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Revisiting the quorum-sensing hierarchy in *Pseudomonas aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors

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Pseudomonas aeruginosa uses the two major quorum-sensing (QS) regulatory systems *las* and *rhl* to modulate the expression of many of its virulence factors. The *las* system is considered to stand at the top of the QS hierarchy. However, some virulence factors such as pyocyanin have been reported to still be produced in *lasR* mutants under certain conditions. Interestingly, such mutants arise spontaneously under various conditions, including in the airways of cystic fibrosis patients. Using transcriptional *lacZ* reporters, LC/MS quantification and phenotypic assays, we have investigated the regulation of QS-controlled factors by the *las* system. Our results show that activity of the *rhl* system is only delayed in a *lasR* mutant, thus allowing the expression of multiple virulence determinants such as pyocyanin, rhamnolipids and C_4-homoserine lactone (HSL) during the late stationary phase. Moreover, at this stage, RhlR is able to overcome the absence of the *las* system by activating specific LasR-controlled functions, including production of 3-oxo-C_{12}-HSL and *Pseudomonas* quinolone signal (PQS). *P. aeruginosa* is thus able to circumvent the deficiency of one of its QS systems by allowing the other to take over. This work demonstrates that the QS hierarchy is more complex than the model simply presenting the *las* system above the *rhl* system.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous and versatile bacterium involved in numerous pathogenic infections affecting immunocompromised individuals and those suffering from cystic fibrosis (Marshall & Carroll, 1991; Pier, 1985; Speert, 1985). This bacterium regulates most of its virulence determinants in a cell-density-dependent manner via a mechanism called quorum-sensing (QS). Such global regulatory systems are found in most bacterial species, and control several and diverse biological functions, such as virulence, bacterial conjugation, bioluminescence and biofilm formation (de Kievit & Iglewski, 2000; Donabedian, 2003; Loh *et al.*, 2002; Miller & Bassler, 2001). QS is mediated by diffusible signalling molecules released into the external environment. These signals, when reaching specific concentrations correlated with specific population cell densities, bind to and activate their respective transcriptional regulators. In *P. aeruginosa*, two conventional complete QS systems are known: the synthases LasI and RhlI produce the *N*-acylhomoserine lactones 3-oxo-C_{12}-HSL and C_4-HSL respectively, which induce their cognate LuxR-type transcriptional regulators LasR and RhlR, responsible for the activation of numerous QS-controlled genes (Juhas *et al.*, 2005; Pesci *et al.*, 1997). Among genes activated by these two regulators are those coding for the LasI and RhlI synthases. Since *N*-acyl-HSLs induce their own production, they are called autoinducers. More recently, a third, distinct QS system has been unveiled. It is composed of a transcriptional regulator from the LysR family, MvfR (PqsR), which directly activates two operons (*phnAB* and *pqsABCDE*) required for the biosynthesis of 4-hydroxy-2-alkylquinolines (HAQs), including molecules involved in 4-quinolone signalling (Déziel *et al.*, 2004; Lépine *et al.*, 2004; Pesci *et al.*, 1999), and for the activation of many QS-controlled genes, via *pqsE* (Déziel *et al.*, 2005; Diggle *et al.*, 2006; Farrow *et al.*, 2008). Among the HAQs, 4-hydroxy-2-heptylquinoline and the *Pseudomonas* quinolone signal (PQS) act as activators of the MvfR regulator, inducing a positive feedback loop typical of QS systems (Xiao *et al.*, 2006a).

QS regulation is a very complex and extensive network influencing, both positively and negatively, the transcription of perhaps 5–10 % of the *P. aeruginosa* genome (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). The LasR regulator is known to initiate the QS regulatory system, as it activates the transcription of a number of other regulators, such as *rhlR*, defining a

Abbreviations: HAQ, 4-hydroxy-2-alkylquinoline; HSL, homoserine lactone; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing.

Two supplementary figures are available with the online version of this paper.

hierarchical QS cascade from the *las* to the *rhl* regulons (Latifi *et al.*, 1996; Pesci *et al.*, 1997). Over the last few years, many whole-genome transcriptomic studies have been published with the aim of identifying genes that are under the control of LasR and/or RhlR (Hentzer *et al.*, 2003; Rasmussen *et al.*, 2005; Schuster *et al.*, 2003; Wagner *et al.*, 2003). Specific directly activated genes were clearly identified as belonging to the *rhl* regulon, such as *rhlAB* (rhamnolipid biosynthesis), *lecA* (lectin), *hcnABC* (HCN production) and both *phzABCDEFG* operons (phenazine biosynthesis) (Latifi *et al.*, 1995; Schuster *et al.*, 2004; Schuster & Greenberg, 2007; Whiteley *et al.*, 1999; Winzer *et al.*, 2000). However, the situation is not as clear for many LasR-controlled genes, for which it has not been possible to define a single consensus LasR binding site sequence in the promoter region, suggesting that some of these genes are activated indirectly (Schuster *et al.*, 2004; Schuster & Greenberg, 2007). Actually, most QS-regulated factors are more or less influenced by both LasR and RhlR, as is the case for the proteases LasA (staphylolytic protease) and LasB (elastase) (Freck-O'Donnell & Darzins, 1993; Hentzer *et al.*, 2003; Nouwens *et al.*, 2003; Schuster *et al.*, 2003; Toder *et al.*, 1994; Wagner *et al.*, 2004). Thus QS plays a predominant role in the regulation of virulence determinants in *P. aeruginosa.* Surprisingly, however, there are increasing reports that *lasR* mutants occur frequently in the natural environment (Cabrol *et al.*, 2003), in airways from individuals with cystic fibrosis (D'Argenio *et al.*, 2007; Smith *et al.*, 2006), in intubated patients (Denervaud *et al.*, 2004) and in individuals suffering from bacteraemia, pneumonia or wound infection (Hamood *et al.*, 1996). This is intriguing, since the LasR regulator is widely considered essential for full *P. aeruginosa* virulence (Preston *et al.*, 1997; Rumbaugh *et al.*, 1999; Storey *et al.*, 1998).

