such compound could synergize with drugs currently used in the CF therapy.

In conclusion, our results contribute to a better understanding of the QS regulatory factors involved in the establishment of the chronic infection caused by *P. aeruginosa*, indicate the RsaL homeostatic regulator of QS as a promising target for drugs specific against this kind of infection, and highlight the importance of carrying out further studies about the role played by QS modulation in mammalian infection models.

Materials and Methods

Bacterial strains and culture conditions

Pseudomonas aeruginosa wild type, substrain PAO1-UW, and its *qteE* and *rsaL* mutant derivatives were supplied by The University of Washington Genome Center (www.genome.washington.edu/UWGC/pseudomonas) [52]. *Escherichia coli* DH5α [53] was used for cloning purposes. Bacterial strains were grown at 37°C in Luria-Bertani broth (LB) [54] with 200 r.p.m shaking; 20 mg/L and 100 mg/L Gentamicin was added to the *E. coli* and *P. aeruginosa* strains for plasmid maintenance, respectively.

Plasmids construction

A DNA region of 1,280 bp encompassing the entire *qteE* gene, including its native promoter region, was amplified from the PAO1-UW genome by PCR and cloned into the KpnI-HindIII

sites of the pBBR1MCS-5 vector [55], generating the pQteE plasmid. The PCR was performed with the following Forward and Reverse oligonucleotides: 5′-CGGGGTACCGAGGACTACCA-GAAAGCCC-3′, and 5′-ATAAAGCTTTCAGGCCAGCCCA-TAGCT-3′; the KpnI and HindIII restriction sites introduced in the oligonucleotides are underlined.

Phenotypic assays

Plasmids pBBR1MCS-5 and pQteE were inserted in the PAO1-UW strain and in the PAO1-UW *qteE* mutant by conjugation, as previously described [56]. Strains were grown 16 hours at 37°C in LB supplemented with 100 μg/ml Gentamicin. For the phenotypic assays, cultures were diluted to an A_{600} of 0.02 in LB and incubated at 37°C with 200 r.p.m shaking. Cell-free supernatants were collected every hour after 3 hours of incubation. The concentration of $3OC_{12}$-HSL, proteases, elastase and pyocyanin in the cell-free supernatants were measured as previously described [24,57–60].

The average measurements and relative standard deviations were calculated from three independent experiments.

Mouse model of *P. aeruginosa* lung infection

C57Bl/6 male mice (20–22 gr) were purchased by Charles River Laboratories (Calco, Italy). All mice were maintained under specific pathogen-free conditions in sterile cages which were put into a ventilated isolator. Fluorescent lights were cycled 12 hours on/12 hours off, and ambient temperature (23±1°C) and relative humidity (40–60%) were regulated. The *P. aeruginosa* agar-beads mouse model was used [37]. PAO1-UW wild type strain and the isogenic *qteE* and *rsaL* mutant strains were used for inclusion in the agar beads, as previously described [38,39,61]. Briefly, mice were anesthetized with 2.5% avertin (2,2,2-tribromethanol, 97%; Sigma Aldrich) in 0.9% NaCl, intubated with a 22-gauge venous catheter and inoculated with *P. aeruginosa* 2×10^6 CFU. Animals were observed twice a day and those showing more than 25% of body weight loss and had evidence of severe clinical disease, such as scruffy coat, inactivity, loss of appetite, poor locomotion, or painful posture, were sacrificed before the termination of the experiments with an overdose of carbon dioxide. Fourteen days after infection, mice were sacrificed by CO_2 administration and murine lungs were excised, homogenized and plated onto Trypticase Soy Agar plates for CFU counting. Recovery of ≥ 1,000 CFU from lung cultures was indicative of chronic infection [37,38]. The results are averages of at least three independent experiments. Overall, 37, 45 and 28 mice were infected with the *P. aeruginosa* wild type, the *qteE* mutant or the *rsaL* mutant strains, respectively (10-12 mice for each experimental group, for experiment).

Statistical analysis was performed using Fisher's exact test (two-tailed) for categorical variables. Differences were considered statistically significant at p value <0.05.

Ethics statement

Animal studies were carried out according to protocols approved by the IRCCS - San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC), and in strict accordance with the Italian Ministry of Health guidelines for the use and care of experimental animals.

Supporting Information

File S1 This file contains Figures S1-S3. Figure S1, Growth curve of *P. aeruginosa* PAO1 wild type, *qteE* and *rsaL* strains. Figure S2, The presence of pBBR1MCS-5 vector does not affect virulence-related phenotypes in *P. aeruginosa*. Figure S3, Bacterial load in the lungs of mice infected with *P. aeruginosa*. (PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: RB MM IDF AB GR LL. Performed the experiments: RB MM IDF. Analyzed the data: RB MM IDF AB GR LL. Contributed reagents/materials/analysis tools: AB GR LL. Wrote the paper: LL.

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Affecting *Pseudomonas aeruginosa* phenotypic plasticity by quorum sensing dysregulation hampers pathogenicity in murine chronic lung infection

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SUPPORTING INFORMATION

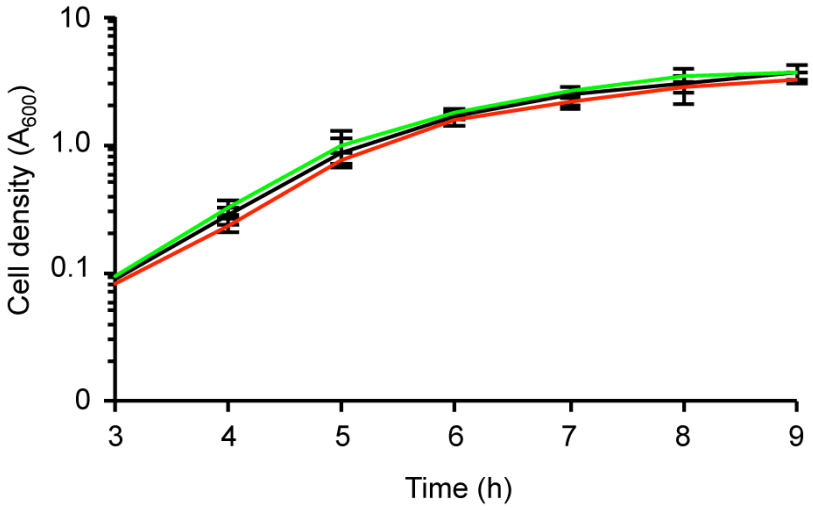

Figure S1. Growth curve of *P. aeruginosa* PAO1 wild type, *qteE* and *rsaL* strains.
Cell density measured in *P. aeruginosa* PAO1 wild type (black line), *qteE* (red line) and *rsaL* strains (green line). Values are the means (± standard deviations) of at least three independent experiments. The pBBR1MCS-5 or pQteE (pBBR1MCS-5-derived) plasmids did not affect the growth curves (data not shown).

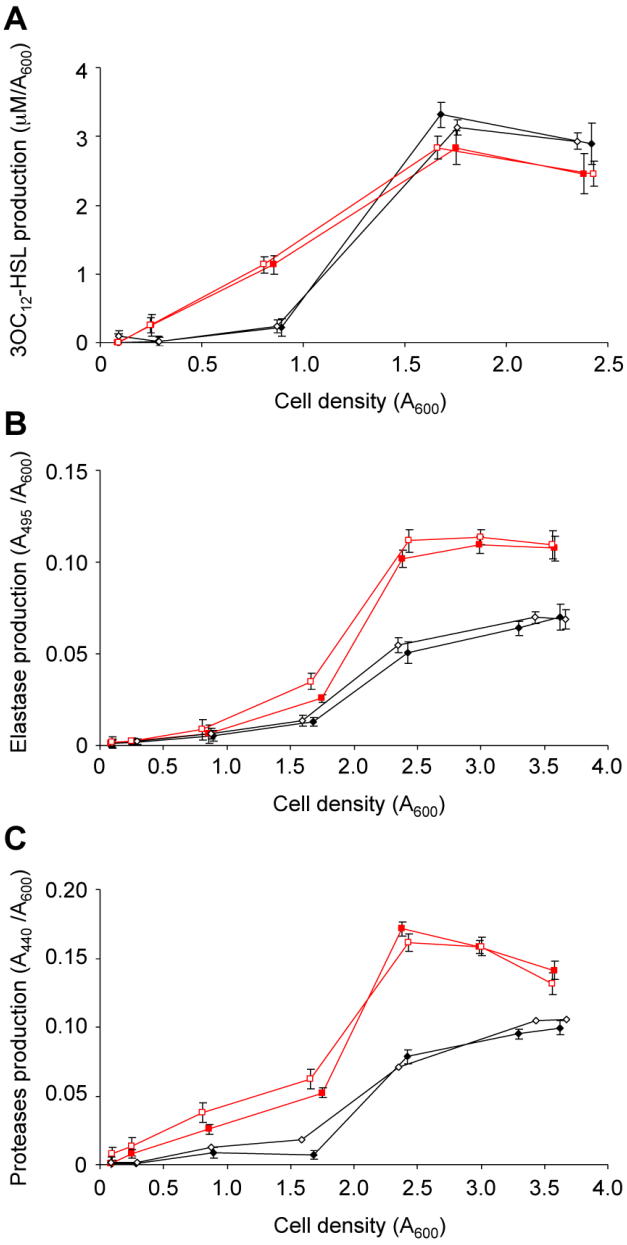

Figure S2. The presence of pBBR1MCS-5 vector does not affect virulence-related phenotypes in *P. aeruginosa.*

Levels of (A) $3OC_{12}$-HSL, (B) elastase, (C) proteases produced along growth by *P. aeruginosa* wild type (black lines) and *qteE* (red lines) strains containing or not the pBBR1MCS-5 empty vector (full and open circles, respectively). Values are the means (± standard deviations) of at least three independent experiments.

