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MicroReview

The virtue of temperance: built-in negative regulators of quorum sensing in *Pseudomonas*

Vittorio Venturi,[1]* Giordano Rampioni,[2] Sándor Pongor[1] and Livia Leoni[2]**
[1]*International Centre for Genetic Engineering and Biotechnology, Padriciano, 99, 34012 Trieste, Italy.*
[2]*Department of Biology, University Roma Tre, Viale Marconi, 446, 00146 Rome, Italy.*

Summary

Many bacteria are now believed to produce small signal molecules in order to communicate in a process called quorum sensing (QS), which mediates cooperative traits and a co-ordinated behaviour. Pseudomonads have been extensively studied for their QS response highlighting that it plays a major role in determining their lifestyle. The main QS signal molecules produced by *Pseudomonas* belong to the family of *N*-acyl-homoserine lactones (AHLs); these are synthesized by a LuxI-family synthase and sensed by a LuxR-family regulator. Most often in *Pseudomonas*, repressor genes intergenically located between *luxI* and *luxR* form an integral part of QS system. Recent studies have highlighted an important role of these repressors (called RsaL and RsaM) in containing the QS response within cost-effective levels; this is central for pseudomonads as they have very versatile genomes allowing them to live in constantly changing and highly dynamic environments. This review focuses on the role played by RsaL and RsaM repressors and discusses the important implications of this control of the QS response.

Introduction

The notion that bacteria co-ordinate their biological functions as members of organized communities introduced the concept of social behaviour in the microbial world, leading to the emergence of sociomicrobiology as a new research topic (Parsek and Greenberg, 2005). The major breakthrough in this field was the discovery that bacteria can communicate, exchanging information aimed at understanding and defining their population structure and dynamics. The best studied example of bacterial social interaction is probably quorum sensing (QS). QS is a communication system that is based on the production, secretion and perception of small signal molecules, allowing a bacterial population to co-ordinate group behaviours as a function of cell density (Fuqua *et al.*, 1994; Bassler, 2002). This model is possibly valid for bacterial species living in homogeneous and stable environments in which QS controls one or few bacterial functions. It is however rather simplistic for bacterial species that experience highly dynamic environments, with a complex and constantly fluctuating chemical, physical and biological surroundings. This selects for bacteria having elastic regulatory networks which allow rapid, reversible and finely tuned responses; these properties can be particularly important for QS systems that affect multiple bacterial phenotypes through major reprogramming of gene expression. This is becoming evident in the QS communication systems of bacteria belonging to the genus *Pseudomonas*. Pseudomonads are Gram-negative bacteria mainly studied for their ability to colonize many different environments, like soil, water, plants and animals, including humans. This broad adaptability is related to their versatile metabolic potential and their ability to finely control gene expression via regulatory elements highly represented in their large genomes. The opportunistic human pathogen *Pseudomonas aeruginosa* is a notorious member of this genus and its QS system is thus far one of the best characterized (Smith and Iglewski, 2003; Schuster and Greenberg, 2006).

Quorum sensing in *P. aeruginosa* is complex, consisting of three interconnected regulatory systems (Smith and Iglewski, 2003; Schuster and Greenberg, 2006; Venturi, 2006; Dubern and Diggle, 2008). The LasI/LasR and RhlI/RhlR QS systems are based on the production and detection of *N*-acyl homoserine lactones (AHLs), the most common signal molecules in Gram-negative bacteria (Fuqua *et al.*, 2001). LasI and RhlI are the synthases

Accepted 13 October, 2011. For correspondence. *E-mail venturi@icgeb.org; Tel. (+39) 040 3757319; Fax (+39) 040 226555; **E-mail leoni@uniroma3.it; Tel. (+39) 06 57336318; Fax (+39) 06 55176321.

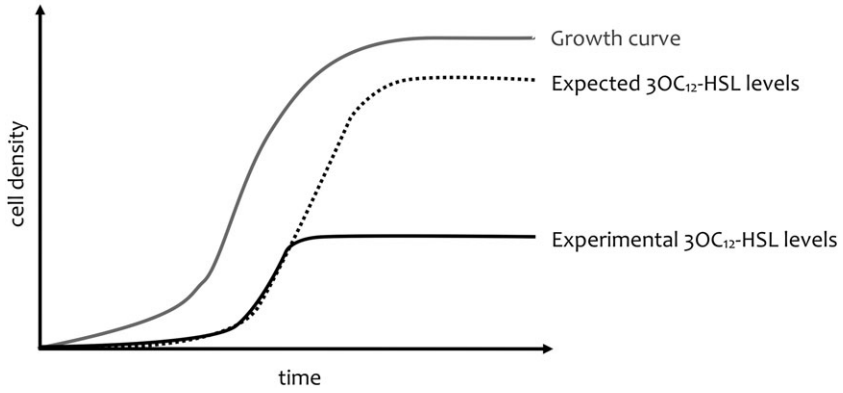

Fig. 1. Schematic representation of $3OC_{12}$-HSL production in *P. aeruginosa* PAO1 during growth (grey line). The levels of signal molecule experimentally measured reach a steady level during exponential growth phase (black solid line), but the expected levels should continue to rise till stationary phase (black dotted line).

which belong to the LuxI-family of genes/proteins and produce the signal molecules *N*-3-oxo-dodecanoyl-homoserine-lactone ($3OC_{12}$-HSL) and *N*-butanoyl-homoserine-lactone (C_4-HSL) respectively. At critical or 'quorum' concentration, $3OC_{12}$-HSL and C_4-HSL bind to the sensors/receptors LasR and RhlR (these belong to the LuxR-family of genes/proteins) respectively, activating them and leading to the regulation of target genes expression. In bacterial AHL QS systems the *luxI* and *luxR* cognate genes are almost always found genetically linked in the genome (Fuqua *et al*., 2001). The LasR/$3OC_{12}$-HSL and RhlR/C_4-HSL complexes also induce transcription of their cognate AHL synthase genes, creating a positive feedback loop that amplifies the production of the signal molecules. A similar regulatory scheme is also found in the third QS system of *P. aeruginosa*, in which the 2-alkyl-4(1*H*)-quinolones HHQ and PQS bind and activate the sensor/receptor PqsR (Dubern and Diggle, 2008). In summary, the three QS systems extensively reprogram the *P. aeruginosa* transcriptome, regulating metabolism and important virulence-related processes, including production of secreted virulence factors, motility and biofilm formation (Adhikari *et al*., 1995; Schuster and Greenberg, 2006; Williams and Camara, 2009 and references therein).