The LasR transcriptional regulator is generally considered to sit at the top of the QS hierarchy in *P. aeruginosa* (Latifi *et al.*, 1996). However, we and others have observed that the phenazine pyocyanin is overproduced by *lasR* mutants at the late stationary phase (Déziel *et al.*, 2005; Diggle *et al.*, 2003). As shown in Fig. 1, a *lasR* mutant produces less pyocyanin during early growth phases, although at the end of exponential growth and during early stationary phase, pyocyanin begins to be produced. During late stationary phase, after 24 h of cultivation, the *lasR* mutant cultures contain much more pyocyanin than cultures of the wild-type strain (35 mg l^{-1} compared to 2.5 mg l^{-1}, respectively). This is unexplained, since pyocyanin production is known to be regulated by QS (Latifi *et al.*, 1995). The regulator of the pyocyanin biosynthesis genes (*phz* genes) is RhlR (Brint & Ohman, 1995), whose transcription is considered to require LasR (de Kievit *et al.*, 2002; Latifi *et al.*, 1996; Pearson *et al.*, 1997; Pesci *et al.*, 1997). In theory, pyocyanin production is thus expected to be absent in *lasR* mutants, whereas experimental data show that it is actually only delayed (Fig. 1).

In order to better understand the specific role of LasR and its involvement in expression of virulence factors, we have characterized the expression of QS-controlled determinants in a *lasR* mutant and have observed that during stationary phase, many QS-regulated virulence factors are expressed. Our data show that at this stage of growth, the RhlR regulon is activated. Moreover, we found that RhlR is able to induce LasR-regulated genes (including some considered specific such as *lasI*) in the absence of *lasR*, unveiling a new mechanism for the bacteria to bypass a defect in their QS regulation, allowing RhlR to induce the *las* system when LasR is non-functional.

METHODS

Strains, plasmids and growth conditions. Table 1 lists strains and plasmids. Bacteria were routinely grown in Tryptic Soy Broth (TSB) medium at 37 °C in a roller drum, with appropriate antibiotics when required (carbenicillin 300 mg l^{-1} and tetracycline 75 mg l^{-1} for *P. aeruginosa*; carbenicillin 100 mg l^{-1} and tetracycline 15 mg l^{-1} for *Escherichia coli*). TSB plates contained 1.5 % agar. For pyocyanin and rhamnolipid detection, King's A medium was used (King *et al.*, 1954). All measurements of optical density and absorbance were obtained with a Thermo Scientific NanoDrop 1000 spectrophotometer.

An isogenic *lasR rhlR* double mutant was generated by allelic exchange of the *rhlR* gene in a *lasR* background with pSB224.10A using sucrose counterselection (Beatson *et al.*, 2002).

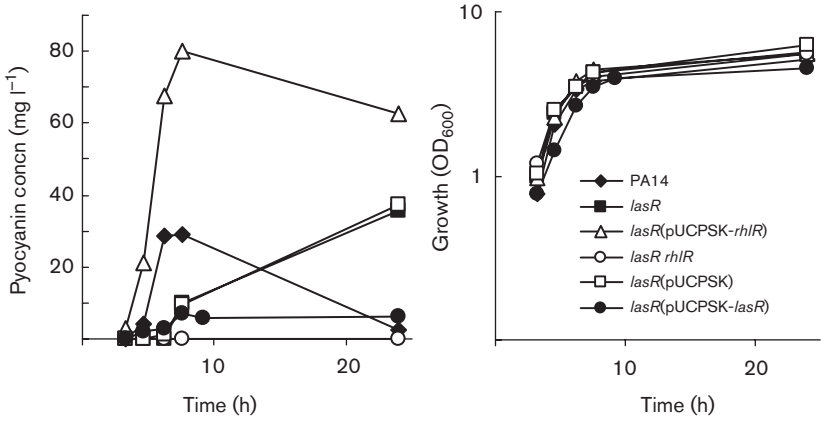

Fig. 1. Expression of pyocyanin is delayed in a *lasR* mutant: *P. aeruginosa lasR* mutant containing a constitutive *rhlR* (pUCPSK*rhlR*) or *lasR* (pUCPSK*lasR*) expression vector, or the same vector without *rhlR* or *lasR* (pUCPSK), compared with the wild-type and the *lasR rhlR* mutant.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Bacteria		
E. coli DH5α	*supE44 ΔlacU169 (ϕ80 lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
P. aeruginosa/lab no.:		
PA14/ED14	Clinical isolate UCBPP-PA14	Rahme *et al.* (1995)
PA14 *lasR*/ED69	*lasR*::Gm derivative of ED14	Déziel *et al.* (2004)
PA14 *lasR rhlR*/ED266	*rhlR*::Tc derivative of ED69	This study
S. aureus Newman	Laboratory strain	ATCC 25904
Plasmids		
pMIC61(pUCPSK-*lasR*)	*lasR* in pUCPSK with *lac* promoter as a *Hin*dIII–*Eco*RI fragment (5′–3′ *lasR*)	John Mattick, Institute of Molecular Bioscience, University of Queensland, Australia
pMIC62(pUCPSK-*rhlR*)	*rhlR* in pUCPSK with *lac* promoter as a *Hin*dIII–*Eco*RI fragment (5′–3′ *rhlR*)	John Mattick
pPCS1002	pLP170 containing *rhlR-lacZ*	Pesci *et al.* (1997)
pSB224.10A	pRIC380 suicide vector carrying *rhlR*::Tc	Beatson *et al.* (2002)
pVD1	pDN19 containing *lasI* with its native promoter, Tc^r	This study
pME3853	pME6010 with a 174 bp *lasI* upstream fragment and translational *lasI*::*lacZ* fusion containing the first 13 *lasI* codons, Tc^r	Pessi *et al.* (2001)
pUCPSK	*E. coli–P. aeruginosa* shuttle vector	Watson *et al.* (1996)
pLJR50	*lasB*p-*lacZ* transcriptional reporter fusion; contains nt −190 to +4 of the *lasB* promoter region, Cb^r	Toder *et al.* (1994)

Standard methods were used to manipulate DNA. Plasmid pDN19 (Nunn *et al.*, 1990) was used to construct pVD1, containing the *lasI* gene under its own promoter. A region spanning from 305 bp upstream to 170 bp downstream of the *lasI* ORF was amplified and inserted between the *Xba*I and *Hin*dIII sites in the pDN19 multiple cloning site. The gene fragment was generated from genomic DNA using PCR with primers 5′-GCTCTAGATTTTGGGGCTGTGTTC-TCTC-3′ and 5′-CCCAAGCTTACTCGAAGTACTGCGGGAAA-3′. The construction was confirmed by effective complementation of a *lasI* mutant. Plasmids were introduced by electroporation (Choi *et al.*, 2006).

lasR mutant subcultures were carried out as follows: a first preculture was made at day 1 and used to inoculate fresh medium for day 2; the latter was used to inoculate fresh medium for day 3. Pyocyanin was measured during each day of culture.

