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# The *Pseudomonas* Quinolone Signal Regulates *rhl* Quorum Sensing in *Pseudomonas aeruginosa*

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**The opportunistic pathogen *Pseudomonas aeruginosa* uses intercellular signals to control the density-dependent expression of many virulence factors. The *las* and *rhl* quorum-sensing systems function, respectively, through the autoinducers *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone ($C_4$-HSL), which are known to positively regulate the transcription of the elastase-encoding gene, *lasB*. Recently, we reported that a second type of intercellular signal is involved in *lasB* induction. This signal was identified as 2-heptyl-3-hydroxy-4-quinolone and designated the *Pseudomonas* quinolone signal (PQS). PQS was determined to be part of the quorum-sensing hierarchy since its production and bioactivity depended on the *las* and *rhl* quorum-sensing systems, respectively. In order to define the role of PQS in the *P. aeruginosa* quorum-sensing cascade, *lacZ* gene fusions were used to determine the effect of PQS on the transcription of the quorum-sensing system genes *lasR*, *lasI*, *rhlR*, and *rhlI*. We found that in *P. aeruginosa*, PQS caused a major induction of *rhlI′-lacZ* and had lesser effects on the transcription of *lasR′-lacZ* and *rhlR′-lacZ*. We also observed that the transcription of both *rhlI′-lacZ* and *lasB′-lacZ* was cooperatively effected by $C_4$-HSL and PQS. Additionally, we present data indicating that PQS was not produced maximally until cultures reached the late stationary phase of growth. Taken together, our results imply that PQS acts as a link between the *las* and *rhl* quorum-sensing systems and that this signal is not involved in sensing cell density.**

*Pseudomonas aeruginosa* is a ubiquitous environmental organism capable of infecting a wide variety of animals, plants, and insects. As a human pathogen, this bacterium is a major source of opportunistic infections in both immunocompromised individuals and cystic fibrosis patients. *P. aeruginosa* is now the leading source of gram-negative nosocomial infections (25) and causes chronic lung infections in approximately 90% of cystic fibrosis patients (7). The ability of this organism to cause devastating infections stems from the production of an arsenal of virulence factors, several of which are controlled according to cell density through an elegant mechanism known as quorum sensing. In gram-negative bacteria, most quorum-sensing systems consist of a LuxR-type transcriptional activator (R protein) and an acylated homoserine lactone signal molecule (autoinducer) (see reference 4 for a review). When a bacterial culture is at a low cell density, basal levels of autoinducer and R protein are produced. As a population grows, the concentration of autoinducer increases with cell density until it reaches a threshold concentration where it binds to and thereby activates an R protein. Activated R protein then activates specific genes, causing cell density-dependent gene expression.

In *P. aeruginosa*, there are at least two quorum-sensing systems, *las* and *rhl*, which control the expression of numerous genes (see reference 21 for a review). The *las* quorum-sensing system consists of the transcriptional activator LasR and the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-$C_{12}$-HSL), the synthesis of which is directed by the LasI autoinducer synthase (5, 15, 16). Similarly, the *rhl* system consists of the transcriptional activator RhlR and the autoinducer *N*-butyryl-L-homoserine lactone ($C_4$-HSL), the synthesis of which is directed by the RhlI autoinducer synthase (12, 13, 17). Together, the *las* and *rhl* systems have been shown to regulate between 1 and 4% of the genes carried by *P. aeruginosa*, demonstrating the global importance of these intercellular signaling systems (27).

While the *las* and *rhl* quorum-sensing systems consist of two separate regulons, their functions are apparently not independent. Both of these systems have been shown to control the transcription of *lasB*, which encodes the major virulence factor LasB elastase (2, 5, 8, 18). It has also been shown that the *las* quorum-sensing system controls the *rhl* quorum-sensing system at both the transcriptional and posttranslational levels (9, 20). The discovery of this link between the systems indicated that they were arranged in a hierarchy where the *las* quorum-sensing system is dominant over the *rhl* quorum-sensing system.