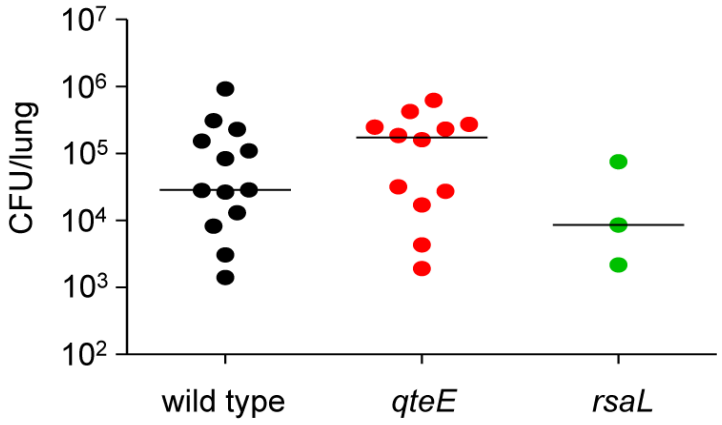

Figure S3. Bacterial load in the lungs of mice infected with *P. aeruginosa.*
Colony-forming units (CFU) per lung were counted after 14 days from challenge. Black full circles, wild type; red full circles, *qteE* mutant; green full circles, *rsaL* mutant. No statistically significant differences between groups were observed ($0.19 \leq p \leq 0.67$; unpaired two-tailed *t* test).

Chapter III

Characterization of the incoherent feedforward loop governing quorum sensing in *Pseudomonas aeruginosa*

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Characterization of the incoherent feedforward loop governing quorum sensing in *Pseudomonas aeruginosa*

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Running title: Regulatory properties of the *las* QS system

Keywords: quorum sensing; gene regulation; *Pseudomonas aeruginosa*; network motifs; incoherent feedforward loop; robustness; *lasR*; *rsaL*; phenotypic plasticity.

SUMMARY

Quorum sensing (QS) is a communication system that controls virulence-related phenotypes in the human pathogen *Pseudomonas aeruginosa*.

The QS receptor LasR responds to the QS signal molecule *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) and promotes signal production by increasing the transcription of the $3OC_{12}$-HSL synthase gene, *lasI*. LasR also activates the expression of other genes, including the *lasI* transcriptional repressor RsaL, generating a regulatory network motif known as type 1 incoherent feedforward loop (IFFL-1) governing $3OC_{12}$-HSL production. Beside *lasI*, RsaL directly represses a set of LasR-activated virulence genes, hence generating a multi-output IFFL-1. Here we demonstrate that the multi-output IFFL-1 built up by LasR and RasL confers robustness with respect to fluctuations in the levels of LasR to the phenotypes controlled by both these transcriptional regulators (*e.g.*, $3OC_{12}$-HSL synthesis and pyocyanin production). On the contrary, other virulence-related phenotypes controlled only by LasR (*e.g.*, elastase and proteases production) are sensitive to changes in LasR levels.

As a whole, the multi output IFFL-1 generated by LasR and RsaL splits the QS regulon in two distinct sub-regulons, ultimately conferring enhanced phenotypic plasticity to the regulation of *P. aeruginosa* virulence-related traits. This is particularly relevant considering the number of environmental and metabolic stimuli known to modulate LasR levels.

INTRODUCTION

Complex biological regulatory networks are made up of simple recurring gene circuits called network motifs, which constitute the "building blocks" of intricate regulative pathways. Network motifs were defined as patterns that occurred in the transcription network much more often than what would be expected for random networks. According with a common lore, these network motifs are overrepresented in the regulatory pathways because they have been evolutionarily selected to perform basic regulatory functions [1-3].

One of the most representative network motif is the type-1 incoherent feedforward loop (IFFL-1) in which an activator X, upon the perception of an input stimulus, exerts an "incoherent" regulation on its output gene Z, by directly activating its transcription, and by simultaneously repressing it *via* the control of a Z-repressor, Y (Fig. 1A). As a result, when the input signal activates X, Z is rapidly produced, but after some time, Y accumulates and reaches the repression threshold for the expression of Z; therefore, Z production decreases and reaches a steady level after an initial expression peak, known as "overshoot" [1,4-5].

Theoretical and computational analyses suggest that the IFFL-1 confers peculiar properties to the expression of its output gene(s). In particular, it has been proposed that IFFLs-1 can generate pulses in the expression of the output gene Z, can accelerate the Z response time, and can confer robustness to Z expression with respect different concentrations of the input signal, within a certain range. These theoretical properties have been in some cases demonstrated with synthetic gene circuits, and rarely also *in vivo*, by investigating the behaviour of natural gene circuits in prokaryotic and eukaryotic cells [6-12]. Both theoretical models and synthetic gene circuit experiments deal with the IFFL-1 as a three gene circuit in isolation. In the real cells, this network motif is usually embedded inside a network of additional regulators and interactors, possibly altering its behaviour with respect to *in silico* or *in vitro* observations. Therefore, it is particularly important to experimentally validate the regulatory properties conferred to gene expression by the IFFL-1 in a natural cell scenario [12].

IFFLs-1 is among the most frequent network motifs in eukaryotic systems, including yeast and animal cells [2,4,13]. As an example, in animal cells IFFLs-1 are involved in the transcriptional networks of embryonic and hematopoietic stem cells, in innate immune regulation, downstream of the Notch signalling pathway, in fly eye development, and in many other processes [14-18]. Moreover, IFFLs-1 are also well represented in bacteria where, among other functions, they have been demonstrated to control galactose metabolism in *Escherichia coli* and sporulation in *Bacillus subtilis* [12,19-20].

In the opportunistic human pathogen *Pseudomonas aeruginosa* the *las* quorum sensing (QS) system is arranged as an IFFL-1, in which the activator LasR simultaneously triggers the expression of the output gene *lasI* and of *rsaL*, a gene encoding the *lasI*-transcriptional repressor RsaL [21-23]. QS is a signalling system enabling the bacterial population to coordinate gene expression in response to cell density and in *P. aeruginosa* it is involved in the expression of the virulence phenotypes [21,24]. The input signal that activates LasR is the QS signal molecule *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) that at low cell density is synthesized at a basal level by the enzyme LasI (encoded by the gene *lasI*); $3OC_{12}$-HSL diffuses outside the producing cells, accumulates in the extracellular milieu until it reaches a threshold concentration, and then it binds to and activates the intracellular receptor LasR. The activated complex LasR/$3OC_{12}$-HSL, besides activating genes involved in *P. aeruginosa* virulence, also promotes *lasI* transcription, thus generating a positive feedback loop which leads to a rapid increase in the production of $3OC_{12}$-HSL [21,25-26]. At the same time, however, the LasR/$3OC_{12}$-HSL complex also activates the expression of the transcriptional repressor RsaL. RsaL accumulates and reaches the threshold concentration at which it binds and represses the *lasI* promoter (P*lasI*), thus counteracting the positive feedback loop generated by the LasR/$3OC_{12}$-HSL complex, and limiting $3OC_{12}$-HSL concentration to a steady level before the end of the exponential phase of growth (Fig. 1B). Besides *lasI*, RsaL negatively regulates also other *P. aeruginosa* virulence genes, such as those involved in pyocyanin production [22-23].

LasR is considered a key element of *P. aeruginosa* virulence since it activates genes involved in virulence factors production (*e.g.* elastase, proteases, pyocyanin) and, at least in some experimental settings, it is also required for full activation of other two *P. aeruginosa* QS systems, the *rhl* and *pqs* systems, both positively contributing to the expression of *P. aeruginosa* virulence-related phenotypes [21]. On this bases, it has been proposed that Vfr, a regulator required for full activation of *lasR* transcription, is a master regulator of QS in *P. aeruginosa*. Indeed, by promoting LasR expression, Vfr is believed to control all the LasR-activated downstream phenotypes. The pivotal role of Vfr in *P. aeruginosa* virulence is supported by the observation that a mutant defective in *vfr* shows a reduction in the activity of the *lasR* promoter (P*lasR*), is impaired in elastase and proteases production, and is less virulent in an *in vivo* infection model of burn mouse with respect to the wild type strain [27-29]. The effect of *vfr* mutation on the expression of elastase and proteases is not surprising if considering that these phenotypes are directly activated by LasR, while they are not repressed by RsaL, *i.e.* they are not controlled by the LasR-RsaL IFFL-1 [22,30]. On the contrary, the transcription of other LasR-output genes, that like *lasI* and pyocyanin genes are simultaneously activated by LasR and repressed by RsaL, has never been investigated so far in a *P. aeruginosa vfr*

deficient background, and their possible response to a reduction of LasR levels consequent to *vfr* mutation is not clearly predictable [22].

Our working hypothesis is that the LasR-RsaL IFFL-1 confers robustness to its output genes, such as *lasI* and pyocyanin genes, where robustness is generically defined as the ability of a system to buffer transient perturbations and maintain the levels of the output unvaried [11]. In particular, in this manuscript we investigate the hypothesis that a reduction in the levels of the activator LasR may be counterbalanced by a consequent reduction in the levels of RsaL, and this would ultimately result in the robustness of the output genes with respect to LasR variations. Altered levels of LasR in *P. aeruginosa* have been achieved by generating mutant strains in which *i)* LasR levels are reduced as a consequence of *vfr* mutation, or *ii)* LasR expression is controlled by an arabinose-dependent promoter. Our results confirm our working hypothesis, and demonstrate that the LasR regulon can be divided in two distinct sub-regulons; the first sub-regulon is sensitive to LasR variations and includes genes only activated by LasR, such as genes involved in elastase and proteases production; the second sub-regulon is robust with respect to LasR variations, and includes genes that are both activated by LasR and repressed by RsaL, such as *lasI* and genes involved in pyocyanin production.

Overall, this study provides the first experimental evidence that an IFFL-1, despite being embedded in a complex regulatory network when in whole cell system, confers robustness to the expression of its output genes with respect to fluctuations in the level of the Z activator, and highlights *P. aeruginosa* phenotypic plasticity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1 (Supplementary Information). *Escherichia coli* S17.1 λ*pir* [31] was used for cloning purposes. All *E. coli* and *P. aeruginosa* strains were routinely grown at 37°C in Luria-Bertani broth (LB), or in LB supplemented with 1.5% (w/v) agar [32]. Unless otherwise stated, antibiotics were added at the following concentrations: *E. coli*: 100 μg/ml ampicillin (Ap), 25 μg/ml kanamycin (Km), 30 μg/ml chloramphenicol (Cm), 10 μg/ml tetracycline (Tc), 15 μg/ml nalidixic acid (Nal); *P. aeruginosa*: 400 μg/ml Cm, 200 μg/ml Tc, 500 μg/ml carbenicillin (Cb). When required, media were supplemented with L-arabinose at the concentration reported in the text.