β-Galactosidase activity assay. Bacteria containing the gene reporter fusions were routinely grown overnight from frozen stocks in TSB with appropriate antibiotics, then subcultured in triplicate at a starting OD_{600} of 0.05 without antibiotic. Culture samples were regularly taken for determination of growth (OD_{600}) and β-galactosidase activity (Miller, 1972). *N*-Butyryl-L-homoserine lactone (C_4-HSL) was purchased from Sigma-Aldrich and the stock solution prepared in acetonitrile.

Quantification of rhamnolipids, pyocyanin, AHLs and HAQs. Detection and measurements were performed by LC/MS. For pyocyanin, AHLs and HAQs, 480 µl culture samples were taken at regular intervals, used for determination of growth (OD_{600}), and mixed with 120 µl acetonitrile containing 50 mg l^{-1} 5,6,7,8-tetradeutero-PQS for a final concentration of 10 mg l^{-1} as internal standard. After centrifugation, 20 µl aliquots of the supernatants were directly injected for LC separation on an Agilent HP1100 HPLC system equipped with a 3 × 150 mm C8 Luna reverse-phase column (Phenomenex). A 1 % acidified water/acetonitrile gradient was used as the mobile phase at a flow rate of 0.4 ml min^{-1}, split to 10 % with a Valco Tee. A Quattro II (Waters) triple-quadrupole MS was used for molecule detection. Data acquisition was performed in full scan mode with a scanning range of 130–350 Da. Precise quantification of C_4-HSL and 3-oxo-C_{12}-HSL was performed by MS/MS, as described previously (Déziel *et al.*, 2005). For rhamnolipid quantification, 500 µl culture samples were taken at regular intervals, used for determination of growth (OD_{600}), and diluted with an equivalent volume of methanol. After centrifugation, 20 µl aliquots of the supernatants were injected for LC/MS analysis as described previously, using 16-hydroxyhexadecanoic acid as internal standard (Déziel *et al.*, 1999; Lépine *et al.*, 2002).

Elastase and protease enzymic assays. TSB plates supplemented with 1 % skim milk were inoculated with 10 µl from cultures at OD_{600} 3. Plates were incubated at 37 °C for 3 days. For specific LasB elastolytic activity, we used a protocol adapted from that of Bjorn *et al.* (1979). Briefly, filter-sterilized culture supernatant samples (100 µl) from late stationary phase cultures were mixed with 5 mg elastin Congo red (Sigma) and 300 µl 0.1 M Tris/HCl pH 7.2. Release of Congo red from degraded elastin was measured as A_{495} after 2 h of incubation at 37 °C followed by centrifugation. For assessment of LasA staphylolytic activity, 4.5 ml of *Staphylococcus aureus* overnight cultures were boiled for 15 min. and 100 µl was mixed with 300 µl of filtered culture supernatants. The OD_{600} was measured after 2 h of incubation at 37 °C with agitation. All experiments were carried out in triplicate.

RESULTS

The expression of RhlR-regulated factors is only delayed in the absence of LasR

Based on previous observations reporting late pyocyanin production in *lasR* mutants, we decided to investigate the

mechanism involved in this phenomenon, as an introduction to exploring QS during the stationary phase.

Since RhlR is the known regulator of the *phz* genes, we hypothesized that late pyocyanin production is due to RhlR activity. In the absence of *lasR*, RhlR should activate the expression of the *phz* genes in the late stationary phase, and in its absence, no pyocyanin should be produced. As shown in Fig. 1, unlike the *lasR* mutant, the *lasR rhlR* double mutant does not produce this phenazine at all. Moreover, *lasR*(pUCPSK-*rhlR*), which constitutively expresses *rhlR* from a plasmid, produces pyocyanin at the same time as the wild-type, confirming that RhlR is responsible for the timing of pyocyanin production. As expected, continued expression of *rhlR* results in higher production of pyocyanin. *lasR*(pUCPSK) acts like the *lasR* mutant, confirming that the vector does not influence pyocyanin expression. Finally, *lasR*(pUCPSK*lasR*) does not overproduce pyocyanin, unlike the *lasR* mutant, showing that the *lasR* mutation is responsible for this phenotype. It is also noteworthy that a *lasI* mutant shows the same pyocyanin overproduction phenotype as the *lasR* mutant (data not shown). To ensure that optical density during all growth stages, and particularly during stationary phase, truly reflected the number of living bacterial cells, we also determined the viable cell counts. This showed that the growth rates and survival of the *lasR* mutant and the wild-type were essentially the same (see Supplementary Fig. S1a, available with the online version of this paper), thus confirming that the difference in pyocyanin production is not the result of variations in the number of viable cells.

To ensure that this late pyocyanin production was not due to a spontaneous mutation that might have occurred in the *lasR* background, we subcultured a culture of the *lasR* mutant on three consecutive days in fresh medium, every time monitoring the production of pyocyanin. Consistently, the cultures had to reach the late stationary phase before producing pyocyanin, indicating that this phenotype in not due to accumulation of secondary mutations during cultivation (see Supplementary Fig. S1b).