Recently, we reported that *P. aeruginosa* produced a third intercellular signal in addition to the two homoserine lactone-type autoinducers. The *Pseudomonas* quinolone signal (PQS), which is capable of inducing *lasB* in *P. aeruginosa*, was identified as 2-heptyl-3-hydroxy-4-quinolone (22). Our initial characterization of PQS indicated that the production of this novel intercellular signal occurred only in the presence of an active form of LasR. We also demonstrated that exogenous PQS exhibited bioactivity in the *lasR* mutant strain PAO-R1 but not in the *lasR rhlR* double mutant strain PAO-JP3. Taken together, these results suggested that LasR was required for PQS production and that RhlR was important for PQS bioactivity (22). This indicated that PQS was intertwined in the quorum-sensing hierarchy, but because of the induction cascade that occurs, its exact role was difficult to determine. Therefore, we set out to further characterize PQS bioactivity and production.

In this report, we present data indicating that PQS provides a link between the *las* and *rhl* quorum-sensing systems. We show that PQS strongly induced *rhlI* in *P. aeruginosa* and had

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant genotype or phenotype | Reference |
|---|---|---|
| *P. aeruginosa* strains | | |
| PAO1 | Wild type | 6 |
| PDO100 | *rhlI*::Tn*501-2*; derived from strain PAO1 | 2 |
| PAO-R1 | *lasR*::Tet; derived from strain PAO1 | 5 |
| PAO-JP2 | *lasI*::Tet *rhlI*::Tn*501-2*; derived from strain PDO100 | 18 |
| Plasmids | | |
| pLPRI | *rhlI′-lacZ* transcriptional fusion | 26 |
| pPCS1001 | *lasR′-lacZ* transcriptional fusion | 20 |
| pPCS1002 | *rhlR′-lacZ* transcriptional fusion | 20 |
| pPCS223 | *lasI′-lacZ* transcriptional fusion | 26 |
| pLP170 | *lacZ* transcriptional fusion vector | 23 |
| pTS400 | *lasB′-lacZ* translational fusion | 15 |
| pECP39 | *trcp-*Δ*lasR*; encodes autoinducer-independent form of LasR | 22 |

lesser positive effects on the transcription of *lasR* and *rhlR*. Our data indicated that *rhlI* and *lasB* are both cooperatively regulated by PQS and $C_4$-HSL. We also determined that PQS was produced maximally in late stationary phase, suggesting that this intercellular signal was not involved in sensing cell density. These exciting results imply an expanded role for *P. aeruginosa* cell-to-cell signaling and suggest a revised model of *P. aeruginosa* quorum sensing.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids utilized in this study are listed in Table 1. For each experiment, strains were plated from 10% milk stocks maintained at −70°C. All *P. aeruginosa* strains were grown in PTSB (5% peptone, 0.25% tryptic soy broth, pH 7.0) (14) supplemented with 200 μg of carbenicillin per ml when appropriate. All cultures were grown at 37°C, and liquid cultures were shaken at 270 rpm. Plasmids were transformed into *P. aeruginosa* by electroporation (24).

**PQS bioassays.** PQS bioassays were performed as described previously (22). Briefly, cells from overnight cultures were harvested by centrifugation and resuspended in PTSB. These washed cells were used to inoculate fresh medium to a starting $A_{660}$ of 0.05. At mid-log phase, subcultures were adjusted (using washed cells) to an $A_{660}$ of 0.02 in fresh medium and added to tubes containing dried signal molecules or extracts. Synthetic PQS was used in all bioassays except those performed with the extracts from the growth curve experiments. The cultures were grown for 18 h and assayed in duplicate for β-galactosidase (β-Gal) activity as described by Miller (10).

**Growth curve extracts.** Freshly plated cells of *P. aeruginosa* strain PAO-JP2(pECP39) were inoculated into 10 ml of medium and grown overnight. Subcultures were started at an $A_{660}$ of 0.05 and grown until they reached the log phase of growth. At that time, the cells were washed with PTSB and used to adjust 400 ml of medium to an $A_{660}$ of 0.05. At specified time points over the next 48 h, a 10-ml aliquot was removed for $A_{660}$ determinations and ethyl acetate extractions. Aliquots were centrifuged at 10,000 × *g* for 10 min at room temperature, and the supernatant was extracted twice with equal volumes of ethyl acetate (22). Water was removed from the extracts by the addition of sodium sulfate followed by evaporation of the solvent in a rotary evaporator. The dried extracts were stored at −20°C until being assayed for PQS activity as described previously, using the bioassay strain *P. aeruginosa* PAO-R1(pTS400) (22). This experiment was repeated four times, and results are presented as the percentage of the maximal activation seen during each separate experiment.