Recombinant DNA techniques

Plasmids used or generated in this study and details of their construction are reported in Table S2 (Supplementary Information). Preparation of plasmid DNA, purification of DNA fragments, restrictions, ligations, and transformations of *E. coli* were carried out by standard procedures [32]. PCR amplifications were performed using Bio Red-Taq DNA polymerase (Bioline) according with manufacturer's instructions. The oligonucleotides used in this study are listed in Table S3 (Supplementary Information). Automated sequencing was performed by Genechron sequence service (Genechron). When required, plasmids were transferred from *E. coli* S17.1 λ*pir* to *P. aeruginosa* PAO1 by conjugation [33].

Construction of *P. aeruginosa* mutants

P. aeruginosa mutant strains were generated using the pDM4- or pEX18-derivative plasmids as previously described [34-35]. For Δ*vfr* and Δ*lasR* mutants plasmids were already available [36-37]. Conversely, the plasmids used to generate the Δ*rsaL* and Δ*lasR*Δ*rsaL* mutant strains have been generated in this study, as described in Table S2 (Supplementary Information). Briefly, the flanking DNA regions of the selected genes were PCR amplified with primers listed in Table S3 (Supplementary Information) and sequentially cloned in pDM4. The resulting pDM4-derivative plasmids were independently introduced in *P. aeruginosa* PAO1 by conjugation. Clones with a chromosomal insertion of the pDM4-derivative plasmids were selected on LB agar plates supplemented with 400 μg/ml Cm and 10 μg/ml Nal. Plasmid excision from the chromosome was subsequently selected on LB agar plates supplemented with 10% (w/v) sucrose. Mutant strains were checked by PCR analysis.

The *P. aeruginosa* strains for arabinose-dependent induction of LasR were obtained by inserting in single copy in a neutral site of the bacterial chromosome the genetic cassette *araC*-P_{BAD}*lasR*, allowing constitutive expression of AraC and carrying the *lasR* gene under the control of the P_{BAD} promoter. To this aim, the mini-CTX1-*araC*-P_{BAD}*lasR* plasmid has been generated as reported in Table S2 (Supplementary Information).

Promoter activity assay

The activities of the *lasR* promoter (P*lasR*) and of the *lasI* promoter (P*lasI*) activity were measured in *P. aeruginosa* strains carrying the pMP-P*lasR*::*lacZ* and pMP-P*lasI*::*lacZ* plasmids, respectively, by standard Miller assay [38]. The pMP-P*lasI*::*lacZ* plasmid was already available [22], while the pMP-P*lasR*::*lacZ* plasmid has been generated in this study as described in Table S2 (Supplementary Information). Briefly, over-night cultures of the PAO1 strains carrying the pMP190-derivative plasmids were diluted to an optical density at 600 nm wavelength of 0.01 (A_{600} = 0.01) in 10 ml of fresh LB and incubated a 37°C with 200 r.p.m. shaking. Promoter activity (Miller units) and cell density (A_{600}) were determined after 9 hours of growth.

Western blot analysis

Western immunoblotting was performed by standard technique [32] with anti-LasR serum (1:1000) [39] and horseradish peroxidase-conjugate anti-rabbit IgG as secondary antibody (1:10000; Promega). Final development was performed with the Amersham ECL chemiluminescent reagents (Amersham Biosciences).

Phenotypic assays

In the phenotypic assays, cultures were diluted to an A_{600} of 0.02 in 50 ml fresh LB, and incubated at 37° C with 200 r.p.m shaking. For the experiments with the strains PAO1 Δ*lasR* *lasR*^{ind} and PAO1 Δ*lasR*Δ*rsaL* *lasR*^{ind} 10 ml of fresh LB was supplemented with different concentrations of L-arabinose ranging from 0.4 to 0.025% (w/v). Cell-free supernatants were collected after 9 hours of incubation. The concentration of $3OC_{12}$-HSL, proteases, elastase and pyocyanin in the cell-free supernatants were measured as previously described [40-43]. The average measurements and relative standard deviations (SD) were calculated from at least three independent experiments. Statistical analysis was performed using Student's *t*-test. Differences were considered statistically significant when p value < 0.05.

RESULTS

The LasR-RsaL IFFL-1 confers robustness to the *las* QS system in *P. aeruginosa*

The *las* system is interwoven in an intricate regulatory network and a number of different regulators are involved in its modulation. However, up to date, only three regulators are known to directly bind and control the activity of the *lasR* promoter (P*lasR*). In detail, Vfr (*virulence factors regulator*) binds to and activates P*lasR*, while AlgR2 (also known as AlgQ) and PA3699 repress *lasR* transcription upon binding to the P*lasR* region [27,36,44]. Therefore, a possible strategy to obtain a *P. aeruginosa* strain expressing low levels of LasR is to inactivate the *vfr* gene. Indeed, it has been reported that in a *P. aeruginosa vfr* mutant strains P*lasR* activity was significantly reduced with respect to the wild type strain [27].

To verify the effect of the *vfr* mutation on P*lasR* activity and on LasR expression also in our strain and experimental settings, a *P. aeruginosa* PAO1 Δ*vfr* in frame clear deletion mutant has been generated, and β-galactosidase assays and western-blot analysis with anti-LasR antibody [37] have been performed on this mutant and on its isogenic wild type strain, both carrying the pMP-P*lasR*::*lacZ* plasmid for the analysis of P*lasR* promoter activity (Table S2, Supplementary Information). First of all, the growth curves of the tested strains were comparable (data not shown), demonstrating that the *vfr* mutation does not affect *P. aeruginosa* growth rate. According to literature data, both P*lasR* activity and LasR protein level were strongly reduced (>80%) in the Δ*vfr* mutant with respect to the wild type strain (Fig. 2), confirming the positive role exerted by Vfr on *lasR* transcription [27].

At this point, the activity of the *lasI* promoter (P*lasI*) was measured in the *P. aeruginosa* wild type and Δ*vfr* strains carrying the pMP-P*lasI*::*lacZ* plasmid, allowing determination of P*lasI* promoter activity (Table S2, Supplementary Information) [22]. In parallel, $3OC_{12}$-HSL synthesis and LasR protein levels were evaluated in the same strains by biosensor-based techniques previously described [40], and by western-blot analysis, respectively. As reported in Fig. 3, *vfr* mutation does not affect P*lasI* activity and $3OC_{12}$-HSL production, despite it causes a strong reduction of LasR levels. These results clearly demonstrate that both the transcription of *lasI* and the LasI-mediated synthesis of the QS signal molecule $3OC_{12}$-HSL are robust with respect to fluctuation in the concentration of their regulator LasR. As previously mentioned, it is likely that this robustness is due to the buffering activity of the LasR-activated repressor RsaL. Indeed, a decreased activation of *lasI* transcription caused by a lower expression of LasR in the *vfr* mutant strain, could be counterbalanced by a decreased repression of *lasI* caused by a concomitant lower expression of RsaL in the same mutant.

To verify this hypothesis, *lasI* transcription, $3OC_{12}$-HSL production, and LasR levels were compared in *P. aeruginosa* Δ*rsaL* and Δ*rsaL*Δ*vfr* mutant strains, both carrying the pMP-P*lasI*::*lacZ* plasmid. As shown in Fig. 3, P*lasI* activity and $3OC_{12}$-HSL production were higher in the Δ*rsaL* mutant with respect to the isogenic wild type strain, in line with the repressive effect directly exerted by RsaL on *lasI* transcription [22,29]. Notably, the reduced LasR levels caused by *vfr* mutation lead to a significant reduction in both P*lasI* activity and $3OC_{12}$-HSL synthesis only in the Δ*rsaL* mutant strains (please compare strains Δ*rsaL* and Δ*rsaL*Δ*vfr*), while no differences were observed in the RsaL-proficient genetic backgrounds (please compare strains wild type and Δ*vfr*). These data demonstrate that the IFFL-1 generated by LasR and RsaL confers robustness with respect to fluctuations in LasR levels to the output gene *lasI*, and consequently to the concentration of the QS signal molecule $3OC_{12}$-HSL.

The LasR-regulon is divided in two sub-regulons with different robustness properties with respect to LasR variations

IFFLs are organized into higher-level motifs: the same X and Y regulators can control multiple output genes, resulting in multi-output IFFLs [45]. The *las* system is a multi-output IFFL-1 because other genes besides *lasI* are contemporary activated by LasR and repressed by RsaL such as the genes involved in pyocyanin production [22]. The effect of the *vfr* mutation on pyocyanin production has been previously determined but it resulted unclear; indeed it was reduced or overexpressed with respect to the wild type strain depending on medium composition [28]. Therefore, in order to asses if the robustness is conserved also in genes regulated by the LasR-RsaL IFFL-1 other than *lasI*, we investigated the effect of reduced LasR levels (caused by Δ*vfr* mutation) on pyocyanin production in a wild type and in a Δ*rsaL* genetic background. Our results show that, as in the case of P*lasI* transcription and $3OC_{12}$-HSL production, pyocyanin production is decreased by the Vfr-dependent LasR reduction only upon the disruption of the *las* IFFL-1 due to the *rsaL* mutation (Fig. 4A). This result demonstrates that, irrespective on the possible influence of different ancillary regulators specifically acting on $3OC_{12}$-HSL or pyocyanin synthesis, the regulatory properties generated by the LasR-RsaL IFFL-1 are conserved on different downstream genes, as expected for an IFFL-1 multi-output network motif.

As previously mentioned, despite some genes belonging to the LasR-regulon are directly controlled by both LasR and RsaL, other LasR-activated genes are not directly repressed by RsaL, as is the case for genes involved in elastase (*lasB*) and protease (*aprA*) production [21,22]. Therefore, despite being activated by LasR, the *lasB* and *aprA* genes are not controlled by the LasR-RsaL IFFL-1. As a consequence, as shown in Fig. 4B and 4C, elastase and protease

production measured in the same cell-free supernatant used in the previous experiments for $3OC_{12}$-HSL and pyocyanin detection, is reduced in the Δ*vfr* mutant with respect to the wild type strain, as a consequence of the lower expression of LasR caused by *vfr* mutation. These results are in agreement with literature data reporting a positive effect of Vfr on the production of elastase and proteases in *P. aeruginosa* PAO1 [27,28,30].