If RhlR is present and active during the late stationary phase in a *lasR* mutant, then we should be able to detect RhlR-regulated factors other than pyocyanin. The *rhlAB* and *rhlC* genes, coding for enzymes involved in rhamnolipid biosynthesis, and *rhlI*, coding for the C_4-HSL synthase, are all directly regulated by RhlR (de Kievit *et al.*, 2002; Medina *et al.*, 2003). We precisely quantified rhamnolipids and C_4-HSL in *lasR*, *lasR rhlR* and *lasR*(pUCPSK-*rhlR*) cultures. As shown in Fig. 2(a, b), the *lasR rhlR* double mutant was unable to synthesize rhamnolipids or C_4-HSL, while the *lasR* mutant produced these molecules with a delay, essentially in late stationary phase. These results support the hypothesis that expression

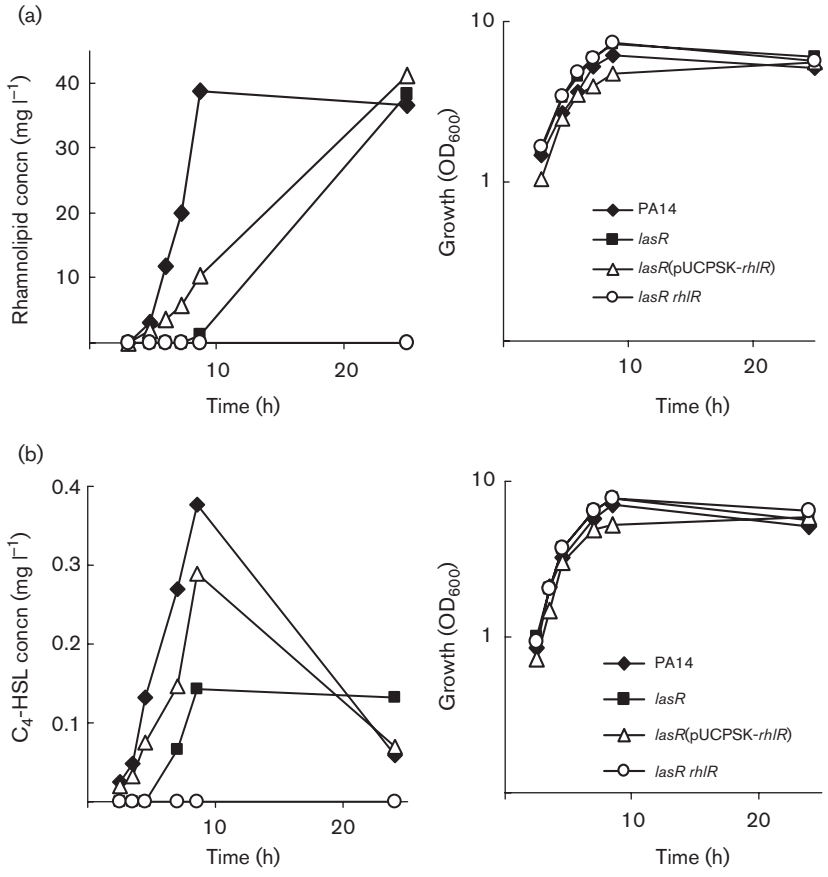

Fig. 2. Expression of RhlR-controlled factors is delayed in a *lasR* mutant. *P. aeruginosa* wild-type and *lasR* mutant containing or not a constitutive *rhlR* expression plasmid (pUCPSK-*rhlR*) are compared. Production of (a) rhamnolipids and (b) C_4-HSL.

of the *rhl* regulon is only delayed in a *lasR* mutant. The production of C_4-HSL and rhamnolipids was restored to levels similar to wild-type when the *lasR* mutant was transformed with an *rhlR* expression vector, confirming that RhlR is responsible for these phenotypes. These results show that the delayed expression of RhlR-controlled phenotypes in a *lasR* background can be restored by expressing *rhlR*.

In order to obtain additional evidence that RhlR is indeed expressed in a *lasR* mutant, we evaluated the transcription of *rhlR* with a *lacZ* fusion reporter. As shown in Fig. 3, maximal *rhlR* transcription occurs at the early stationary phase in the wild-type strain. Furthermore, it follows a similar expression pattern in the *lasR* mutant background, but at lower levels. Still, during late stationary phase, level of *rhlR* expression slightly increases in the *lasR* mutant, while it decreases in the wild-type. These data support the significant presence of RhlR in *lasR* mutants during late stationary phase, as previously reported (Diggle *et al.*, 2003).

It is well established that the production of proteolytic enzymes such as LasA and LasB, responsible for staphylolytic and elastolytic activities respectively, is under LasR regulation (Rust *et al.*, 1996; Storey *et al.*, 1998; Toder *et al.*, 1991). However, there are indications that production of these enzymes might also be under partial RhlR control (Brint & Ohman, 1995; Diggle *et al.*, 2003; Pearson *et al.*, 1997). To evaluate global protease activity of the strains, the wild-type strain, and *lasR* and *lasR rhlR* mutants, were inoculated on solid medium containing skim milk. Protease activity was visible for the *lasR* mutants while the double mutant was unable to degrade milk proteins (see Supplementary Fig. S2). Since this test only indicates general proteolytic activity, it was interesting to target specific proteases. Fig. 4(a) shows that the *lasR* mutant is able to activate *lasB* expression late in stationary phase, while the double *lasR rhlR* mutant cannot. Detection of LasB activity confirmed these results. During late stationary phase, the *lasR* mutant shows significant elastolytic activity, which is nearly as high as that in *lasR*(pUCPSK-*rhlR*) (Fig. 4b). Finally, Fig. 4(c) shows that the wild-type and the *lasR* mutant, complemented with *rhlR* or not, express LasA activity, while the *lasR rhlR* double mutant does not. Taken together, all these results indicate that the expression of many QS-controlled factors is only delayed when LasR is defective.