**Viability growth curves.** Washed cells from late-log-phase cultures of *P. aeruginosa* strains PAO-JP2(pECP39) and PAO1 were used to start a 35-ml subculture at an $A_{660}$ of 0.05. At specified intervals, 1 ml of each culture was removed and the $A_{660}$ was determined. Serial dilutions were spread on duplicate plates which were incubated overnight at 37°C. Colonies on plates with between 30 and 300 colonies were counted, and these data were used to produce viability curves. Viability curves were repeated at least twice, and results are presented as the mean CFU/ml $\pm \sigma^{n-1}$.

## RESULTS

**PQS induces *rhlI* in *P. aeruginosa*.** Previous research has shown that PQS was capable of inducing *lasB* expression in *P. aeruginosa* (22). During the initial characterization of PQS, it was also suggested that active LasR protein is required for PQS production and that RhlR is required for PQS bioactivity (22). The latter conclusion was based on the fact that exogenous PQS induced a *lasB′-lacZ* fusion in a *lasR* mutant but not in a *lasR rhlR* double mutant. This inability to activate *lasB* in the absence of RhlR suggested that PQS may be acting directly or indirectly through *rhl* quorum sensing. While PQS appeared to be an integral part of the *P. aeruginosa* quorum-sensing hierarchy, its exact role in cell-to-cell signaling was not apparent. To determine if PQS was affecting the components of *rhl* or *las* quorum sensing, we monitored β-Gal activity in strain PAO-R1 (*lasR*) containing *rhlI′-lacZ* (pLPRI), *rhlR′-lacZ* (pPCS1002), *lasI′-lacZ* (pPCS223), or *lasR′-lacZ* (pPCS1001) reporter fusions. (The *lasR* mutant strain PAO-R1 was used because it does not produce PQS [22], which allows the effects of exogenously added PQS to be determined.) These experiments produced a very interesting result. We discovered that PQS caused a major induction of *rhlI′-lacZ* in strain PAO-R1 (pLPRI) (Fig. 1). The addition of 50 μM PQS to this strain led to the production of 48,428 ± 7,610 Miller units of β-Gal activity, a notable increase over the 2,380 ± 611 Miller units of β-Gal activity produced in the absence of PQS. In contrast, the *lasI′-lacZ* fusion was not affected by exogenously added PQS, and as expected, β-Gal expression from strain PAO-R1 containing the vector control, pLP170, was unaffected by the addition of PQS (Fig. 1). This suggested that the production of the $C_4$-HSL signal, but not the 3-oxo-$C_{12}$-HSL signal, was positively regulated by PQS. It should be noted here that the induction of *rhlI* caused by the addition of PQS to strain PAO-R1(pLPRI) shown in Fig. 1 (48,428 ± 7,610 Miller units) does not fully complement *rhlI′-lacZ* expression to the level seen in the wild-type strain containing pLPRI (139,797 ± 10,291 Miller units [see Table 2]). This indicates that either LasR or a LasR-controlled factor (in addition to PQS) is required for complete induction of *rhlI* in *P. aeruginosa*. We also found that the expression of *lasR′-lacZ* and *rhlR′-lacZ* increased approximately twofold in the presence of PQS (Fig. 1). This was interesting because the activation of *lasR* by a LasR-controlled factor such as PQS indicates that PQS may be part of a positive feedback loop within the quorum-sensing hierarchy. However, we must point out that both *lasR* and *rhlR* are partially expressed in the absence of PQS and LasR (Fig. 1), demonstrating that other factors are important for their expression.

The results presented in Fig. 1 show that the addition of PQS had the greatest effect on the expression of *rhlI* compared to the other quorum-sensing system genes. It has previously been reported that *rhlI* is positively regulated by both $C_4$-HSL–RhlR and 3-oxo-$C_{12}$-HSL–LasR (9). To learn more about the factors affecting *rhlI* induction, we monitored the expression of *rhlI′-lacZ* from plasmid pLPRI contained in the *P. aeruginosa* wild-type strain PAO1 and the defined mutant strains PDO100 (*rhlI*) and PAO-R1 (*lasR*). In the presence or absence of $C_4$-HSL and/or PQS, the expression of *rhlI′-lacZ* in strain PDO100(pLPRI) was very similar to that in the wild-type strain (Table 2). This indicated that both $C_4$-HSL and PQS did not affect *rhlI* expression in an *rhlI* mutant, implying that elements capable of inducing full *rhlI* expression were present in strain PDO100 (*rhlI*). However, examination of the expression of *rhlI′-lacZ* in the *lasR* mutant strain PAO-R1 indicated that both $C_4$-HSL and PQS are important regulators of *rhlI*. The addition of 5 μM $C_4$-HSL or 50 μM PQS to strain PAO-R1 (pLPRI) caused the expression of *rhlI′-lacZ* to be 20 or 35%, respectively, of that seen in strain PAO1(pLPRI) (Table 2). Most interestingly, the addition of both $C_4$-HSL and PQS