Overall, our data indicate that the LasR regulon is divided in two sub-regulons with different robustness with respect to LasR variations. Indeed, RsaL counterbalances a perturbation in the levels of LasR (in this case a mutation in *vfr*) in the sub-group of genes controlled by the LasR-RsaL IFFL-1, while this perturbation affects the sub-group of genes that are only activated by LasR but not repressed by RsaL.

In the above described experiments, the reduction of LasR levels has been achieved by mutating the *vfr* gene. To exclude possible effects of *vfr* mutation on the tested phenotypes beyond the reduced expression of LasR, we generated a *P. aeruginosa* PAO1 engineered strain with governable levels of LasR. To this aim, a mini-CTX1-*araC*-P_{BAD}*lasR* plasmid has been generated. This plasmid allows inserting in a neutral site of *P. aeruginosa* PAO1 chromosome in single copy of a genetic cassette for constitutive expression of the AraC repressor, coupled to a transcriptional fusion between the P_{BAD} promoter and the *lasR* gene. Therefore, in absence of the L-arabinose inducer, the P_{BAD} promoter is repressed by AraC, and *lasR* is not transcribed; conversely, exogenous provision of L-arabinose induces a conformation change in AraC, and consequent activation of the P_{BAD} promoter. As a result, in a *P. aeruginosa* PAO1 strain containing the *araC*-P_{BAD}*lasR* cassette the expression levels of LasR are proportional to the concentration of L-arabinose present in the medium. The mini-CTX1-*araC*-P_{BAD}*lasR* plasmid was independently introduced in the mutant strain *P. aeruginosa* PAO1 Δ*lasR* and in the double mutant strain *P. aeruginosa* PAO1 Δ*lasR*Δ*rsaL,* generating the Δ*lasR lasR*^{IND} and Δ*lasR*Δ*rsaL lasR*^{IND} strains, respectively. In this way the effect of L-arabinose-induced variations in LasR levels could be compared in a *rsaL* proficient and deficient genetic background. As shown in Fig. 5A, the levels of $3OC_{12}$-HSL produced by the mutant strain Δ*lasR lasR*^{IND} are maintained almost to their maximum value (considered as 100%) for L-arabinose concentrations ranging from 0.4% to 0.1% (W/V), and $3OC_{12}$-HSL production is significantly reduced only for L-arabinose concentrations ≤ 0.05 % (W/V). On the contrary, in the Δ*lasR*Δ*rsaL lasR*^{IND} strain, in which the buffering effect exerted by RsaL on *lasI* expression is missing, $3OC_{12}$-HSL production follows a linear dose-response decrease as a function of reduced L-arabinose concentration. Indeed, if considering as 100% the level of $3OC_{12}$-HSL produced in the presence of the maximal L-arabinose concentration 0.4% (W/V), $3OC_{12}$-HSL levels are significantly reduced of about 20% and 40% for L-arabinose concentration of 0.2% and 0.1% (W/V), respectively. Notably,

this different regulative behaviour is not apparent for elastase and proteases production, since L-arabinose reduction leads to a similar decrease in the levels of these two virulence factors in both the Δ*lasR lasR*^{IND} and Δ*lasR*Δ*rsaL lasR*^{IND} strains (Fig. 5B and 5C).

These data ultimately demonstrate that only a subset of the LasR-controlled genes are robust with respect to variations in the levels of this regulator, and that this property is dependent on the negative regulator RsaL, or in other terms, on the LasR-RsaL IFFL-1. As expected, the latter experiment also highlights that the buffering capacity exerted by RsaL on the expression of genes controlled by both LasR and RsaL (*i.e. lasI*) is restricted within a defined range of LasR fluctuations. Indeed, when the concentration of L-arabinose is ≤ 0.05 % (W/V), the reduction of the RsaL-mediated repression is no longer sufficient to counterbalances the reduction of the LasR-dependent activation on the target genes.

DISCUSSION

The main result of this work has been the demonstration that the IFFL-1 generated by LasR and RsaL confers robustness to the expression of QS output genes. This has been shown by using two different strategies to achieve fluctuations in the levels of LasR. In the first case, a mutation in the *vfr* gene, that codes for the activator of LasR, caused a reduction in LasR absolute levels. In the second case, the expression levels of LasR were controlled and modulated on purpose by using an L-arabinose inducible system. The use of a *vfr* mutant strain allowed to test the behaviour of the IFFL-1 in a "natural" scenario, since Vfr is an activator of *lasR* transcription. If considering that the Vfr-mediated control of LasR level is dependent on the intracellular levels of the second messenger cyclic AMP (cAMP), a co-activator of Vfr [27-29], it is likely that the control exerted by Vfr on *lasR* transcription is missing in growth conditions leading to low intracellular levels of cAMP. Our results suggest that *P. aeruginosa* might respond to fluctuating levels of cAMP by altering the expression of a subset of genes controlled only by LasR, while buffering the expression level of the genes controlled by both LasR and RsaL. Unfortunately, cAMP levels in other bacteria like *E. coli* are known to be dependent on the metabolic status of the cell, mainly as a consequence of differences in carbon sources [46], while the environmental stimuli modulating cAMP levels in *P. aeruginosa* are still poorly understood. In the future, it would be interesting to test if an artificial modulation of the *P. aeruginosa* cAMP levels, obtained by altering the expression of the cAMP synthase adenilate cyclase, would lead to a differential regulation of the two subset of LasR- and LasR-RsaL-controlled genes.

For what concern the results obtained in the LasR-inducible strain, their importance relies on the observation that the regulatory properties of the LasR-RsaL IFFL-1 are conserved irrespective on *vfr* mutation, allowing to exclude possible side effect exerted by Vfr and other possible *lasR* transcriptional regulators on the tested phenotypes. Notably, the conserved robustness of the output gene observed when using both the Δ*vfr* mutant and the LasR-inducible genetic background is not trivial if considering that these strains might be not comparable in terms of absolute LasR levels. In this context, it is interesting to mention that the IFFL-1 has been previously proposed to provide a response to fold-changes in the input signal, rather than to its absolute levels. This "fold-change detection property" means that the dynamics of the output gene expression (*e.g.* amplitude and duration of the output gene Z transcription) depend on the relative changes in input signal, rather than on its absolute levels [9]. In line with this hypothesis our data demonstrate that the buffering effect exerted by RsaL on the output gene *lasI* is conserved, irrespective on the possibly different absolute levels of LasR in the Δ*vfr* mutant with respect to the LasR-inducible strains. This is

probably due to the fact that, since RsaL itself is LasR-controlled, RsaL levels are expected to vary as a function of LasR levels, extending the range of LasR concentration for which robustness of the output gene is conserved.

Previous works investigated the role of the IFFL-1 in conferring robustness to output genes with respect to fluctuations in different cues, such as different levels of the input signal. This has been mainly done in theoretical studies, and has been confirmed only in few cases with synthetic gene circuits in natural cells [10,11,47-49]. At the best of our knowledge, this is the first case in which the robustness with respect variations in the levels of the activator X has been reported for an IFFL-1 in natural bacterial cells, and for a gene circuit involved in communication and expression of virulence.

From a physiological perspective, the relevance of this work is the demonstration that *P. aeruginosa* has the ability to modulate with different dynamics distinct subsets of LasR-controlled genes. In other terms, by altering the levels of a single intracellular receptor, LasR, *P. aeruginosa* can modulate the expression level of only the subset of the LasR-controlled genes that are not simultaneously regulate by RsaL. This complex regulatory behaviour likely enhances *P. aeruginosa* phenotypic plasticity, and might be relevant also during the infection process. *P. aeruginosa* can establish both acute and chronic infections, characterised by different behaviours and distinctive phenotypes. An acute infection is rapid, systemic and carried out by a planktonic bacterial community expressing high levels of virulence factors. Conversely, in a chronic infection bacterial proliferation is limited to a specific host tissue such as the lung of cystic fibrosis patients, and bacteria can persist in the host for extended periods of time, forming biofilm and adopting a slow-growing sessile lifestyle [50]. Despite the different patterns of phenotypes expressed by *P. aeruginosa* during the acute and the chronic infections, both are positively controlled by QS. Our results suggest that the different response of the two LasR sub-regulons to external cues affecting LasR may be involved in the different levels of expression of the virulence-related genes required in the acute and in the chronic infection. Indeed some LasR-controlled virulence factors, such as elastase and proteases, are largely expressed during acute infections, while their expression level is reduced during chronic infections [50]. Conversely, other LasR-controlled virulence factors, such as pyocyanin, are expressed in both the acute and chronic infections [28]. Notably, high levels of $3OC_{12}$-HSL and pyocyanin have been recovered in the sputum of cystic fibrosis patients with *P. aeruginosa* chronic lung infection [51]. In this scenario it is tempting to speculate that the virulence phenotypes involved in the acute infection require a rapid regulation to allow the bacterial population to face environmental changes that occur in a planktonic lifestyle, and therefore their expression level is promptly adjusted in response to variations in LasR levels. Conversely, virulence

phenotypes required during chronic infections likely need to be steadily expressed despite spurious environmental and metabolic fluctuations possibly affecting LasR levels, and for this reason they might be under the control of the LasR-RsaL IFFL-1. This hypothesis is strengthened by our recent data demonstrating that RsaL is required for full potential of *P. aeruginosa* to establish chronic lung infection in a murine model system [52].

Since biofilm is one of the virulence-related phenotypes controlled by the *las* QS system in *P. aeruginosa* [24], it would be interesting in the future to investigate the role played by the LasR-RsaL IFFL-1 on biofilm formation. In the eukaryotic cells an IFFL-1 has been described in which a microRNA confers robustness to the regulatory network controlling cell differentiation. In this case, the IFFL allows the cells to maintain their differentiation programme despite possible fluctuations in the environmental cues triggering this developmental process [10]. Since in the biofilm bacteria live in close proximity and differentiate in distinct cell types, biofilm formation is often compared to the differentiation process that occurs in multicellular eukaryotic organisms. In this context, it is interesting to mention that both *P. aeruginosa* mutant strains impaired in *lasI* and *rsaL* are impaired in biofilm formation [43,53,54]. Therefore, it is likely that, similarly to the developmental processes in eukaryotic cells, also biofilm formation in *P. aeruginosa* requires the robustness regulatory properties conferred by IFFL-1 network motifs.