RhlR controls factors generally considered to be solely regulated by LasR

Another observation we and others have made is that not only pyocyanin but also PQS is produced during late stationary phase by a *lasR* mutant (Déziel *et al.*, 2004; Diggle *et al.*, 2003). This was unexpected, since the final step in PQS synthesis is catalysed by the *lasR*-dependent PqsH enzyme (Déziel *et al.*, 2004; Gallagher *et al.*, 2002; Whiteley *et al.*, 1999; Xiao *et al.*, 2006b). It is of note that there is a close correlation between the timing of production of both PQS and pyocyanin in *lasR* mutant backgrounds (Déziel *et al.*, 2005; Diggle *et al.*, 2002, 2003). To test if RhlR might also be responsible for this effect, we quantified PQS production by the wild-type and the *lasR*, *lasR rhlR* and *lasR*(pUCPSK-*rhlR*) mutants. As shown in Fig. 5(a), during the exponential and early stationary growth phases, PQS production is totally absent in the double mutant and barely detectable in the *lasR* mutant unless *rhlR* is expressed, which leads to a substantial reduction in the delay observed for that mutant. The same reduction of PQS is observed in a *lasI* mutant, and can also be restored by overexpressing RhlR in that mutant (data not shown). At the late stationary phase, however, the concentration of PQS in *lasR* mutant cultures is similar to the wild-type, while the double mutant still shows no detectable production. These data explain the late PQS production in a *lasR* mutant by the activity of RhlR.

We then asked whether *lasI*, probably the most specific LasR-regulated gene, which codes for the autoinducer synthase producing 3-oxo-C_{12}-HSL, might also be regulated by RhlR. As expected from the above data, we found that 3-oxo-C_{12}-HSL production is greatly increased in *lasR*(pUCPSK-*rhlR*) compared to the wild-type strain, at the same optical density (Fig. 5b). It also shows that 3-oxo-C_{12}-HSL is eventually produced in a *lasR* mutant at late stationary phase, but is totally absent if *rhlR* is also defective.

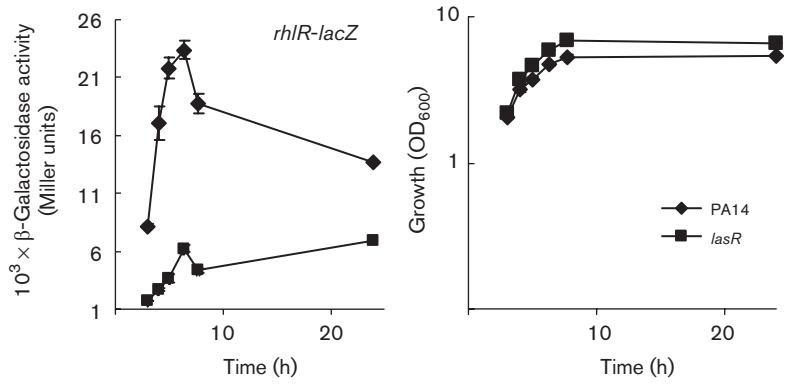

Fig. 3. *rhlR* transcription in a *lasR* mutant increases during late stationary phase. β-Galactosidase activity using the pSC1002 vector containing the *rhlR-lacZ* transcriptional reporter.

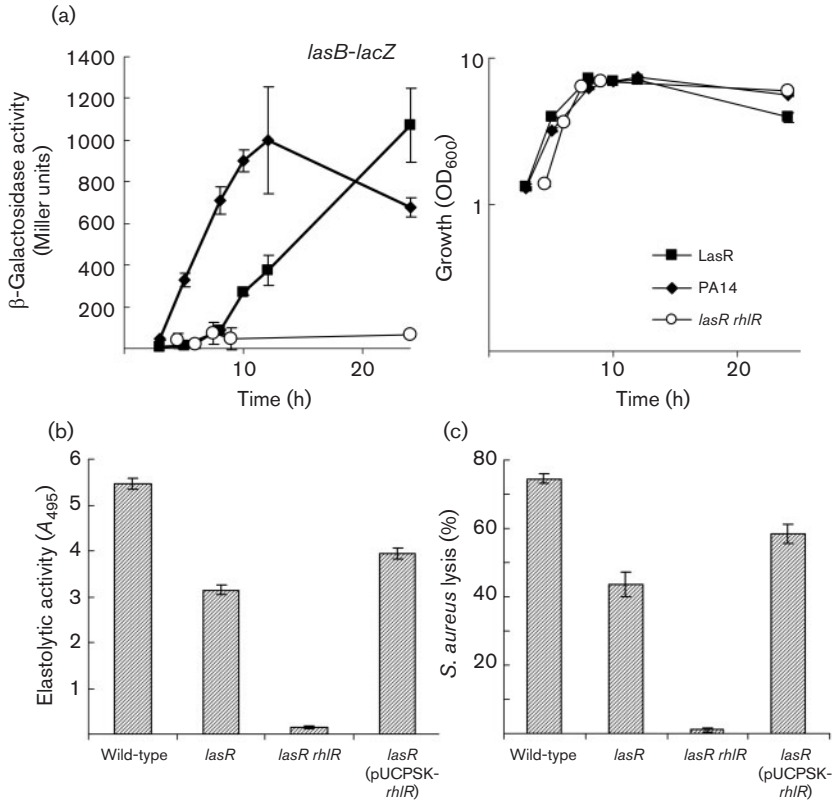

Fig. 4. LasA and LasB are activated late in a *lasR* mutant but not in a *lasR rhlR* double mutant. (a) Transcription of the *lasB* gene; (b) elastolytic (LasB) activity; (c) staphylolytic (LasA) activity.