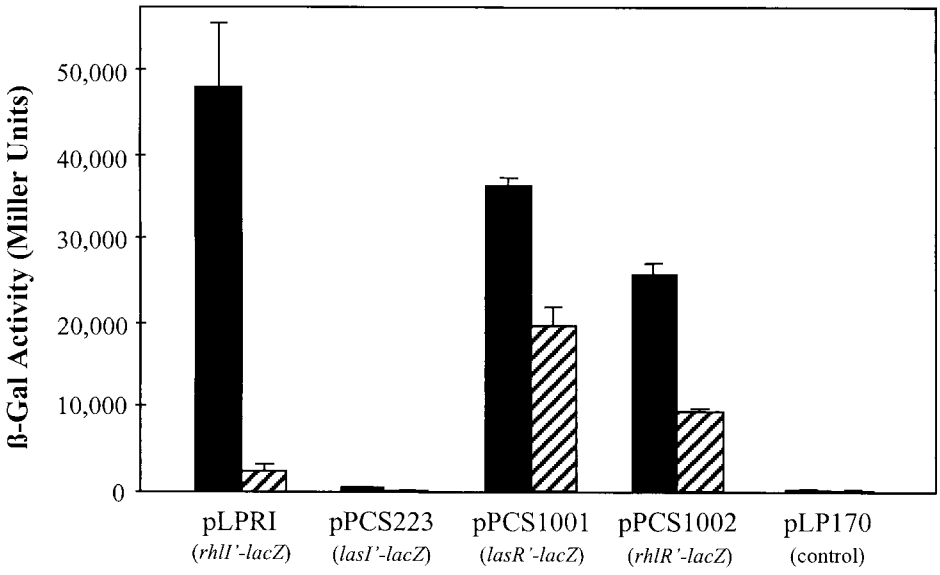


FIG. 1. Effect of PQS on expression of quorum-sensing components in *P. aeruginosa*. Cultures of *P. aeruginosa* strain PAO-R1 (*lasR*) containing the indicated plasmid were grown for 18 h in the presence (black bars) or absence (hatched bars) of 50 μM PQS and assayed for β-Gal activity. Results are expressed in Miller units ± $\sigma^{n-1}$ and are the means for duplicate β-Gal assays from at least three separate experiments. Reporter fusions contained on plasmids: pLPRI, *rhlI'-lacZ*; pPCS223, *lasI'-lacZ*; pPCS1001, *lasR'-lacZ*; pPCS1002, *rhlR'-lacZ*; pLP170, control vector with promoterless *lacZ*.

caused *rhlI'-lacZ* to be expressed at approximately the wild-type level, indicating that these signals cooperatively effect *rhlI* expression. Taken together, the data presented in Fig. 1 and Table 2 indicate that *rhlI* expression falls under the control of both PQS and $C_4$-HSL. In addition, these data confirm the results of Latifi et al. (9), who reported that both the *las* and *rhl* quorum-sensing systems affect the expression of *rhlI*.