FIGURE LEGENDS

Fig. 1: Schematic representation of an IFFL-1 and of the *las* QS system.

(A) The activator X, upon the perception of an input signal, activates the transcription of the output gene Z and of the repressor of Z, Y. Arrows indicate positive regulations; T-bar represents negative regulation. **(B)** The activator LasR, upon the perception of the input signal $3OC_{12}$-HSL, activates the transcription of the output gene *lasI* and of the repressor of *lasI*, RsaL. Arrows indicate positive regulations; T-bar represents negative regulation; dashed arrow represents information flow.

Fig. 2: Effect of *vfr* mutation on P*lasR* activity and LasR levels.

(A) P*lasR* activity measured by a β-galactosidase assay in the indicated PAO1 strains carrying the transcriptional fusions P*lasR*::*lacZ*. M.u., Miller units. The average values from three independent experiments are reported with SD. **(B)** Western-blot analysis performed with anti-LasR polyclonal serum on protein crude extracts from the PAO1 indicated strain; a *P. aeruginosa* Δ*lasR* strain was used as negative control. The western-blot is representative of three independent experiments.

Fig. 3: Effect of *vfr* and *rsaL* mutations on P*lasI* activity, $3OC_{12}$-HSL production and LasR levels.

(A) P*lasI* activity measured by a β-galactosidase assay in the indicated PAO1 strains carrying the transcriptional fusions P*lasI*::*lacZ*. M.u., Miller units. **(B)** $3OC_{12}$-HSL concentration measured in the cell-free supernatant of the PAO1 indicated strains. **(C)** Western-blot analysis performed with anti-LasR polyclonal serum on protein crude extracts from the PAO1 indicated strains; a *P. aeruginosa* Δ*lasR* strain was used as negative control. For both **(A)** and **(B)**, the average values from three independent experiments are reported with SD. The western-blot is representative of three independent experiments.

Fig. 4: Effect of *vfr* and *rsaL* mutations on pyocyanin, elastase and proteases production.

(A) Pyocyanin, **(B)** elastase and **(C)** proteases production measured in the cell-free supernatant of the PAO1 indicated strains. For the three assays, the average values from three independent experiments are reported with SD.

Fig. 5: Effect of LasR variations on $3OC_{12}$-HSL, elastase and proteases production.

(A) $3OC_{12}$-HSL, **(B)** elastase and **(C)** proteases production measured in the PAO1 Δ*lasR* $lasR^{IND}$ strain (white bars) and in the PAO1 Δ*lasR*Δ*rsaL* $lasR^{IND}$ strain (grey bars) grown in LB supplemented with different L-arabinose concentrations, as indicated below the histograms. For both the strains, 100% of $3OC_{12}$-HSL, elastase and proteases production was considered as the maximum values measured with 0.4% (w/v) L-arabinose. For the three assays, the average values from five independent experiments are reported with SD. *, $p < 0.05$ with respect to 0.4% (w/v) L-arabinose in the same strain; **, $p < 0.01$ with respect to 0.4% (w/v) L-arabinose in the same strain.

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Figure 1

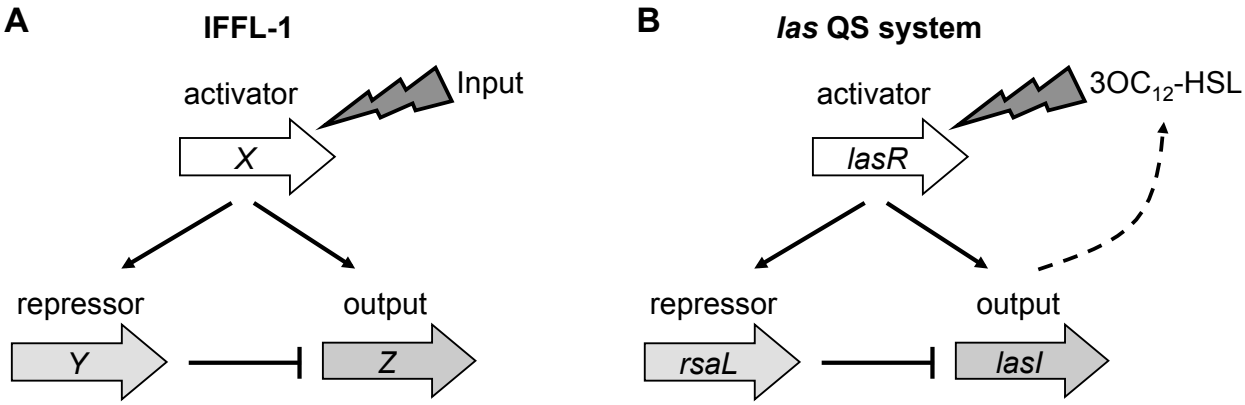

A
IFFL-1
activator
Input
X
repressor
output
Y
Z
B
las QS system
activator
3OC12-HSL
lasR
repressor
output
rsaL
lasI

Figure 2

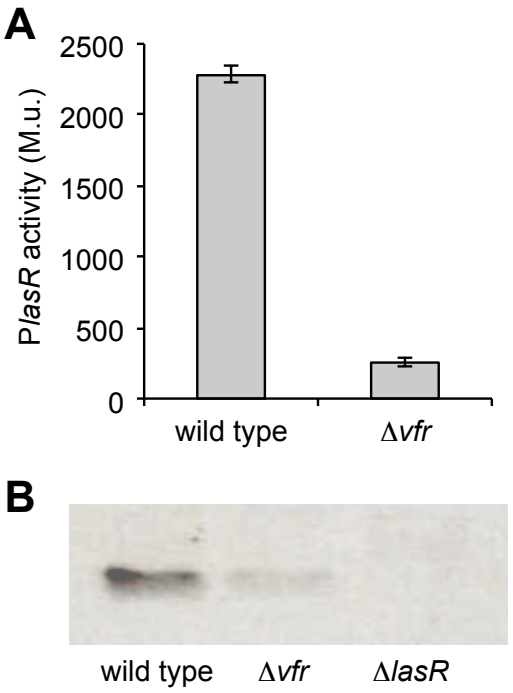

A
2500
2000
1500
1000
500
0
P*lasR* activity (M.u.)
wild type
Δ*vfr*
B
wild type
Δ*vfr*
Δ*lasR*

Figure 3

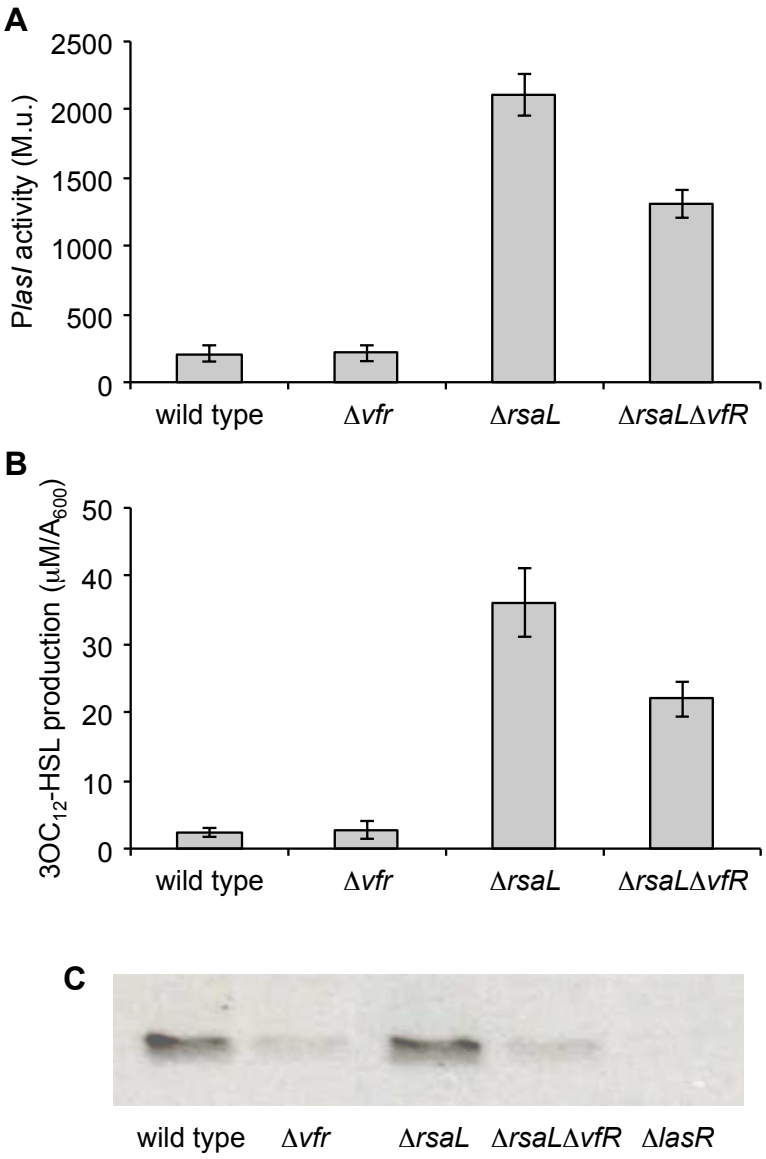

A
P*lasI* activity (M.u.)
2500
2000
1500
1000
500
0
wild type
Δ*vfr*
Δ*rsaL*
Δ*rsaL*Δ*vfR*
B
3OC12-HSL production (μM/A600)
50
40
30
20
10
0
wild type
Δ*vfr*
Δ*rsaL*
Δ*rsaL*Δ*vfR*
C
wild type
Δ*vfr*
Δ*rsaL*
Δ*rsaL*Δ*vfR*
Δ*lasR*