Importantly, QS in *P. aeruginosa* does not rely only on cell density. For example, the QS-response for most genes cannot be anticipated under several environmental conditions by early exposure of the bacterial cells to high levels of LuxR-family proteins and to the 'quorum' concentration of signal molecule (Whiteley *et al*., 1999; Schuster and Greenberg, 2007). Moreover, a plethora of transcriptional and post-transcriptional regulators has been shown to affect the timing and extent of QS gene expression, likely integrating cell density dependency of QS to other environmental signals. However, the biological relevance, the extent and possible direct or indirect regulation of AHL QS genes/proteins by many of these elements remains far from clear. The complicated network of regulators affecting QS in *P. aeruginosa* has been the subject of several recent reviews (Juhas *et al*., 2005; Schuster and Greenberg, 2006; Venturi, 2006; Williams and Camara, 2009).

Two important regulators affecting the AHL-based QS systems in pseudomonads are the RsaL and RsaM repressors; interestingly these are genetically linked to the signal synthase and signal receptor genes. These regulators are essential to restrain the QS response within physiological profitable levels, contrasting the positive feedback loop that leads to signal molecule accumulation. If QS is important to exploit environmental resources, its control is key to avoid energy waste and ensure the optimal adaptive response. In this context the repressive role exerted by RsaL and RsaM might confer temperance to the QS systems in which they are encased, a virtue fundamental in social behaviour. This MicroReview intends to review and discuss current knowledge of these two repressors, which in our view stand out and merit special attention.

RsaL, a regulator of QS homeostasis in *P. aeruginosa*

The amplification of signal production via the positive feedback loop in *Vibrio fischeri* provides hysteresis to QS-dependent gene expression; once a critical concentration of signal is reached, signal production per cell increases, and thus small decreases in population density do not reverse the QS response (Williams *et al*., 2008). Interestingly, in *P. aeruginosa* PAO1, levels of $3OC_{12}$-HSL attain a steady state level in the range of a few μM prior to the onset of stationary phase, despite the fact that expression of the $3OC_{12}$-HSL synthase gene, *lasI*, is autoregulated by the LasR/$3OC_{12}$-HSL-dependent positive feedback (Fig. 1). This implies that there is another regulatory element that balances the positive autoregulation to provide $3OC_{12}$-HSL homeostasis (Ward *et al*., 2004).

The first study on *rsaL* dates back to 1999 when Iglewski and co-workers identified an ORF encoding a predicted protein of only 80 amino acids in the intergenic region between the *lasR* and *lasI* genes of *P. aeruginosa*

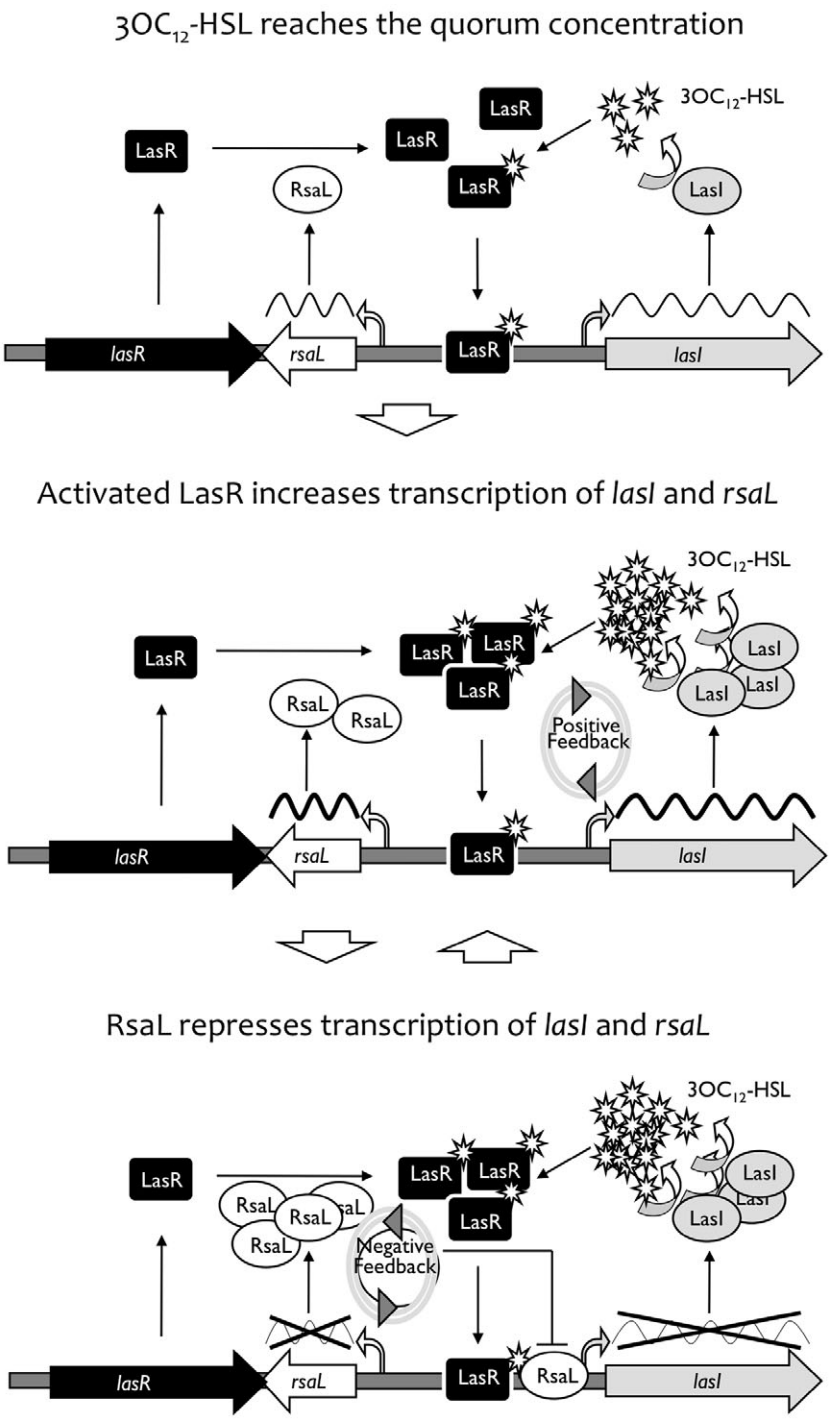

Fig. 2. Schematic model of the molecular mechanism underlying QS homeostasis in *P. aeruginosa* PAO1. The interplay of the positive feedback loop generated by LasR and the negative feedback loop generated by RsaL leads to steady level of $3OC_{12}$-HSL before entry in stationary phase of growth. Modified from Rampioni *et al.* (2007a).