**PQS and $C_4$-HSL cooperatively induce *lasB*.** Previous data have shown that three intercellular signals, 3-oxo-$C_{12}$-HSL, $C_4$-HSL, and PQS, are each capable of activating *lasB* in *P. aeruginosa* (15, 17, 22). The fact that these signals are not produced independently of each other has made it difficult to determine the role of each individual signal with regard to *lasB* activation. Research focused on the role of PQS in *lasB* expression had indicated that *lasB* induction by PQS required a functional *rhl* quorum-sensing system (22). This led us to speculate that PQS and $C_4$-HSL were functioning in a cooperative manner to activate *lasB*. In order to determine whether $C_4$-HSL and PQS have cooperative effects on *lasB* expression, the activation of *lasB'-lacZ* in strains PDO100 and PAO-R1 was monitored in the presence of PQS and/or $C_4$-HSL. In strain PAO-R1, PQS caused *lasB'-lacZ* to be induced to 28% of the wild-type level seen in strain PAO1(pTS400) (Table 3). The addition of $C_4$-HSL alone had a minor effect on *lasB'-lacZ* in this strain, but the addition of $C_4$-HSL and PQS together caused *lasB'-lacZ* to be induced to 58% of the wild-type level (Table 3). This indicated that these two signals had a cooperative effect on *lasB* induction. A similar effect was seen in strain PDO100(pTS400), where the lack of a functional *rhl* quorum-sensing system caused *lasB'-lacZ* to be expressed at 36% of the wild-type level seen in strain PAO1(pTS400) (Table 3). The addition of PQS alone caused an increase of *lasB'-lacZ* expression to 64% of the wild-type level, and the addition of $C_4$-HSL alone restored *lasB'-lacZ* expression to the expected wild-type level (Table 3). Addition of PQS and $C_4$-HSL together had an additive effect on the induction of *lasB'-lacZ*, which was expressed at approximately twice the wild-type level seen in strain PAO1(pTS400).

**PQS is not constitutively produced.** The production of the $C_4$-HSL and 3-oxo-$C_{12}$-HSL signals is initiated during the log phase of growth. Pearson et al. (16) purified 3-oxo-$C_{12}$-HSL from late-log-phase cultures of strain PAO1, and $C_4$-HSL has

TABLE 2. Regulation of *rhlI* by PQS and/or $C_4$-HSL[a]

| Signal added | β-Gal activity[b] of the following strain containing pLPRI (*rhlI'-lacZ*) | |
|---|---|---|
| | PAO-R1 (*lasR*) | PDO100 (*rhlI*) |
| PQS (50 μM) | 48,428 ± 7,610 | 141,151 ± 27,930 |
| $C_4$-HSL (5 μM) | 27,433 ± 1,797 | 136,143 ± 26,227 |
| PQS (50 μM) and $C_4$-HSL (5 μM) | 125,537 ± 11,596 | 126,468 ± 7,633 |
| None | 2,380 ± 611 | 123,873 ± 10,798 |

[a] Cultures were grown for 18 h in the presence of the indicated signal, and β-Gal activity was assayed.

[b] Data are the means for duplicate β-Gal assays from at least three separate experiments and are presented in Miller units ± $\sigma^{n-1}$. As a reference for these experiments, we determined that the wild-type *P. aeruginosa* strain PAO1 containing pLPRI produces 139,797 ± 10,291 U of β-Gal activity.

TABLE 3. Regulation of *lasB* by PQS and/or $C_4$-HSL[a]

| Signal added | β-Gal activity[b] of the following strain containing pTS400 (*lasB'-lacZ*) | |
|---|---|---|
| | PAO-R1 (*lasR*) | PDO100 (*rhlI*) |
| PQS (50 μM) | 8,907 ± 2,020 | 20,488 ± 2,908 |
| $C_4$-HSL (5 μM) | 831 ± 50 | 42,277 ± 4,503 |
| PQS (50 μM) and $C_4$-HSL (5 μM) | 15,170 ± 1,519 | 64,569 ± 6,884 |
| None | 16 ± 4 | 11,340 ± 3,427 |

[a] Cultures were grown for 18 h in the presence of the indicated signal, and β-Gal activity was assayed.

[b] Data are the means for duplicate β-Gal assays from at least three separate experiments and are presented in Miller units ± $\sigma^{n-1}$. As a reference for these experiments, we determined that the wild-type *P. aeruginosa* strain PAO1 containing pTS400 produces 31,857 ± 5,834 U of β-Gal activity.