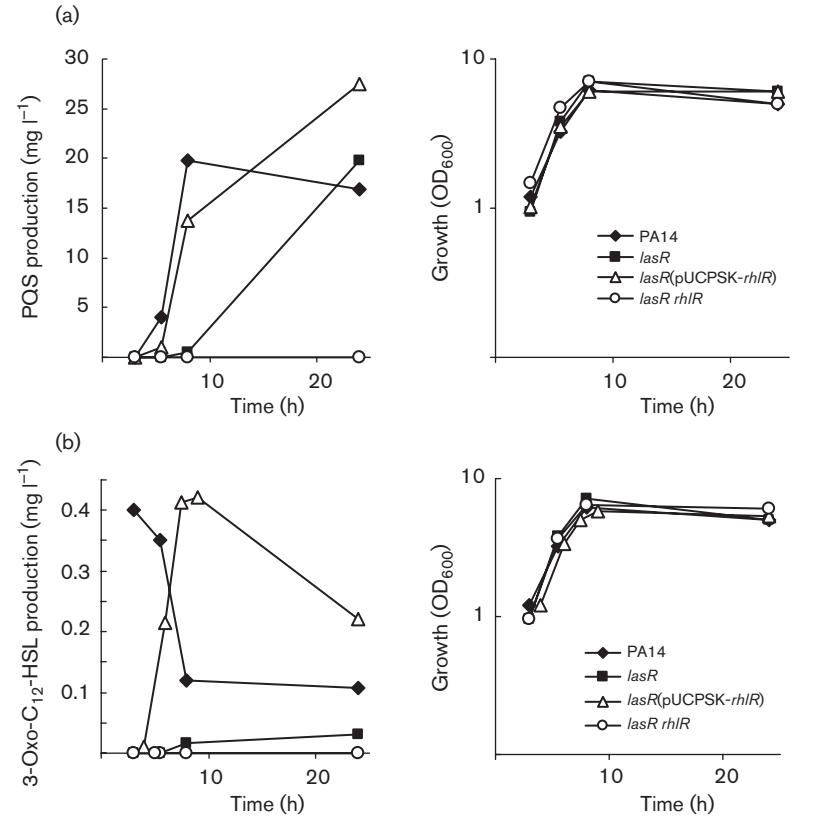

Fig. 5. Production of PQS (a) and 3-oxo-C_{12}-HSL (b) requires *rhlR* in the absence of *lasR*. LC/MS analysis from culture supernatants.

RhlR controls *lasI* in a heterologous system

In order to further identify RhlR as an alternative activator of *lasI* transcription in the absence of a functional LasR, we constructed a heterologous system in *E. coli*. A vector (pME3853) carrying the *lasI-lacZ* gene reporter was introduced into *E. coli* DH5α. In the presence of the *rhlR* gene constitutively expressed on another compatible plasmid, and with addition of its autoinducer C_4-HSL, β-galactosidase activity was greatly enhanced in the *E. coli* strain, while only basal expression was detected in absence of *rhlR* or C_4-HSL (Fig. 6a). To confirm 3-oxo-C_{12}-HSL production through activation by RhlR, a vector containing *lasI* under its native promoter was introduced into *E. coli* DH5α. 3-Oxo-C_{12}-HSL was detected in this heterologous system only in the presence of both RhlR and its autoinducer C_4-HSL (Fig. 6b).

DISCUSSION

P. aeruginosa is an opportunistic pathogen that relies on its impressive ability to coordinate gene expression in order to compete against other species for nutrients or colonization. QS appears essential for this bacterium for competitiveness in clinical or environmental niches. The QS LasR transcriptional regulator is known to control a wide array of *P. aeruginosa* virulence-associated factors. Nevertheless, several reports mention the high frequency of *lasR* mutations among clinical and environmental isolates (Cabrol *et al.*, 2003; D'Argenio *et al.*, 2007). Most intriguingly, some *lasR* mutants still produce QS-regulated virulence factors such as pyocyanin (Heurlier *et al.*, 2005), and naturally occurring *lasR* mutants have been isolated from wounds or intubated patients (Denervaud *et al.*, 2004; Hamood *et al.*, 1996). It was thus interesting to analyse the involvement of LasR in the expression of QS-regulated virulence determinants in more detail.

This study provides new insights into the interplay between the *las* and the *rhl* QS systems in *P. aeruginosa*, and demonstrates that a *lasR* mutation does not lead to loss of virulence factors. Expression of the *rhl* regulon is delayed until the late stationary phase in a *lasR* mutant, and is thus responsible for the late production of virulence factors in this background, such as pyocyanin, QS signalling molecules and proteases. These observations provide a solid basis allowing us to explain numerous inconsistencies in previous reports, and bring some clarifications to the *P. aeruginosa* QS model, as summarized in Fig. 7.

RhlR-regulated factors are expressed late in a *lasR* mutant

The delayed production of pyocyanin in a *lasR* mutant background has been anecdotally observed in numerous reports (Déziel *et al.*, 2005; Diggle *et al.*, 2002, 2003; Heurlier *et al.*, 2005; Kohler *et al.*, 2001; Lujan *et al.*, 2007; Salunkhe *et al.*, 2005). It has been suggested that RhlR might be involved in that production, although no evidence was presented (Diggle *et al.*, 2003). Here we present evidence for the role of the RhlR regulator in pyocyanin production in a *lasR* mutant, since no production can be observed in a *lasR rhlR* double mutant and production is advanced in a *lasR* mutant complemented with *rhlR*.

The activity of RhlR during stationary phase in a *lasR* mutant was confirmed by the delayed production of other RhlR-controlled factors, C_4-HSL and rhamnolipids. Others

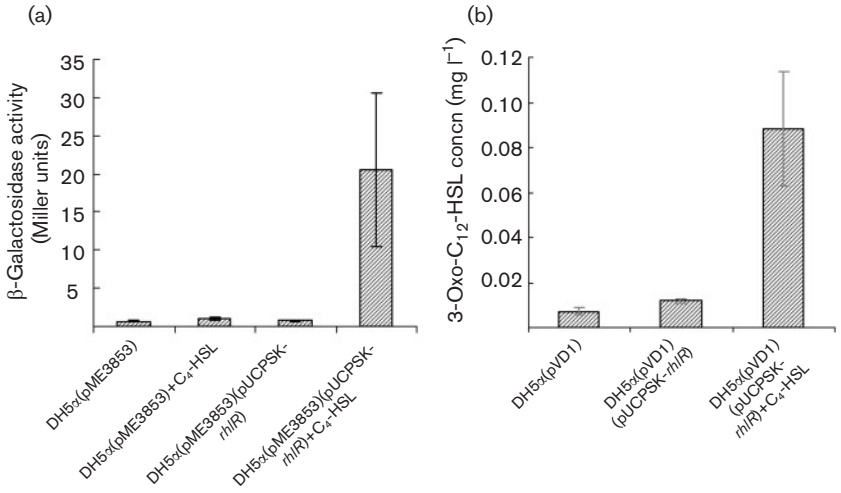

Fig. 6. LasI is activated by RhlR in a heterologous *E. coli* DH5α system in the presence of either or both C_4-HSL (5 mg l^{-1}) and *rhlR* (pUCPSK-*rhlR*). (a) *lasI-lacZ* expression (pME3853); (b) 3-oxo-C_{12}-HSL production in the presence of the *lasI* gene with its native promoter (pVD1).