PAO1 (Fig. 2). Since the overexpression of this ORF resulted in a strong reduction of $3OC_{12}$-HSL production, the authors could foresee the importance of this negative regulator as an antagonist of LasR, and named this newly identified gene *rsaL,* from the inverted spelling of *lasR* (de Kievit *et al.*, 1999).

The demonstration that RsaL is the regulator responsible for $3OC_{12}$-HSL homeostasis in *P. aeruginosa* was provided a few years later since levels of $3OC_{12}$-HSL in *rsaL* mutant cultures were seen to continue to rise in late logarithmic and stationary phase to a level about 10-fold higher than in wild-type cultures (Rampioni *et al.*, 2007a).

The divergent orientation of the adjacent *rsaL* and *lasI* genes plays a key role in the $3OC_{12}$-HSL homeostasis mechanism depicted in Fig. 2; LasR triggers the transcription of *rsaL* and *lasI*, while RsaL represses the transcrip-

tion of both genes, by binding to their respective target sites on the *rsaL-lasI* bidirectional promoter. Despite the RsaL binding site on P*lasI* being very close to the *las*-box recognized by LasR on the same promoter, the two proteins can bind their target DNA sequence simultaneously, and the binding of one protein does not alter the affinity of the other (Schuster *et al*., 2004; Rampioni *et al*., 2007a). However, when both proteins are simultaneously engaged on the promoter, the RsaL repressive effect is dominant (Rampioni *et al*., 2007a). The dependency of *rsaL* transcription upon the LasR/3OC_{12}-HSL complex generates a negative feedback loop, which counteracts the positive feedback loop mediated by LasR/3OC_{12}-HSL for QS signal molecule production. Moreover, autoregulation of RsaL expression ensures the maintenance of this negative regulator within confined levels.

Homeostasis most probably allows a population of *P. aeruginosa* PAO1 cells to maintain 3OC_{12}-HSL at appropriate levels in a given environment and to change steady-state levels of 3OC_{12}-HSL as conditions change. Other mechanisms can also participate in controlling 3OC_{12}-HSL homeostasis, as for example signal-molecule-degrading enzymes and import-export pumps (Kohler *et al*., 2001; Aendekerk *et al*., 2002; Huang *et al*., 2006; Sio *et al*., 2006).

The observation that in *P. aeruginosa* PAO1 3OC_{12}-HSL reaches a plateau concentration during the logarithmic phase of growth raises the question of how this bacterium can sense further increases in cell density. Considering that the levels of the QS signal molecule C_4-HSL continue to increase during late-logarithmic growth (Chugani *et al*., 2001; Kohler *et al*., 2001), it is reasonable to assume that the expression of QS genes controlled by both 3OC_{12}-HSL and C_4-HSL can be connected to cell density through the first signal molecule till a certain point of the growth curve, and through C_4-HSL at higher cell density.

RsaL, a global regulator of gene expression and virulence in *P. aeruginosa*

The determination of the RsaL regulon showed that it comprises over 200 genes, half of which are also QS-controlled (Rampioni *et al*., 2009). The reason for this overlap is not surprising, since RsaL itself is QS regulated and the *rsaL* mutant produces significant higher levels of 3OC_{12}-HSL than the parental strain (Rampioni *et al*., 2007a). Additional transcriptomic studies, which uncoupled the regulatory relationship between RsaL and QS, showed that RsaL controls 130 genes independently from its effect on 3OC_{12}-HSL levels. Some of these genes are important in pathogenicity, such as those involved in pyocyanin and hydrogen cyanide production; RsaL was shown to directly bind the promoter region of pyocyanin and hydrogen cyanide genes *in vitro* (Rampioni *et al*., 2009). Considering that pyocyanin and hydrogen cyanide genes respond to both 3OC_{12}-HSL and C_4-HSL (Pessi and Haas, 2000; Whiteley and Greenberg, 2001), the direct repression exerted by RsaL on these genes could reflect the need for keeping the concentration of their products at a steady level even if the population density continues to rise. The overproduction of virulence factors could be in some circumstances counterproductive, for instance by eliciting an effective host immune response. Moreover, at high concentrations hydrogen cyanide and pyocyanin could be toxic for *P. aeruginosa* itself.

More than 90% of the genes controlled by RsaL are repressed, including six genes encoding putative or confirmed transcriptional factors, thus many genes could be controlled by RsaL indirectly through auxiliary regulators (Rampioni *et al*., 2007a). RsaL therefore negatively controls the expression of many genes through different and not mutually exclusive mechanisms: i.e. (i) repression of 3OC_{12}-HSL signal molecule production, (ii) direct binding of target genes, and (iii) indirect control of some genes via auxiliary regulators.

The broad range of RsaL functions expands the QS regulon and increases its complexity, indicating that it could have a strong impact on *P. aeruginosa* PAO1 physiology. Indeed, the *rsaL* mutant displays enhancement of surface motility (twitching and swarming) and of secreted virulence factors production (i.e. elastase, haemolysins and hydrogen cyanide). In accordance, this mutant is also hypervirulent in an insect virulence model, indicating that RsaL is a repressor of *P. aeruginosa* PAO1 virulence, at least in this experimental model of acute infection (Rampioni *et al*., 2009). Interestingly, the *rsaL* mutant produces less biofilm than the wild-type strain and displays enhanced antibiotic susceptibility to many antibiotics, with respect to the wild-type (Rampioni *et al*., 2009).

A *P. aeruginosa* population can adopt distinct behaviours in acute and chronic infections. An acute infection is rapid, systemic, and likely to be carried out by a planktonic (motile) bacterial community, requiring high-level expression of virulence factors (Furukawa *et al*., 2006). It is believed that in this kind of infection, QS could be important to allow the massive production of virulence factors when the bacterial community has reached a sufficient density to overcome the host immune system (Smith and Iglewski, 2003). The chronic infection is usually localized in a specific host tissue; bacteria adopt a slow-growing sessile life style (biofilm), they show high intrinsic resistance to antibiotics and can persist in the host for extended periods of time. In addition they produce limited amounts of virulence factors, despite high cell densities, in order to avoid and resist the host immune system and prolonged antibiotic therapies (Furukawa *et al*., 2006). The phenotype of the *rsaL* mutant suggests it could play a positive role in chronic infections. In agreement with this

hypothesis, *rsaL* expression was found to be constitutively upregulated in *P. aeruginosa* isolated from chronically infected cystic fibrosis (CF) lungs, suggesting that during the adaptation of *P. aeruginosa* to the CF lung there is a positive selection for cells expressing high levels of RsaL (Son *et al*., 2007).