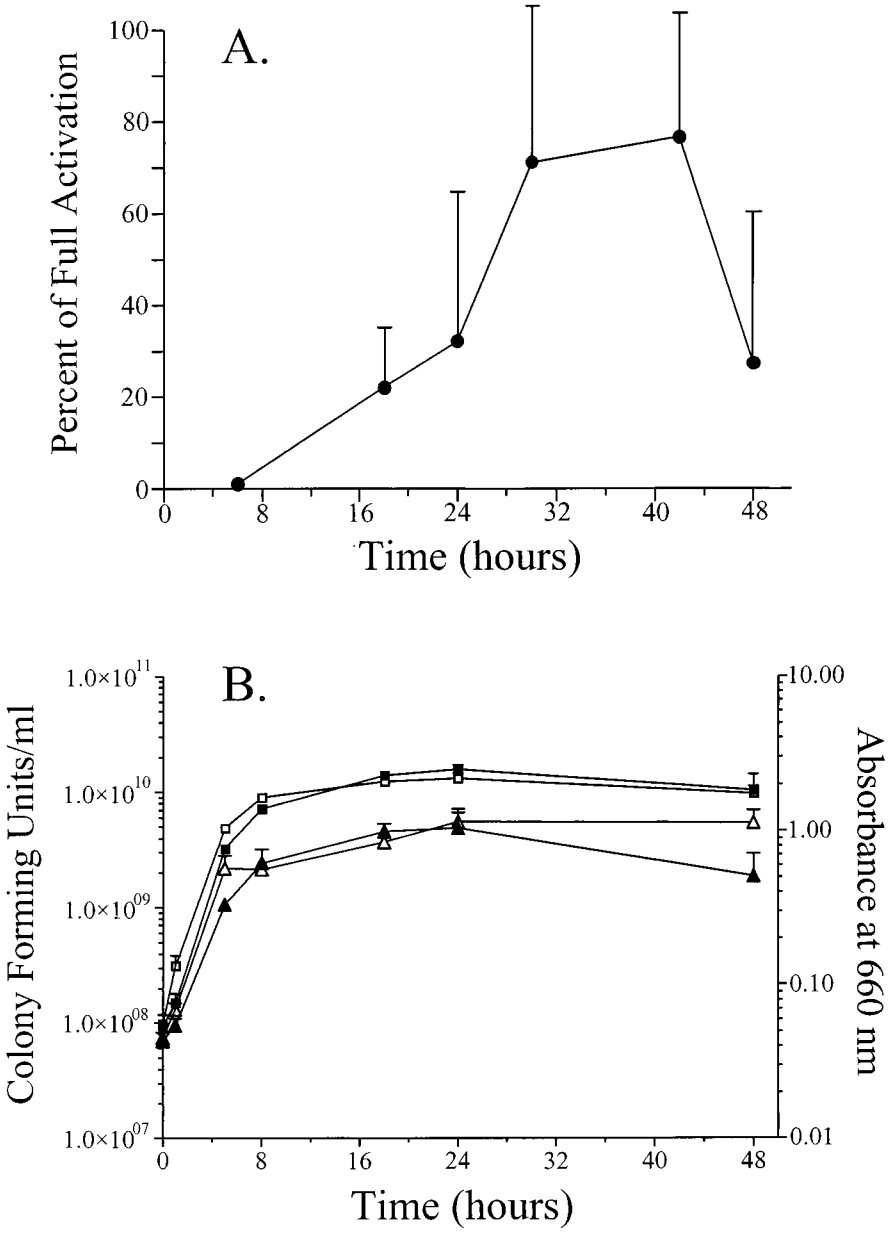


FIG. 2. PQS production is initiated in early stationary phase. (A) PQS bioassays (see Materials and Methods) were performed on culture supernatant extracts prepared throughout the growth cycle of *P. aeruginosa* strain PAO-JP2(pECP39). Results were derived from duplicate β-Gal assays from at least four separate experiments and are expressed as the mean percentage + $\sigma^{n-1}$ of the maximal activation seen during each separate growth curve experiment. (B) Viability and optical density curves for strains PAO-JP2(pECP39) (closed symbols) and PAO1 (open symbols). Cultures were sampled at various times during the growth cycle, and optical density (absorbance at 660 nm) was determined (squares). Samples were also serially diluted and plated to determine CFU per milliliter (triangles). Data are from at least two separate experiments performed in duplicate and are presented as the mean ± $\sigma^{n-1}$.

been isolated from extracts of early-stationary-phase cultures of strain PAO1 (17). It has also been shown that *lasR* and *rhlR* are induced during the last half of log-phase growth (20). These data combined with the fact that the production of PQS required active LasR led us to speculate that PQS would be produced during the late log phase of growth. In order to learn more about the production of PQS, we monitored its synthesis throughout the growth cycle. The PQS-producing strain PAO-JP2(pECP39) (22) was grown in PTSB for 48 h, and crude PQS was recovered from aliquots removed at specific intervals.