Figure 4

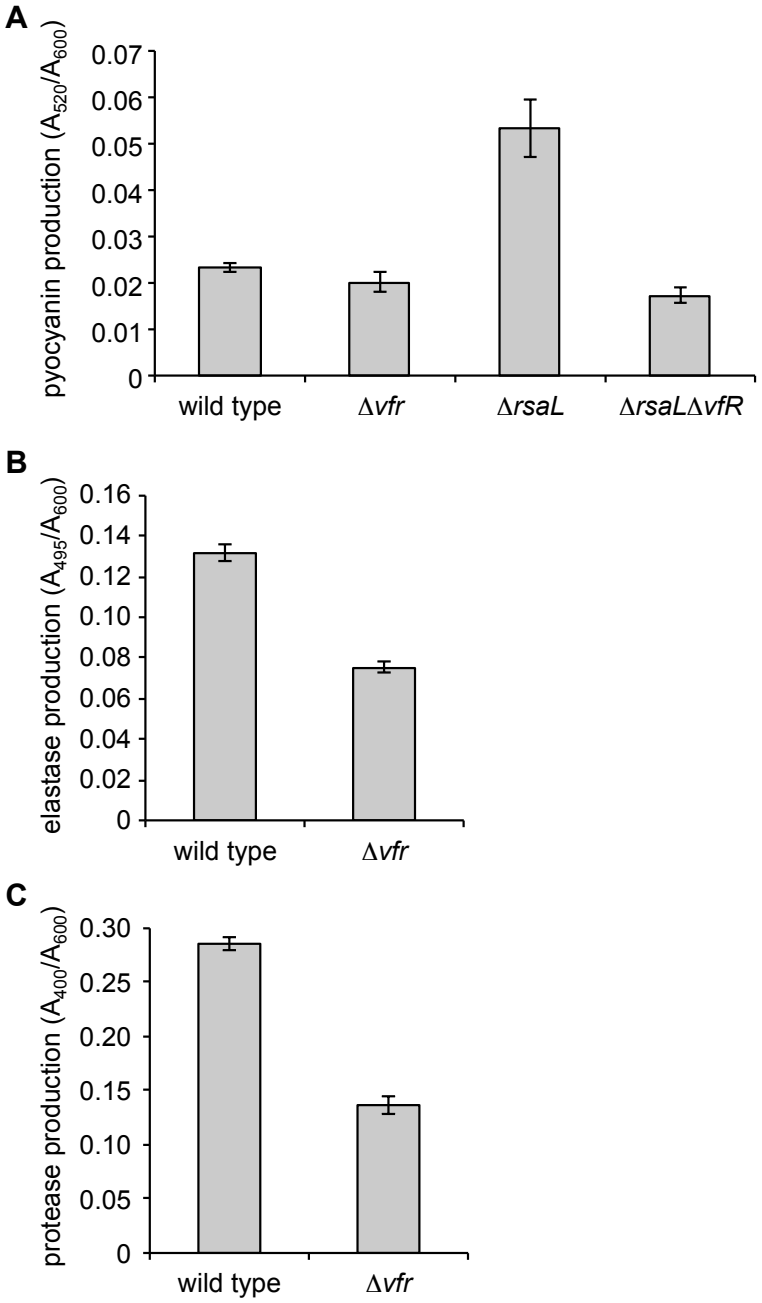
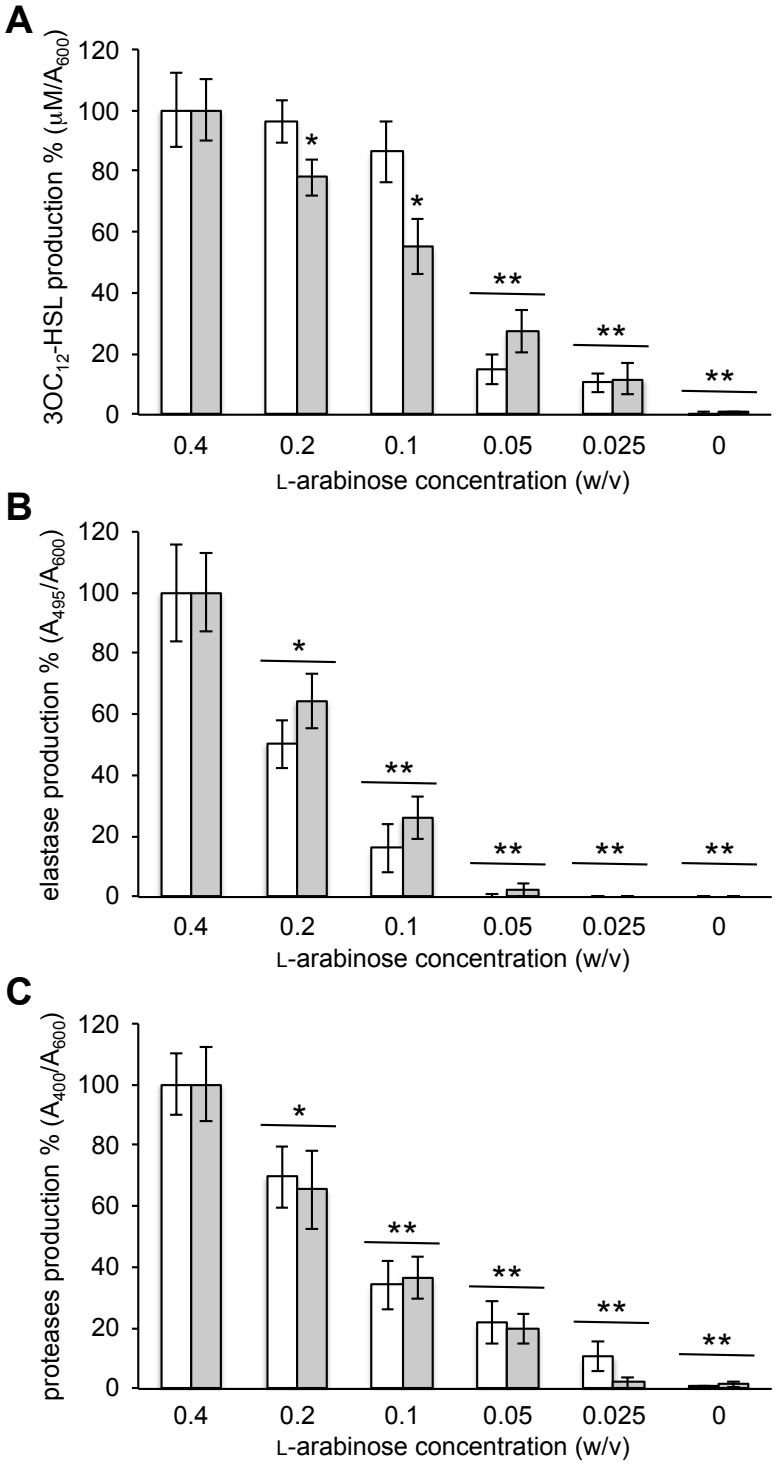

A
pyocyanin production (A_{520}/A_{600})
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
wild type
Δvfr
ΔrsaL
ΔrsaLΔvfR
B
elastase production (A_{495}/A_{600})
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
wild type
Δvfr
C
protease production (A_{400}/A_{600})
0.30
0.25
0.20
0.15
0.10
0.05
0
wild type
Δvfr

Figure 5


A
3OC12-HSL production % (µM/A600)
120
100
80
60
40
20
0
*
*
**
**
**
0.4
0.2
0.1
0.05
0.025
0
L-arabinose concentration (w/v)
B
elastase production % (A495/A600)
120
100
80
60
40
20
0
*
**
**
**
**
0.4
0.2
0.1
0.05
0.025
0
L-arabinose concentration (w/v)
C
proteases production % (A400/A600)
120
100
80
60
40
20
0
*
**
**
**
**
0.4
0.2
0.1
0.05
0.025
0
L-arabinose concentration (w/v)

SUPPLEMENTARY INFORMATION

Characterization of the incoherent feedforward loop governing quorum sensing in *Pseudomonas aeruginosa*

Roslen Bondí [1], Marco Messina [1], Francesca Longo [1 §], Livia Leoni [1] and Giordano Rampioni [1 *]

Table S1. Bacterial strains used in this study.

Strain	Relevant characteristics	Reference/Source
E. coli		
S17.1 λ*pir*	Conjugative strain for suicide plasmids.	[31]
SM10 (pFLP2)	Strain carrying the pFLP2 plasmid (FLP; Ap^R/Cb^R) for Flp-mediated recombination.	[35]
P. aeruginosa		
PAO1	wild type strain ATCC15692.	American Type Culture Collection
PAO1 Δ*vfr*	*P. aeruginosa* PAO1 mutant strain with in frame clear deletion deletion of the *vfr* gene.	[36]
PAO1 Δ*rsaL*	*P. aeruginosa* PAO1 mutant strain with in frame clear deletion of the *rsaL* gene, obtained by using the pDM4Δ*rsaL* plasmid (Table S2).	This study
PAO1 Δ*vfr* Δ*rsaL*	*P. aeruginosa* PAO1 double mutant strain with in frame clear deletion of both the *rsaL* and *vfr* genes, obtained by using the pDM4Δ*rsaL* plasmid (Table S2).	This study
PAO1 Δ*lasR*	A 344 bp region encompassing the *lasR* gene, corresponding to nucleotides 1558474-1558819 of *P. aeruginosa* PAO1 genome, has been deleted from *P. aeruginosa* PAO1 chromosome by using the pEX18Δ*lasR* plasmid (Table S2).	This study

PAO1 Δ*lasR* Δ*rsaL*	A 685 bp region encompassing the *lasR* and *rsaL* gene, corresponding to nucleotides 1558473-1559112 of *P. aeruginosa* PAO1 genome, has been deleted from *P. aeruginosa* PAO1 chromosome by using the pDM4Δ*lasR*Δ*rsaL* plasmid (Table S2).	This study
PAO1 Δ*lasR lasR*^{ind}	*P. aeruginosa* PAO1 Δ*lasR* mutant strain carrying the *araC*-P_{BAD}*lasR* transcriptional fusion for the L-arabinose-dependent induction of *lasR* integrated into the chromosome. Generated by using the mini-CTX1-*araC*-P_{BAD}*lasR* (Table S2).	This study
PAO1 Δ*lasR*Δ*rsaL* *lasR*^{ind}	*P. aeruginosa* PAO1 Δ*lasR*Δ*rsaL* mutant strain carrying the *araC*-P_{BAD}*lasR* transcriptional fusion for the L-arabinose-dependent induction of *lasR* integrated into the chromosome. Generated by using the mini-CTX1-*araC*-P_{BAD}*lasR* (Table S2).	This study

Table S2. Plasmids used in this study.