Pseudomonas aeruginosa PUPa3 is a plant growth-promoting rice rhizosphere isolate. In this strain the *las* QS system is important for rice root colonization, for full virulence in a nematode model of infection, for swimming motility and production of secreted factors. The *rsaL* mutant of PUPa3 produces very high levels of the QS signal molecule $3OC_{12}$-HSL, 100 times more than the wild-type strain, indicating that RsaL in PUPa3 is more effective in repressing *lasI* gene expression than RsaL in PAO1 ($3OC_{12}$-HSL levels are 10 times higher in the PAO1 *rsaL* mutant than in the wild-type strain). Interestingly, despite the role of RsaL as a negative regulator of QS, the *rsaL* and *lasI* mutants are both impaired in root colonization, a process related to the ability to form biofilm (Steindler *et al*., 2009). This observation suggests that QS is required for root colonization, but excess production of QS signal molecules might be counterproductive. A similar phenomenon was observed in *P. aeruginosa* PAO1, where biofilm formation was negatively affected both in a *lasI* and in an *rsaL* mutant (Davies *et al*., 1998; Rampioni *et al*., 2009). Therefore, also in the environmental rizhosphere isolate *P. aeruginosa* PUPa3, RsaL-mediated restraint of $3OC_{12}$-HSL production seems to play a central role in biofilm-dependent host colonization.

Differences in RsaL mechanism in *P. putida* and *P. aeruginosa*

A QS locus highly homologous to the *las* system of *P. aeruginosa* has been characterized also in the *Pseudomonas putida* strains PCL1445, IsoF and WCS358, and designated *ppuR*/*rsaL*/*ppuI* (Steidle *et al*., 2002; Bertani and Venturi, 2004; Dubern *et al*., 2006). In these plant growth-promoting rhizobacteria QS positively regulates processes important for root colonization, such as biofilm formation (Steidle *et al*., 2002; Dubern *et al*., 2006; G. Rampioni *et al*., unpublished) and siderophore production (G. Rampioni *et al*., unpublished). Unfortunately, no *rsaL* mutant has been constructed in the IsoF strain, and consequently no role for RsaL with respect to QS can be hypothesized (Steidle *et al*., 2002). In *P. putida* PCL1445 RsaL strongly represses signal molecule production and is involved in the regulation of biosurfactant and biofilm production; however, its precise mechanism of action is currently unknown (Dubern *et al*., 2006). Conversely, RsaL has been extensively studied in *P. putida* WCS358. As in *P. aeruginosa*, also in this system the LasR-homologue PpuR triggers the transcription of the $3OC_{12}$-HSL synthase gene *ppuI*, generating a positive feedback loop, while RsaL ($RsaL_{WCS}$) strongly represses *ppuI* transcription and $3OC_{12}$-HSL production (Bertani and Venturi, 2004). The mechanism of action of RsaL in repressing the transcription of the synthase genes *ppuI* and *lasI* seemed to be conserved in *P. putida* WCS358 with respect to *P. aeruginosa* PAO1, since in both strains RsaL was shown to bind *in vitro* the cognate promoter (P*ppuI* or P*lasI*) on a region overlapping the −10 consensus sequence for sigma 70 (Rampioni *et al*., 2006; G. Rampioni *et al*., unpublished). The comparative analysis of RsaL from *P. putida* WCS358 and *P. aeruginosa* discloses new interesting properties of this regulator.

In *P. aeruginosa* PAO1 the $RsaL_{PAO}$ binding site on P*lasI* is a palindromic motif that allows $RsaL_{PAO}$ dimerization on the target promoter (Rampioni *et al*., 2007b). This motif is absent in the $RsaL_{WCS}$ binding site on P*ppuI*, that contains only an emi-palindromic site conserved with respect to the $RsaL_{PAO}$ binding site on P*lasI*. This difference results in the inability of purified $RsaL_{WCS}$ to form stable protein/DNA complexes with a P*ppuI* probe *in vitro*, while the same protein can bind the P*lasI* promoter. Moreover, $RsaL_{WCS}$ has a strong repressing effect on *ppuI* transcription in *P. putida* WCS358, while it does not repress the same promoter in the heterologous host *P. aeruginosa* PAO1 (G. Rampioni *et al*., unpublished). These interesting observations suggest that, while $RsaL_{PAO}$ represses *lasI* transcription as a homodimer, $RsaL_{WCS}$ most likely needs a molecular interactor/cofactor specific to *P. putida* WCS358 to repress *ppuI* transcription. An *in silico* modelling performed to determine $RsaL_{PAO}$ and $RsaL_{WCS}$ structure supports this hypothesis. This analysis revealed that the overall structure of the RsaL proteins is very similar to the *N*-terminal domain of the lambda cI repressor (λcI-NTD) and to the POU specific domain of the mammalian transcription factor Oct-1 (Oct-1 POUs), and clustered the RsaL proteins in the tetra-helical superclass of helix–turn–helix proteins (Rampioni *et al*., 2007b). λcI-NTD binds to a palindromic sequence of DNA as a homodimer, and dimer formation in this protein is driven by the α-helix 5 (Jain *et al*., 2004). This α-helix is absent in Oct-1 POUs, a protein domain that binds to DNA in association with the POU homeodomain (Phillips and Luisi, 2000). Therefore, the observation that the α-helix 5 is present also in $RsaL_{PAO}$, while this structural element is lacking in $RsaL_{WCS}$ (Rampioni *et al*., 2007b), supports the idea that $RsaL_{PAO}$, similarly to λcI-NTD, binds to a palindromic DNA sequence as a homodimer, while $RsaL_{WCS}$ binding to DNA requires the association with another DNA-binding protein, similarly to Oct-1 POUs (Phillips and Luisi, 2000).

The evidence that QS in *P. putida* WCS358 is repressed by $RsaL_{WCS}$ in association with another factor raises the possibility that the cell density-dependent regulation of QS in this bacterium is integrated with other

environmental cues at the level of RsaL. In this context, it is important to consider that in PAO1 the expression of $RsaL_{PAO}$ is dependent on LasR, while in WCS358 the transcriptional regulator PpuR does not affect $RsaL_{WCS}$ expression (Bertani and Venturi, 2004; Rampioni *et al*., 2007a). While in *P. aeruginosa* PAO1 the levels of $RsaL_{PAO}$ are cell density dependent, and $RsaL_{PAO}$ alone is sufficient to repress signal molecule production, in *P. putida* WCS358 $RsaL_{WCS}$ and its putative interactor might be expressed or active only in defined conditions, not necessarily related to cell density. Therefore, the RsaL-mediated repression of QS that in *P. aeruginosa* PAO1 is an integral part of the QS circuit, in *P. putida* WCS358 could constitute a key element to connect the production of the QS signal molecule with other metabolic or external stimuli.