The results presented in Fig. 2 indicate that PQS was not found in strain PAO-JP2(pECP39) culture medium until the end of the log phase of growth. The maximal amount of this signal was found in cultures that were in the late stationary phase of growth (30 to 42 h after inoculation), and the concentration decreased after 48 h of growth (Fig. 2A). These results showed that PQS was produced maximally long after the *las* and *rhl* quorum-sensing systems had been activated, which suggested that its production required a factor, in addition to the *las* quorum-sensing system, that is not available until the stationary phase of growth. These results are especially interesting when one considers that the genes controlled by the *las* quorum-sensing system will be induced in strain PAO-JP2(pECP39) because the autoinducer-indepen-

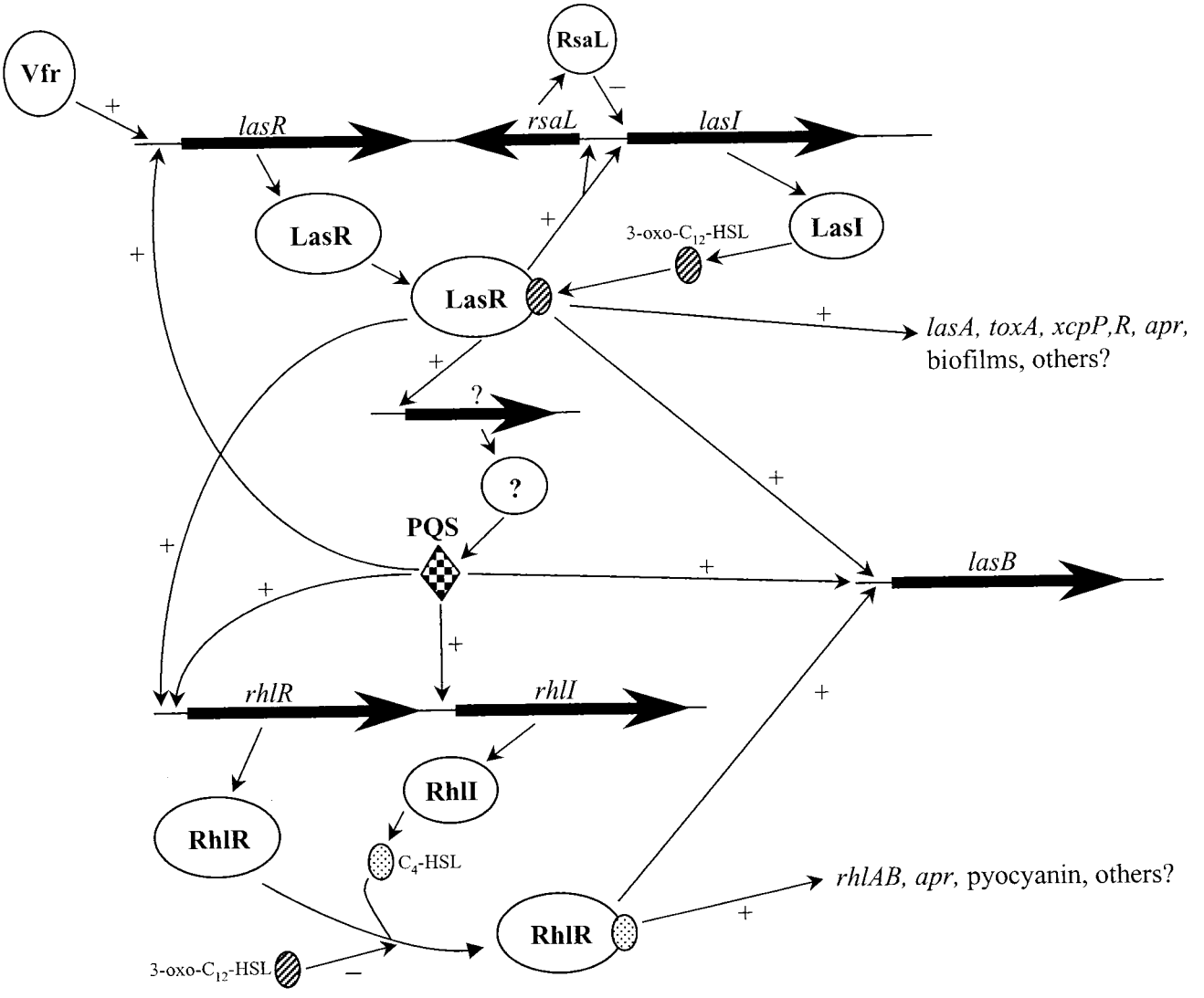


FIG. 3. Model of the *P. aeruginosa* quorum-sensing hierarchy. The quorum-sensing cascade begins with the induction of the *las* quorum-sensing system when cells reach a threshold density. Vfr induces *lasR* (1), and the concentration of 3-oxo-$C_{12}$-HSL increases to the point where it binds to and activates LasR. The LasR–3-oxo-$C_{12}$-HSL complex induces genes controlled by the *las* quorum-sensing system, including a negative regulator gene (*rsaL*) (3), *rhlR*, and an unidentified gene required for PQS production. PQS either directly or indirectly induces *rhlI*, which leads to the production of $C_4$-HSL that binds to and activates RhlR. The RhlR–$C_4$-HSL complex can then induce genes controlled by the *rhl* quorum-sensing system. At this time it is not known whether PQS is capable of directly activating RhlR or acts through another regulator. (Two additional unanalyzed LuxR homologs, which may play a role in the activity of PQS, are encoded by *P. aeruginosa* [www.pseudomonas.com].) Genes and proteins are indicated by thick arrows and unfilled circles, respectively. Plus or minus symbols indicate transcriptional activation or repression of the gene(s) at the end of an arrow, respectively. Blocking of the association between RhlR and $C_4$-HSL by 3-oxo-$C_{12}$-HSL is indicated by a minus symbol next to the arrow between 3-oxo-$C_{12}$-HSL and $C_4$-HSL at the bottom of the figure. Question marks indicate an unknown member(s) of the PQS synthesis pathway that is affected by LasR–3-oxo-$C_{12}$-HSL.