Plasmid	**Relevant characteristics and plasmid construction**	**Reference/Source**
pDM4	Suicide vector for construction of deletion mutants; *sacBR*; *oriR6K*; Cm^R.	[34]
pDM4Δ*vfr*	pDM4 derivative plasmid for in frame clear deletion of the *vfr* gene.	[36]
pDM4Δ*rsaL*	pDM4 derivative plasmid for in frame clear deletion of the *rsaL* gene. The upstream (UP) and the downstream (DW) DNA regions of *rsaL* were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW61G/RV62G and FW63G/RV64G, respectively (Table S3). The UP and DW PCR fragments were subsequently cloned in pDM4 by XhoI-BamHI and BamHI-XbaI restriction, respectively.	This study

pEX18Δ*lasR*	pEX18Tc derivative plasmid for in frame deletion of the *lasR* gene from nucleotides 1558474-1558819 of *P. aeruginosa* PAO1 genome.	[37]
pDM4Δ*lasR*Δ*rsaL*	pDM4 derivative plasmid for in frame deletion of the *lasR* and *rsaL* genes from nucleotides 1558473-1559112 of *P. aeruginosa* PAO1 genome. The upstream (UP) and downstream (DW) regions of the genetic *locus* containing the *lasR* and *rsaL* genes were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW675/RV765 and RV62G/ FW61G, respectively (Table S3). The UP and DW PCR fragments were subsequently cloned in pDM4 by SpeI-BamHI and BamHI-XhoI restriction, respectively.	This study
mini-CTX1-*araC*-P_{BAD}*tolB*	mini-CTX1 derivative plasmid carrying the *araC*-P_{BAD}*tolB* cassette from pBEM9, cloned by XhoI/EcoRI restriction.	[55]
mini-CTX1-*araC*-P_{BAD}*lasR*	mini-CTX1-*araC*-P_{BAD} derivative plasmid containing a 720 bp DNA fragment encompassing the *lasR* gene. *lasR* was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW763 and RV764 (Table S3) and cloned in the mini-CTX1-*araC*-P_{BAD}*tolB* plasmid by HindIII-EcoRI restriction.	This study
pMP190	Promoter probe vector; IncQ; 15 kb; Sm^r Cm^r.	[56]
p*lasR*190	A DNA fragment encompassing the *lasR* promoter region (P*lasR*) was PCR amplified from *P. aeruginosa* PAO1 genome with primers FW324 and RV325 (Table S3) and cloned by SalI-BglII restriction in the pMP190 plasmid upstream of the promoterless *lacZ* gene.	This study
p*lasI*190	A DNA fragment encompassing the *lasI* promoter region (P*lasI*) was PCR amplified and cloned in the pMP190 plasmid upstream of the promoterless *lacZ* gene.	[22]

Table S3. Oligonucleotides used in this study.

Name	Sequence (5'-3') [a]	Position [b]	Restriction site
FW324	NNNACGC**GTCGAC**GGTCGCACGCGTGGCG	1557812	SalI
RV325	NNNNGGA**AGATCT**CAACCAAGGCCATAGCGC	1558166	BglII
FW675	GG**ACTAGT**ACCTATGCGCCGCCGTTG	1557572	SpeI
RV676	CGC**GGATCC**AGCGCTACGTTCTTCTTAAACT	1558149	BamHI
RV765	GCG**GGATCC**ACTCGTGCTGCTTTCGCGT	1558454	BamHI
FW763	TAT**AAGCTT**ATGGCCTTGGTTGACGGTTT	1558171	HindIII
RV764	TAT**GAATTC**TCAGAGAGTAATAAGACCCAAA	1558869	EcoRI
FW61G	CCG**CTCGAG**CGCATCGCCTCCAGCGT	1559614	XhoI
RV62G	TAT**GGATCC**GTGTGAAGCCATTGCTCTG	1559111	BamHI
FW63G	TAT**GGATCC**TTGCATTTCTATATAGAAGG	1558932	BamHI
RV64G	TGC**TCTAGA**CTGGGAACCGTCCATCTAC	1558441	XbaI

[a] restriction sites are in bold face.

[b] Position with respect to *P. aeruginosa* PAO1 chromosome (www.pseudomonas.com) [57].

Additional references [a]:

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[a] Please note that other references cited in this Supplementary Information file are listed in the Reference Section of the manuscript main body.

CONCLUSIONS

Traditional approaches to combat bacterial infections rely on the disruption of essential functions by preventing the synthesis and assembly of key components of bacterial processes, such as cell wall and protein synthesis, or DNA replication. These strategies, and the resulting antibiotic compounds, are highly effective, but they result in substantial stress on the targeted bacterium, which rapidly selects for resistant subpopulations. Indeed, in a population of billions of bacteria under antibiotic selective pressure, if a small fraction develops a resistance mechanism due to the natural mutation rate or to horizontal gene transfer from other resistant bacteria, it may quickly grow and become the dominant proportion of the population, while the majority of the non-resistant cells are killed (Rasko and Sperandio, 2010).

Since their introduction into clinical practice at the middle of the 20th century, the use of antibiotics was followed by the arising of resistant strains: once one antibiotic was used widely, resistant strains capable of inactivating the drug became prevalent. So the studies in the antibiotic field from the so called “golden age” in 1950s to the “genomic era” in 1990s mainly aimed at improving their use, at modifying their chemical structure to bypass the resistance mechanism, and at evaluating possible new targets for the design of new effective compounds. Nowadays, it is clear that any attempt to identify new antibiotic compounds is vanished by the ability of bacteria to develop resistance mechanisms. Consequently, we are now in the post-antibiotic era, with limited treatment options for a growing number of resistant bacteria, and we are going back to the situation of the “dark age”, that is the period before the introduction of the antibiotics in clinical practise (Davies and Davies, 2010; Fig. 7).

A possible alternative to the use of antibiotics relies on strategies based on the inhibition of virulence rather than of bacterial growth. Virulence traits are not essential for bacterial survival and therefore an anti-virulence drug should exert a milder evolutionary pressure for the development of resistance (Cegelski *et al*., 2008; Rasko and Sperandio, 2010; Imperi *et al*., 2013). The goal of an anti-virulence drug is to disarm bacteria from virulence factors reducing their adaptability to the host environment, and therefore facilitating the host immune system to clear the infections. For bacteria, the first step for the instauration of an infection is sensing the environment once in the host and, consequently, respond with the activation of virulence traits. Among the possible anti-virulence strategies, targeting the signalling processes may specifically interfere with the ability of bacteria to recognize the site of infection and to consequently activate specific virulence traits that are needed to establish the infection (Rasko and Sperandio, 2010).

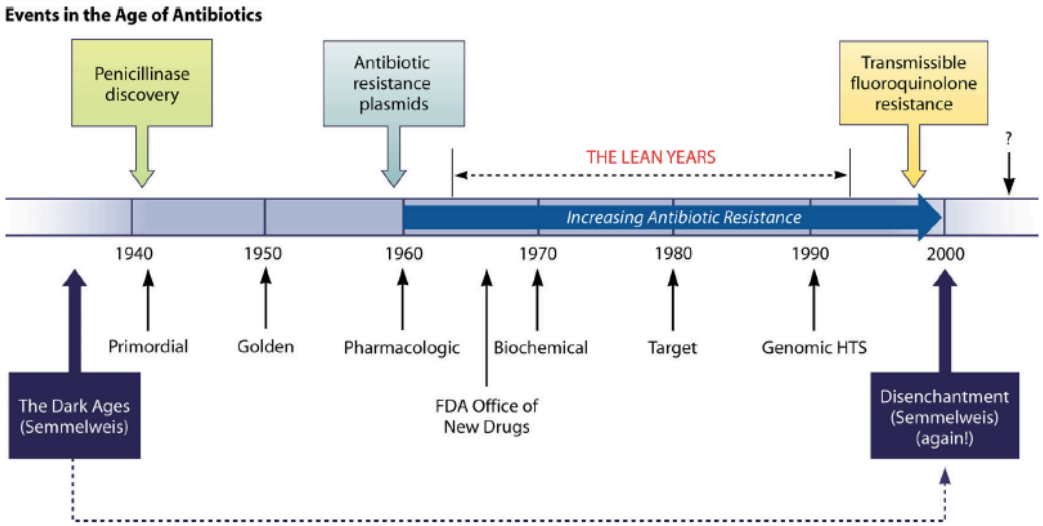

Fig.7. History of antibiotic discovery and concomitant development of antibiotic resistance. The dark ages, the preantibiotic era; primordial, the advent of chemotherapy *via* the sulfonamides; golden, the halcyon years when most of the antibiotics used today were discovered; the lean years, the low point of new antibiotic discovery and development; pharmacologic, attempts were made to understand and improve the use of antibiotics by dosing, administration, etc.; biochemical, knowledge of the biochemical actions of antibiotics and resistance mechanisms led to chemical modification studies to avoid resistance; target, mode-of-action and genetic studies led to efforts to design new compounds; genomic/HTS, genome sequencing methodology was used to predict essential targets for incorporation into high-throughput screening assays; disenchantment, with the failure of the enormous investment in genome-based methods, many companies discontinued their discovery programs. Other milestones in this history include the creation of the FDA Office of New Drugs after the thalidomide disaster led to stricter requirements for drug safety, including the use of antibiotics. This slowed the registration of novel compounds. Before antibiotics were discovered, Semmelweis advocated hand washing as a way of avoiding infection; this practice is now strongly recommended as a method to prevent transmission. Modified from Davies and Davies, 2010.

The anti-virulence strategy targeting signalling processes is particularly promising for bacteria whose infections involve a large number of virulence factors, such as *P. aeruginosa.* Indeed, *P. aeruginosa* is resistant to most antibiotic therapies, and its pathogenicity relies on the expression of an arsenal of virulence factors; for this reason, a useful anti-virulence drug should target the regulatory mechanisms governing the expression of multiple virulence traits, rather than a single virulence factor. In particular, since QS in *P. aeruginosa* controls virulence factors production and biofilm formation, this signalling system is considered a good candidate for the development of effective anti-virulence drugs against this bacterium (Cegelski *et al*., 2008; Rasko and Sperandio, 2010).