Despite the different regulation underlying the activation of *rsaL* transcription, it is significant that RsaL has a negative effect on its own expression in both *P. aeruginosa* PAO1 and *P. putida* WCS358 strains (Bertani and Venturi, 2004; Rampioni *et al*., 2007a). This feature could constitute an important control mechanism to limit RsaL accumulation, so to ensure the possibility of a quick switch from the QS-repressed to the QS-activated state in function of variable environmental conditions.

RsaL in *Burkholderia* and the curious case of *Pseudomonas fuscovaginae*

The RsaL protein has recently been reported in a *Burkholderia* species cluster which is associated to plants (Suarez-Moreno *et al*., 2008; 2010). This cluster contains 30 species, all are non-pathogenic and most of them can fix atmospheric nitrogen. One characteristic is that they share a QS system, called BraI/RsaL/BraR, which is highly conserved among all the species and is homologous to the LasI/RsaL/LasR of *P. aeruginosa* and PpuI/RsaL/PpuR of *P. putida*. In one species, namely *B. kururiensis*, *rsaL* has been inactivated, revealing that this mutant produces 2000 times more AHLs than the wild-type strain (Suarez-Moreno *et al*., 2008). This increase is far more of what observed for *P. aeruginosa* and *P. putida*, and raises the question on the role of RsaL in these species. Indeed, the potential of the *B. kururiensis rsaL* mutant to produce levels of QS signal molecule considerably higher than the wild-type strain suggests that defined environmental conditions (for example plant signals) can result in the exploitation of this potentiality by repressing RsaL expression or activity. It is also possible that in this strain QS signal molecules might have additional role apart from cell-density signalling.

Pseudomonas fuscovaginae is an important plant-pathogen possessing two AHL QS systems, both required for full pathogenicity in plant infection models. The PfvI-PfvR system is homologous to the LasI-LasR system of *P. aeruginosa* and produces and responds to $3OC_{10}$-HSL and $3OC_{12}$-HSL whereas the PfsI-PsfR system is homologous to the BviI-BviR system of *Burkholderia vietnamiensis* and produces and responds to C_{10}-HSL and C_{12}-HSL. PfvR can be activated by the signals produced by PfsI, while the AHLs synthesized by PfvI are not recognized by PfsR (Mattiuzzo *et al*., 2011). Therefore, the PfsI-PfsR system can positively influence the activity of the PfvI-PfvR system but not vice versa. In general, PfvR has broader signal specificity with respect to PfsR, indicating that it could also respond to AHLs produced by neighbouring bacteria of different species (Fig. 3).

Importantly, both *P. fuscovaginae* QS loci contain an additional gene located between the AHL synthase and the response regulator genes (Mattiuzzo *et al*., 2011). The gene located between *pfvI* and *pfvR* was named *rsaL,* because encodes for a protein that is homologous to *P. aeruginosa* PAO1 RsaL. Surprisingly, inactivation of *rsaL* in *P. fuscovaginae* does not result in increased *pfvI* transcription or AHL production under standard laboratory growth conditions, and has no effect on the pathogenic potential of *P. fuscovaginae* in plant-infection models. However, RsaL represses *pfvI* transcription when the entire system (i.e. PfvI, RsaL and PfvR) is expressed in *E. coli* in the presence of the AHLs recognized by PfvR (Mattiuzzo *et al*., 2011). The *pfvI* gene is poorly expressed under standard laboratory growth conditions and the notion that PfvR can respond to a wide range of AHLs leads to speculate that this system could be active in polymicrobial communities. In this view, the RsaL protein of *P. fuscovaginae* might have a role in 'adjusting the volume' of bacterial conversation in the environment. This interesting possibility deserves future investigations.

The real surprise and major novelty in the study by Mattiuzzo and co-workers was the discovery of the *rsaM* gene, located between *pfsI* and *pfsR*, and encoding a protein of 167 amino acids with no homology with functionally characterized proteins (Mattiuzzo *et al*., 2011). The *rsaM* mutant produced fourfold more C_{10}-HSL and displayed attenuation of virulence in a rice infection model, with respect to the wild-type. It is important to notice that C_{10}-HSL activates also the receptor of the other QS system PfvR, beside its cognate receptor PfsR. Moreover, RsaM represses the PfvR-dependent *pfvI* transcription in *E. coli* (Mattiuzzo *et al*., 2011), highlighting that *pfvI* is negatively regulated by both RsaL and RsaM. RsaM tightly controls the expression of the *pfv* system both directly and indirectly, through its repressive effect on C_{10}-HSL production (Fig. 3). This dual control could be particularly important in the presences of AHL produced by other bacteria in a polymicrobial community. Overall, RsaM appears to be the major temperance actor of the two AHL QS systems in *P. fuscovaginae*.