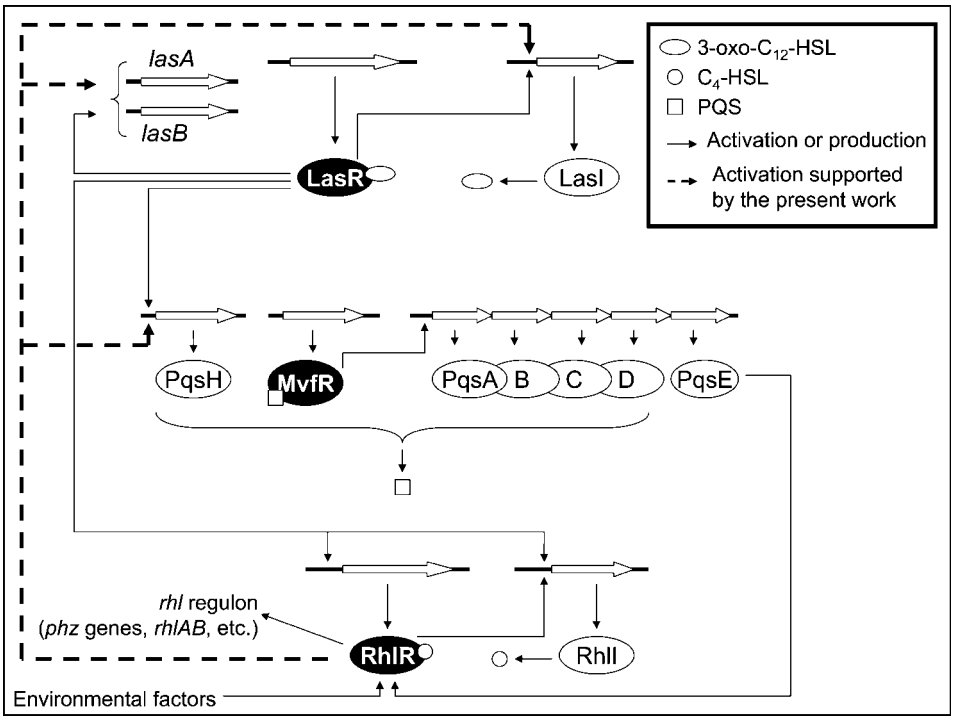

Fig. 7. Proposed model for the influence of RhlR on the *las* regulon. Basal expression of *lasI* leads to weak production of 3-oxo-C_{12}-HSL. This signalling molecule binds to some LasR regulators produced due to basal transcription of *lasR*. Once LasR is bound to its autoinducer, it activates the *las* regulon, leading to increased *lasI* transcription and activation of the *rhl* regulon. Our present work reveals an overlap between the *las* and *rhl* regulons where the *las* system is activated by the RhlR regulator. In the presence of a *lasR* mutation leading to loss of its function, residual *rhlI* and *rhlR* transcriptions, perhaps combined with environmental factors, will allow activation of the *rhl* regulon, but with a marked delay due to lack of *rhlI* and *rhlR* transcriptional activation by LasR. Eventually, when the bacteria reach stationary phase, RhlR is present and functional to activate numerous virulence factors such as pyocyanin, rhamnolipids, proteases and signalling molecules including PQS and 3-oxo-C_{12}-HSL.

have noticed the presence of rhamnolipids during this stage. Van Delden *et al.* (1998) proposed that environmental factor(s) could be responsible for the induction of some virulence factors in a *lasR* mutant, and that this induction should be mediated by RhlR. In another study, Kohler *et al.* (2001) reported rhamnolipid production by *lasR* and *lasI* mutants. Interestingly, the delayed production of pyocyanin and rhamnolipids in a *lasR* mutant background observed in our work seems to correlate with the delayed accumulation of C_4-HSL and PQS. These signals both positively upregulate the *rhl* system. Our results provide new evidence that LasR mainly acts as an exponential growth phase activator on several RhlR-dependent factors.

The expression of the *rhl* system is maintained in a *lasR* mutant

The above results can be explained by the fact that RhlR is expressed in a *lasR* mutant, as confirmed by a transcriptional *rhlR-lacZ* reporter, showing reduced but sustained transcription of *rhlR*. This finding contradicts the prevailing concept that the *rhl* system is inactive in the absence of a functional LasR (Latifi *et al.*, 1996). However, the available literature on the QS system in *P. aeruginosa* is largely based on experiments carried out during early growth stages. Indeed, Van Delden *et al.* (1998) had previously noticed significant *rhlR* expression in a *lasR* mutant background during the stationary phase, using the same reporter that we did. Perhaps the *lacZ* reporter used by Latifi *et al.* (1996) was less sensitive or a difference in growth conditions modified the response. Nevertheless, our results agree that the *rhl* system is indeed underexpressed during the exponential and early stationary growth phase in a *lasR* mutant, but importantly seems to maintain a higher level of activity than in the wild-type during late stationary phase when *lasR* is absent. Still, our results are in agreement with induction of the *rhl* system by LasR (Latifi *et al.*, 1996). Most probably, basal *rhlR* and *rhlI* transcription leads to the autoinduction of the *rhl* QS system in a *lasR* mutant background. It is also possible that environmental conditions are involved in that induction, since factors such as starvation (Van Delden *et al.*, 1998), phosphate and iron (Jensen *et al.*, 2006) have an influence on RhlR activation.