As described in the introduction, *P. aeruginosa* has four QS systems that are responsible for the signalling involved in the activation and maintenance of virulence factors production during the

infections: the *las*, the *rhl*, the *pqs* and the IQS systems. In rich medium, these systems are hierarchically organized with the *las* system at the top of the hierarchy (Lee and Zhang, 2015). Anti-virulence strategies against *P. aeruginosa* QS systems, in particular targeting the *las* QS system, have been already investigated; indeed the anthelminthic drug niclosamide has been recently repurposed for its QS inhibitory activity (Imperi *et al*., 2013), and a further optimization of this molecule for drug administration *via* aerosol will pave the way for it use in humans (Costabile *et al*., Submitted to Molecular Pharmaceutics. Supplementary material).

It has been suggested that not only genes involved in the synthesis and perception of signal molecules are feasible targets for anti-virulence drugs, but also their regulators (Hirakawa and Tomita, 2013). The *las* QS system, besides sensing the cell density, is embedded in a complex regulatory network that responds to different environmental cues (*e.g.*, temperature, pH, osmolarity, oxidative stress, nutrient starvation). This complexity allows to fine-tune *las* system expression to determine the optimal survival strategy (Williams *et al*., 2007). However, the proteins involved in this regulation and their mechanisms of action are largely unknown (Venturi, 2006; Williams and Camara, 2009).

In this work (Chapter I) a new direct repressor of *lasR* has been identified, the protein PA3699. EMSA experiments demonstrated the specific binding of PA3699 to the *lasR* promoter region (P*lasR*), and the overexpression of PA3699 strongly repressed P*lasR* transcription and the expression of LasR-dependent virulence phenotypes, such as $3OC_{12}$-HSL, elastase, proteases and pyocyanin production. However, as for other regulators of QS, the environmental stimulus activating PA3699 has not been identified. Despite PA3699 overexpression represses P*lasR* activity, a mutation in the gene coding for PA3699 does not have any evident effect in the cultural condition tested. A possible explanation for this discrepancy is that PA3699 is not sufficiently expressed of active in our experimental settings to exert its repressive role. This hypothesis is supported by the observation that PA3699 belongs to the TetR family of transcriptional regulators, which are usually activated upon binding of a specific ligand to an allosteric site. Thus, it is likely that, when expressed at a physiological level, PA3699 regulates *lasR* transcription only in the presence of a specific still unknown ligand (Longo *et al*., 2013).

The identification of PA3699 as a new direct repressor of *lasR* represent a further step in the understanding the intricate network of regulators and signalling systems that modulate QS activity in *P. aeruginosa*, and hopefully will lead to the identification of new promising targets for future drug-research programmes. Further studies aiming at the identification of the physiological conditions in which PA3699 exerts its repressive action on *lasR* might shed light on the complete pathway that connects metabolic and environmental cues to the downstream LasR-mediated

phenotypes, and might also extend our knowledge on the environmental conditions that *P. aeruginosa* is able to face by modulating the QS response.

As described in the introduction, the complex regulatory network in which the *las* system is embedded includes also three post-translational regulators of LasR, QteE, QslA and QscR, that are required for avoiding premature activation of the QS response before the "quorum" cell density is reached (Chugani *et al*., 2001; Sienhel *et al.*, 2010; Seet *et al.*, 2011). In this context, it is interesting to mention that one of the most recognised hypothesis to explain the importance of QS during the infection process is that QS allows bacteria to restrain the production of immunogenic virulence factors until enough bacterial cells amass to overcome the immune system response (Fuqua *et al.*, 1994; Sienhel *et al.*, 2010). However, the effect of the anticipation of QS response on *P. aeruginosa* ability to establish infections has been poorly investigated *in vivo* so far.

To clarify this issue (Chapter II), we characterized the *qteE* mutant for its ability to anticipate the production of $3OC_{12}$-HSL and of major QS-controlled virulence phenotypes such as elastase, proteases and pyocyanin production. Moreover, the effect of the *qteE* mutation has been investigated in a murine model of infection that allows evaluating the ability of *P. aeruginosa* to establish both the acute and the chronic infection. Despite the *qteE* mutation anticipates the QS response in test tubes, it does not have statistically significant effects on the ability of *P. aeruginosa* to establish both the acute, and the chronic infection, at least in this infection model (Bondì *et al*., 2014).

This result is quite surprisingly considering that the *las* system has at least three regulators involved in the pre-quorum repression suggesting that the timing of the QS response is important for *P. aeruginosa*. However, it is possible that our infection model is not appropriate for discriminating this fine-tuning, or that, since *P. aeruginosa* is an opportunistic pathogen, the timing of QS response is not important during the infection processes in mammals, while it could be relevant for *P. aeruginosa* adaptability in a different ecological niche (Brown *et al.*, 2012). In any case, in the absence of other evidences demonstrating that a dysregulation in the timing of the QS response confers an advantage *in vivo*, our results strongly suggest that a premature QS activation is not detrimental for *P. aeruginosa* pathogenic potential. Moreover, our data indicate that regulators controlling the timing of QS response are not suitable targets for the development of anti-virulence drugs.

This thesis also deals with the repressor of the *las* QS system RsaL, whose regulative function and its implications during the infections have been discussed in both Chapter II and III.

RsaL counteracts the positive feedback loop generated by the LasR/$3OC_{12}$-HSL on *lasI* transcription, thus limiting of $3OC_{12}$-HSL accumulation in the post-quorum phase of growth

(Rampioni *et al.,* 2007; Fig. 4). The arrangement of genes in the *las* QS system resemble the genetic structure of the type-1 incoherent feed forward loop (IFFL-1), a network motif that is conserved from bacteria to humans, and which confers peculiar properties to the expression of the genes that it regulates (output genes), including pulse generation, acceleration of the response time, fold-change detection and others (Alon, 2007; Bondì *et al.,* in preparation).

In Chapter III of this PhD thesis we demonstrated that the output gene *lasI*, as well as other outputs genes controlled by the LasR-RsaL IFFL-1 are robust with respect to LasR variations, and that this is due to the RsaL repressor. Indeed, since LasR itself controls RsaL levels, a decreased *lasI* expression due to lower LasR levels are counterbalanced by a concomitant decreasing repression of *lasI* due to lower RsaL levels. Accordingly, in a *P. aeruginosa rsaL* mutant strain robustness of the output phenotypes is lost, and their expression level become sensitive to LasR variations.

Interestingly, phenotypes like $3OC_{12}$-HSL and pyocyanin production that are controlled by the LasR-RsaL ILLF-1, and which expression is consequently robust with respect to LasR fluctuations, are involved in the chronic infection process, as suggested by the presence of $3OC_{12}$-HSL and pyocyanin in sputa of CF patients with *P. aeruginosa* chronic lung infection (Winstanley and Fothergill, 2009). Conversely, other phenotypes activated by LasR, but not repressed by RsaL and associated with the acute infection, such as elastase and proteases production, are not controlled by the LasR-RsaL IFFL-1. As a consequence, these phenotypes are sensitive to LasR variations, and this might indicate the need to rapidly alter their expression level during the progression of acute infections.

Overall, our results lead to the conclusion that the LasR-regulon can be divided into two sub-regulons, one that is robust and the other that is sensitive to variations in the intracellular levels of LasR. If considering that the *las* QS system is involved in the positive regulation of both acute and chronic infections, and that these infection strategies are characterized by different expression levels of virulence factors (Smith *et al*., 2004; Furukawa *et al*., 2006), it is tempting to speculate that the division of the LasR regulon into two sub-regulons caused by the LasR-RsaL IFFL-1 could act as a switch in the "choice" of *P. aeruginosa* to establish the acute or the chronic infection, depending on environmental signals affecting LasR expression. As an example, an external stimulus causing a decrease in LasR level might affect the expression of phenotypes that are only activated by LasR and that are required for acute virulence, while the same level of LasR could be sufficient to maintain unaltered the expression of phenotypes that are controlled by both LasR and RsaL, and that are required for chronic virulence. This hypothesis is supported by the following evidences:

- a mutant defective in Vfr, that is the activator of LasR, strongly affects the transcription of *lasR* and also the levels of LasR in the cells and the acute virulence phenotypes (Albus *et al.*, 1997; Bondì *et al.*, in preparation); therefore a *vfr* mutation can be considered as an insult leading to a reduction in the levels of LasR. A *vfr* mutant, in which the levels of LasR are reduced, is less virulent with respect to the wild type strain in a murine model of acute pneumonia, suggesting that a mutation in Vfr, affects the acute virulence phenotypes enabling *P. aeruginosa* to establish the acute infection (Smith *et al*., 2004);
- The disruption of the *las* IFFL-1 by mutating *rsaL*, also disrupts the robustness of the output genes with respect to LasR variations (Bondì *et al.*, in preparation; Chapter III). Disrupting the robustness conferred by RsaL, negatively affects *P. aeruginosa* biofilm formation and, notably, decreases *P. aeruginosa* ability to establish a chronic infection (Rampioni *et al.*, 2009; Bondì *et al*., 2014; Chapter II). This suggests that upon the disruption of the *las* IFFL-1 the chronic phenotypes become sensitive to environmental changes affecting LasR, and that consequently they are not maintained within the levels necessary to establish a chronic infection.

  In the evolution of the *P. aeruginosa* chronic infection in CF lung, it has been reported the emergence of mutants in *lasI* and *lasR*, while there is a positive selection for cells expressing high levels of RsaL (Son *et al.*, 2007; Starkey *et al.*, 2009); conversely, mutations in *rsaL* are not commonly isolated in CF clinical samples, and this reinforces the hypothesis that its presence is critical for the instauration and maintenance of the virulence phenotypes. However, in a *lasR* mutant also RsaL function is missing, and this suggest that a mutation in the *rsaL* gene can be tolerated only when associated to the lack of expression of the entire LasR regulon.

  On the whole this PhD thesis contributes to fill in the gap of knowledge on the *las* system regulatory network and its relevance also relies in the possibility that all the information provided could be used for the development of anti-virulence drugs. Indeed, targeting a bacterial process such as the *las* QS system that is embedded in an intricate regulatory network, require a deep knowledge of the processes to target. For this reason, the knowledge of the mechanisms of regulation of the *las* system and of their importance for pathogenesis goes in parallel with its application in the drug research programs.

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