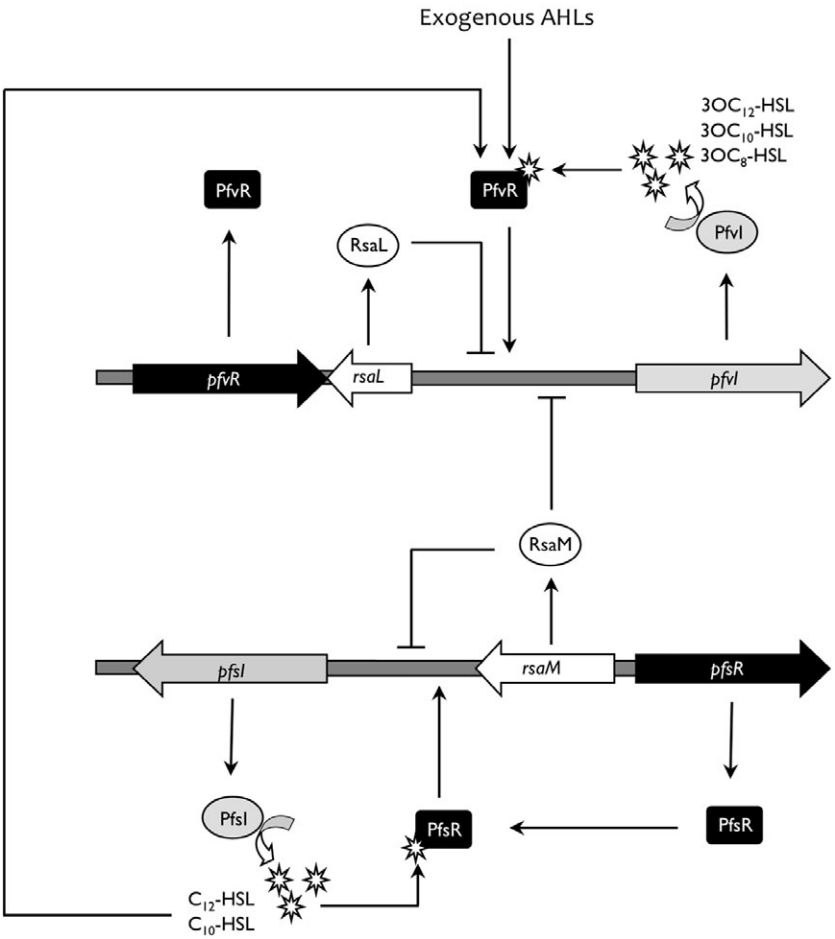

Fig. 3. Schematic model of the two AHL-QS systems of *P. fuscovaginae*. The PfvR receptor responds to the AHLs produced by the PfvI and PfsI synthases, while PfsR only responds to PfsI-produced AHLs. The response of PfvR to a wide range of long-chain AHLs at high concentration could indicate a response to exogenously AHLs produced by other bacteria. RsaL represses *pfvI* transcription, while RsaM represses both *pfvI* and *pfsI*. Modified from Mattiuzzo *et al.* (2011).

Since RsaM shares no homology with functionally characterized proteins, other studies will be required to understand its molecular mechanism of action. It is possible that RsaM belongs to a new class of transcription factors, and that it directly represses transcription of target genes, or it could repress PfvI and PfsI expression indirectly, for instance by inhibiting the functionality of the signal receptor required for their activation.

Distribution and synteny of *rsaL* and *rsaM* genes in bacterial genomes

It was of interest to determine how commonly found are *rsaL*- and *rsaM*-like genes in bacteria. In a previous analysis, 12 ORFs encoding homologues of the *P. aeruginosa* RsaL protein were found in sequence data banks. Among these, only the *rsaL*-like genes belonging to *P. aeruginosa, P. putida and Burkholderia xenovorans* were linked to a *luxI*/*luxR*-family QS locus (Rampioni *et al.*, 2007b). Here, we carried out a thorough updated analysis aimed at understanding the distribution of *rsaL*-like and *rsaM*-like genes in bacterial genomes, and how widespread is the presence of these genes within genetic loci encoding AHL-based QS systems.

Overall, 97 ORFs showing a significant level of homology with *P. aeruginosa* RsaL have been found; interestingly, some bacterial genomes contain more than one *rsaL*-like gene, for instance *Thauera* sp. MZ1T and *Laribacter hongkongensis* HLHK9 contain three and four *rsaL*-like paralogues respectively. In summary, we found at least one *rsaL*-like gene in 39 different bacterial species belonging to β, γ and δ divisions (Table S1).

The *rsaL* gene is found in 14 complete bacterial genomes that harbour *luxI*/*luxR*-family genes – we term these AHL-QS genomes – and 17 genomes that do not possess the AHL QS genes (non-AHL-QS genomes) (Table S1). In the AHL-QS genomes, the *rsaL* gene is almost exclusively found in the canonical arrangement already described in pseudomonads (Fig. 4A). Only two AHL-QS genomes, namely *Chromobacterium violaceum* and *Nitrosospira multiformis* are exceptions to this rule as they contain an *rsaL* gene, which is unlinked to the *luxI*/*luxR* pair (Fig. 4B). According to sequence similarity, the RsaL proteins encoded by genes located within the

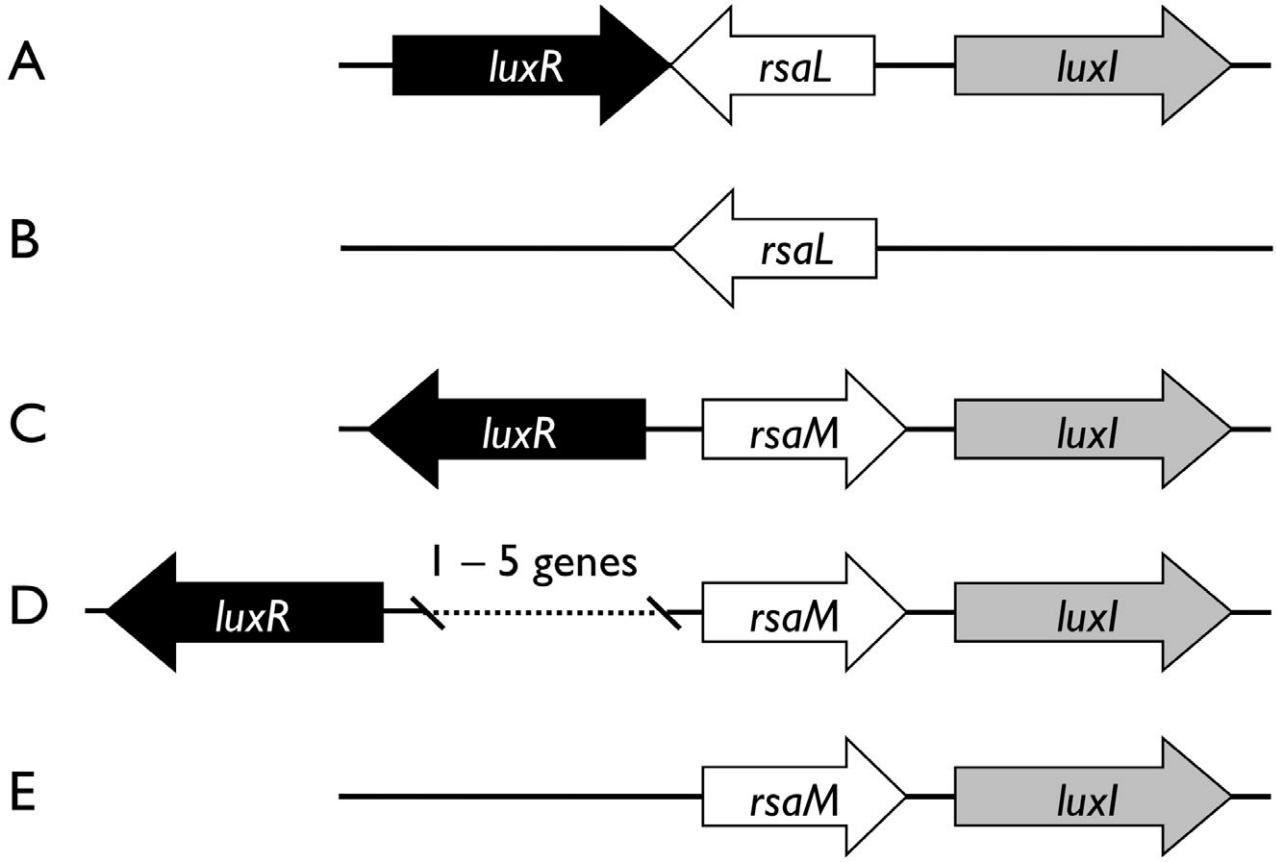

Fig. 4. Genetic organization of the *rsaL* and *rsaM* genes with respect to QS loci in sequenced genomes. In 14 genomes *rsaL* has been found in the canonical arrangement, intergenically located in between the *luxI*- and *luxR*-family genes (A), while in 17 genomes *rsaL* is localized independently from the AHL-QS system (B). In most part of the investigated genomes *rsaM* is found in the canonical arrangement, intergenically located in between the *luxI*- and *luxR*-family genes (C); in few genomes a second *rsaM* gene is present, but in these cases 1–5 other ORFs are also found intergenically located (D); in *B. ambifaria* MC40-6 and AMMD the *rsaM* gene is in tandem with the *luxI*-family gene, but no cognate *luxR*-family gene is in the vicinity (E).