RhlR partially controls LasR-specific factors in the absence of a functional LasR regulator

Some LasR-dependent factors can be expressed in the absence of this regulator, RhlR apparently acting as a surrogate activator. Indeed, PQS was detected in late stationary phase of a *lasR* mutant (Déziel *et al.*, 2005; Diggle *et al.*, 2003). Using a heterologous system, we found that the gene coding for the specific autoinducer synthase of LasR, *lasI*, is also transcriptionally activated by RhlR, and this is accompanied by the production of the corresponding AHL. It has previously been observed that some activities known to be LasR regulated are also affected by RhlR. Numerous proteolytic enzymes responsible for elastolytic (via the *lasB* gene) and staphylolytic (via the *lasA* gene) activities were initially reported to be specifically under the control of LasR (Rust *et al.*, 1996; Storey *et al.*, 1998; Toder *et al.*, 1991), although these activities were also reported to be also partially under RhlR regulation (Brint & Ohman, 1995; Diggle *et al.*, 2003; Pearson *et al.*, 1997). The absence of a correlation between *lasR* and *lasB* transcription in some clinical and environmental *P. aeruginosa* strains was noted by Cabrol *et al.* (2003). Our data suggest that this is explained by the additional control of *lasB* by RhlR.

In agreement with the work presented here, evidence for production of 3-oxo-C_{12}-HSL in some *lasR* mutants was reported by Sandoz *et al.* (2007). However, they suggested that this phenotype was due to compensatory mutations, while this is not the case in the present work. Production of 3-oxo-C_{12}-HSL in a *lasR* mutant would suggest that the bacterium is wasting resources, since no LasR protein is present to be activated by this autoinducer. However, it is known that this molecule also plays a role in *P. aeruginosa* pathogenicity as a virulence factor inducing inflammation *in vivo* (Qazi *et al.*, 2006; Shiner *et al.*, 2006; Smith *et al.*, 2002) and accelerates apoptosis in macrophages and neutrophils (Tateda *et al.*, 2003; Vikstrom *et al.*, 2005). *N*-Acyl-HSLs are also signalling molecules involved in intra- and inter-species communication, which allow *P. aeruginosa* to compete or collaborate with other bacterial species (Eberl & Tummler, 2004; Juhas *et al.*, 2005; Qazi *et al.*, 2006; Riedel *et al.*, 2001; Shiner *et al.*, 2005; Williams, 2007). Interestingly, another QS regulator, QscR, can bind to 3-oxo-C_{12}-HSL (Ledgham *et al.*, 2003) and requires this signalling molecule to regulate some genes (Lee *et al.*, 2006; Lequette *et al.*, 2006). 3-Oxo-C_{12}-HSL can thus be valuable even in absence of the LasR regulator. PQS production in a *lasR* mutant is also important, since it allows the activation of the third QS system in *P. aeruginosa* (Diggle *et al.*, 2006; Xiao *et al.*, 2006a), which affects expression of multiple virulence factors (Calfee *et al.*, 2001; Déziel *et al.*, 2005; Xiao *et al.*, 2006b), with a positive effect on the *rhl* regulon (Diggle *et al.*, 2003; Jensen *et al.*, 2006; McKnight *et al.*, 2000).

LasR is a direct and indirect regulator of QS-controlled genes

In light of these elements, it appears that at least some of the known LasR-regulated genes are not strictly controlled only by this regulator. Although direct binding of LasR to some promoters such as that from *lasB* has been reported (Schuster *et al.*, 2004), here we confirm that at least *lasA*, *lasB*, *pqsH* and *lasI* (Pearson *et al.*, 1997; Pesci *et al.*, 1997; this study) can also be controlled via RhlR, and are activated late in the absence of LasR. Although unique binding sites have been identified in several LasR-regulated promoters (Schuster *et al.*, 2004), it has not been possible to define a single consensus binding site sequence in the promoter of QS-controlled genes, or to differentiate LasR- vs RhlR-specific promoters (Anderson *et al.*, 1999; Schuster *et al.*, 2003; Wagner *et al.*, 2003; Whiteley *et al.*, 1999; Whiteley & Greenberg, 2001). Our data suggest that RhlR can efficiently recognize LasR boxes, since RhlR is able to activate some LasR-specific genes.

Finally, considering the fact that 3-oxo-C_{12}-HSL production begins to decrease early and thus does not correlate with *lasB* transcriptional activation or PQS production, and that LasR is inactive in absence of its autoinducer (Schuster *et al.*, 2004), it is reasonable that other regulators are able to take over and/or supplement LasR in order to express these factors. Since C_4-HSL, unlike 3-oxo-C_{12}-HSL, accumulates during growth, it appears that RhlR is active and functional for a much longer period than LasR. RhlR is thus a good candidate to itself activate some LasR-regulated factors.

Finally, it is noteworthy that we have repeated most of the experiments reported here with different *P. aeruginosa* PAO1 strains, leading to the same conclusions. We have therefore no indication that our results are restricted to the PA14 strain.

Spontaneous emergence of *lasR* mutants during infections would preclude targeting the *las* system for antivirulence QS therapy

Understanding why supposedly non-virulent mutants might be selected in infected patients is an intriguing question. After quantification of pyocyanin, rhamnolipids and protease activities, our results show that during late stationary phase, a *lasR* mutant is able to produce at least as much of these virulence factors as the wild-type. This might help to explain the occurrence of such mutants among clinical isolates (Heurlier *et al.*, 2006).

Over the last few years, research has been carried out to identify drugs targeting QS to prevent virulence instead of bacterial survival, in order to circumvent the risks of resistance observed with antibiotic treatments (Hentzer *et al.*, 2002, 2003; Smith *et al.*, 2003; Wu *et al.*, 2004). Until now, these therapies have mostly targeted the *las* system, because blocking the activity of LasR is thought to inactivate all *P. aeruginosa* QS. However, we have shown here not only that the *rhl* system is expressed late in a *lasR* mutant but also that RhlR is able to overcome the *las* system when the latter is deficient, by activating specific LasR-controlled functions. This suggests that *P. aeruginosa*

can circumvent the deficiency of one of its QS systems by allowing the other to take over. This should be taken into account in the light of new therapies directed against QS in *P. aeruginosa*. In this respect, targeting other levels of the QS circuitry, such as PQS/4-quinolone signalling (Lesic *et al.*, 2007) or the RhlR/C_4-HSL system, might represent interesting alternatives.

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