luxI/luxR pair form a large cluster; in contrast, the RsaL proteins in the other genomes are more diverse (Fig. S1).

The *rsaM* gene is only found in genomes from β and γ proteobacteria containing AHL-QS systems (Fig. S2; Table S2). Eight of the 14 *rsaM*-containing species (represented by 30 genomes) belong to the *Burkholderia* genus. The *rsaM*-like gene is invariably found adjacent to the *luxI*-family gene and oriented in the same direction (Fig. 4C). *Burkholderia pseudomallei* and *Burkholderia thailandiensis* genomes have two AHL-QS-loci containing an *rsaM*-like gene. One locus harbours *rsaM* in the canonical arrangement, and the second one has 1–5 genes between the *luxR* and *rsaM* gene (Fig. 4D). Finally, *Burkholderia ambifaria* also has two AHL-QS-loci containing an *rsaM*-like gene. One rsaM gene is in the canonical arrangement and the second one lacks a *luxR*-family gene in the immediate genomic neighbourhood (Fig. 4E). Interestingly, in the *Burkholderia* genomes containing two AHL-QS loci, the RsaM proteins encoded by genes not immediately linked to a *luxR*-family gene form a separate cluster. This cluster is most closely related to the RsaM protein of *P. fuscovaginae* (Fig. S2). *P. fuscovaginae* represents so far the only case in which *rsaL* and *rsaM* are found within the same genome.

In conclusion, so far *rsaL*-like genes can be found either linked or un-linked to a *luxI*-family gene, in the first case the two genes are always divergently transcribed. The conservation of a divergent orientation of *rsaL*-like and *luxI*-family genes suggests that this genetic organization could play a functional role in RsaL-mediated regulation of AHL production. All the *rsaM*-like genes identified so far are adjacent to a *luxI*-like gene, invariantly in the same orientation. This suggests that although the mechanism of action of RsaM could differ from that of RsaL, this protein could play a major role in regulating AHL production in a number of β and γ proteobacteria. Interestingly, neither *rsaL*- or *rsaM*-like genes have been found so far in α-proteobacteria genomes.

Concluding remarks

Regulation via QS in *Pseudomonas* results in a major change of gene expression profile; it is therefore to be expected that bacteria have evolved mechanisms to adjust/control this response. In this MicroReview we have highlighted the important 'temperance' role that built-in negative regulators play in controlling this energetically very expensive commitment. In *P. aeruginosa,* RsaL is a fundamental part of a homeostatic circuit guarding the transcription of the AHL synthase. This mechanism maintains AHL production within optimal levels and provides an option for allowing its variation under certain environmental conditions.

RsaL is almost always present in *Pseudomonas* which harbour *luxI/R* genes, thus suggesting that in this genus, the presence of an AHL QS system lacking RsaL is counter-selected. From initial comparisons on the mode of action of RsaL in two *Pseudomonas* species, it is likely that its molecular mechanisms can differ from species to species. It is too early to soundly speculate on the reasons for these differences; further work is required in order to determine if RsaL plays different or more roles in *Pseudomonas* spp. In addition, the stringency of repression exerted by RsaL can also differ; again this could imply additional roles of RsaL and also of the AHL since in some cases *rsaL* mutants result in production of unusually very high AHL concentrations. Moreover, interesting questions are whether RsaL controls AHL homeostasis also in other QS systems and if its expression/activity is influenced by other environmental cues.

The recent finding of RsaM is evidence that built-in negative regulators could become a common scenario of AHL QS circuits. The current lack of similarity of RsaM to known proteins and of data on its mechanism of action makes it more difficult at this stage to conjecture on its precise role. From a first genetic study and since it is very commonly found within AHL QS systems in *Burkholderia* spp. (including opportunistic pathogens belonging to the *Burkholderia* cepacia complex), it is reasonable to speculate that RsaM is involved in QS homeostasis in *Burkholderia* genus. It is therefore likely that *Pseudomonas* spp. have evolved RsaL whereas *Burkholderia* RsaM as a built-in negative regulator of AHL QS circuits. Other bacterial genera or species could possess other types of negative regulators that are closely connected to the AHL QS systems. For example in *Agrobacterium tumefaciens*, a negative regulator called TraM binds to TraR (the LuxR family component of the AHL QS system), and prevents it from binding to target gene promoters thus modulating the QS response (Luo *et al*., 2000; Piper and Farrand, 2000; Qin *et al*., 2007). A negative regulator which also binds the two LuxR-family proteins has recently been found in *P. aeruginosa* and has been designated QteE; this regulator plays an important role in controlling LasR and RhlR levels ensuring the correct timing of the QS response (Siehnel *et al*., 2010). Studying these regulators that are an integral part of the AHL QS circuits is important in understanding the QS mechanism, which determines the fate of a bacterial population.

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References

Adhikari, T.B., Cruz, C., Zhang, Q., Nelson, R.J., Skinner, D.Z., Mew, T.W., and Leach, J.E. (1995) Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* in Asia. *Appl Environ Microbiol* **61:** 966–971.

Aendekerk, S., Ghysels, B., Cornelis, P., and Baysse, C. (2002) Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium. *Microbiology* **148:** 2371–2381.

Bassler, B.L. (2002) Small talk. Cell-to-cell communication in bacteria. *Cell* **109:** 421–424.

Bertani, I., and Venturi, V. (2004) Regulation of the N-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA. *Appl Environ Microbiol* **70:** 5493–5502.

Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C., and Greenberg, E.P. (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **98:** 2752–2757.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., and Greenberg, E.P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280:** 295–298.

Dubern, J.F., and Diggle, S.P. (2008) Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Mol Biosyst* **4:** 882–888.

Dubern, J.F., Lugtenberg, B.J., and Bloemberg, G.V. (2006) The ppuI-rsaL-ppuR quorum-sensing system regulates biofilm formation of *Pseudomonas putida* PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II. *J Bacteriol* **188:** 2898–2906.

Fuqua, C., Parsek, M.R., and Greenberg, E.P. (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35:** 439–468.

Fuqua, W.C., Winans, S.C., and Greenberg, E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176:** 269–275.

Furukawa, S., Kuchma, S.L., and O'Toole, G.A. (2006) Keeping their options open: acute versus persistent infections. *J Bacteriol* **188:** 1211–1217.

Huang, J.J., Petersen, A., Whiteley, M., and Leadbetter, J.R. (2006) Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1. *Appl Environ Microbiol* **72:** 1190–1197.

Jain, D., Nickels, B.E., Sun, L., Hochschild, A., and Darst, S.A. (2004) Structure of a ternary transcription activation complex. *Mol Cell* **13:** 45–53.

Juhas, M., Eberl, L., and Tummler, B. (2005) Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* **7:** 459–471.

de Kievit, T., Seed, P.C., Nezezon, J., Passador, L., and Iglewski, B.H. (1999) RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* **181:** 2175–2184.

Kohler, T., van Delden, C., Curty, L.K., Hamzehpour, M.M., and Pechere, J.C. (2001) Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. *J Bacteriol* **183:** 5213–5222.

Luo, Z.Q., Qin, Y., and Farrand, S.K. (2000) The antiactivator TraM interferes with the autoinducer-dependent binding of TraR to DNA by interacting with the C-terminal region of the quorum-sensing activator. *J Biol Chem* **275:** 7713–7722.

Mattiuzzo, M., Bertani, I., Ferluga, S., Cabrio, L., Bigirimana, J., Guarnaccia, C., *et al*. (2011) The plant pathogen *Pseudomonas fuscovaginae* contains two conserved quorum sensing systems involved in virulence and negatively regulated by RsaL and the novel regulator RsaM. *Environ Microbiol* **13:** 145–162.

Parsek, M.R., and Greenberg, E.P. (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* **13:** 27–33.

Pessi, G., and Haas, D. (2000) Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J Bacteriol* **182:** 6940–6949.

Phillips, K., and Luisi, B. (2000) The virtuoso of versatility: POU proteins that flex to fit. *J Mol Biol* **302:** 1023–1039.

Piper, K.R., and Farrand, S.K. (2000) Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. *J Bacteriol* **182:** 1080–1088.

Qin, Y., Su, S., and Farrand, S.K. (2007) Molecular basis of transcriptional antiactivation. TraM disrupts the TraR-DNA complex through stepwise interactions. *J Biol Chem* **282:** 19979–19991.

Rampioni, G., Bertani, I., Zennaro, E., Polticelli, F., Venturi, V., and Leoni, L. (2006) The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the lasI promoter. *J Bacteriol* **188:** 815–819.

Rampioni, G., Schuster, M., Greenberg, E.P., Bertani, I., Grasso, M., Venturi, V., *et al*. (2007a) RsaL provides quorum sensing homeostasis and functions as a global regulator of gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* **66:** 1557–1565.

Rampioni, G., Polticelli, F., Bertani, I., Righetti, K., Venturi, V., Zennaro, E., and Leoni, L. (2007b) The *Pseudomonas* Quorum-Sensing Regulator RsaL Belongs to the Tetrahelical Superclass of H-T-H Proteins. *J Bacteriol* **189:** 1922–1930.

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

Schuster, M., and Greenberg, E.P. (2006) A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* **296:** 73–81.

Schuster, M., and Greenberg, E.P. (2007) Early activation of quorum sensing in *Pseudomonas aeruginosa* reveals the architecture of a complex regulon. *BMC Genomics* **8:** 287.

Schuster, M., Urbanowski, M.L., and Greenberg, E.P. (2004) Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci USA* **101:** 15833–15839.

Siehnel, R., Traxler, B., An, D.D., Parsek, M.R., Schaefer, A.L., and Singh, P.K. (2010) A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **107:** 7916–7921.

Sio, C.F., Otten, L.G., Cool, R.H., Diggle, S.P., Braun, P.G., Bos, R., *et al*. (2006) Quorum quenching by an N-acylhomoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. *Infect Immun* **74:** 1673–1682.

Smith, R.S., and Iglewski, B.H. (2003) *P. aeruginosa* quorum-sensing systems and virulence. *Curr Opin Microbiol* **6:** 56–60.

Son, M.S., Matthews, W.J., Jr, Kang, Y., Nguyen, D.T., and Hoang, T.T. (2007) *In vivo* evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. *Infect Immun* **75:** 5313–5324.

Steidle, A., Allesen-Holm, M., Riedel, K., Berg, G., Givskov, M., Molin, S., and Eberl, L. (2002) Identification and characterization of an N-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Appl Environ Microbiol* **68:** 6371–6382.

Steindler, L., Bertani, I., De Sordi, L., Schwager, S., Eberl, L., and Venturi, V. (2009) LasI/R and RhlI/R quorum sensing in a strain of *Pseudomonas aeruginosa* beneficial to plants. *Appl Environ Microbiol* **75:** 5131–5140.

Suarez-Moreno, Z.R., Devescovi, G., Myers, M., Hallack, L., Mendonca-Previato, L., Caballero-Mellado, J., and Venturi, V. (2010) Commonalities and differences in regulation of N-acyl homoserine lactone quorum sensing in the beneficial plant-associated *Burkholderia* species cluster. *Appl Environ Microbiol* **76:** 4302–4317.

Suarez-Moreno, Z.S., Caballero-Mellado, J., and Venturi, V. (2008) The new group of non-pathogenic plant-associated nitrogen-fixing *Burkholderia* spp. shares a conserved quorum sensing system, which is stringently regulated by the RsaL repressor. *Microbiology* **154:** 2048–2059.

Venturi, V. (2006) Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol Rev* **30:** 274–291.

Ward, J.P., King, J.R., Koerber, A.J., Croft, J.M., Sockett, R.E., and Williams, P. (2004) Cell-signalling repression in bacterial quorum sensing. *Mathods Med Biol* **21:** 169–204.

Whiteley, M., and Greenberg, E.P. (2001) Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. *J Bacteriol* **183:** 5529–5534.

Whiteley, M., Lee, K.M., and Greenberg, E.P. (1999) Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **96:** 13904–13909.

Williams, J.W., Cui, X., Levchenko, A., and Stevens, A.M. (2008) Robust and sensitive control of a quorum-sensing circuit by two interlocked feedback loops. *Mol Syst Biol* **4:** 234.

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

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