dent LasR encoded by pECP39 is expressed constitutively (data not shown). The determination of CFU and $A_{660}$ for cultures of strain PAO-JP2(pECP39) indicated that bacteria were viable and in the stationary phase of growth from approximately 8 to 48 h after inoculation (Fig. 2B). The CFU and optical density of strain PAO-JP2(pECP39) were very similar throughout the growth curve to those of the wild-type strain PAO1 (Fig. 2B), indicating that strain PAO-JP2(pECP39) was growing normally.

## DISCUSSION

We have discovered that the intercellular signal PQS controls the *rhl* quorum-sensing system at a transcriptional level. PQS was found to have a major positive effect on *rhlI* induction and lesser positive effects on the induction of *rhlR* and *lasR* (Fig. 1). These results, along with previous data which showed that PQS production required an active LasR protein (22), indicate that PQS may act as an additional connecting signal between the *las* and *rhl* quorum-sensing systems by transcriptionally regulating the autoinducer synthase gene, *rhlI*. This would be the third link between these two systems. LasR and 3-oxo-$C_{12}$-HSL were shown to positively regulate the transcription of *rhlR* (9, 20) and *rhlI* (9) in *P. aeruginosa* and *Escherichia coli*, and 3-oxo-$C_{12}$-HSL posttranslationally affected RhlR by blocking the interaction of $C_4$-HSL and RhlR in *E. coli* (20). The purpose of PQS acting as an additional linking signal between the two quorum-sensing systems is not known. Spec-

ulatively, the upregulation of *rhlI* by PQS (which implies a subsequent increase in $C_4$-HSL production) may be necessary to overcome the inhibitory effect of 3-oxo-$C_{12}$-HSL on the *rhl* quorum-sensing system. It is also interesting that Pearson et al. (19) showed that 3-oxo-$C_{12}$-HSL accumulated inside *E. coli* cells, while $C_4$-HSL freely diffused to reach an internal-external equilibrium. This could lead to an imbalance of autoinducer concentration that would require the upregulation of *rhlI* in order to provide enough intracellular $C_4$-HSL to compete with 3-oxo-$C_{12}$-HSL for binding to RhlR.

Another interesting finding with regard to *rhlI* regulation is that it was induced by PQS or $C_4$-HSL in our *lasR* mutant, but neither signal had an effect in our *rhlI* mutant (Table 2). Additionally, when added together PQS and $C_4$-HSL had a cooperative effect on *rhlI* induction in strain PAO-R1 (*lasR*) (Table 2). The reason that *rhlI* expression was not affected in strain PDO100 (*rhlI*) is not clear, but there is a plausible explanation. Since both PQS and the *las* quorum-sensing system have been shown to regulate *rhlI* (Fig. 1) (9), it is most likely that these elements are able to complement the *rhlI* mutation with regard to *rhlI′-lacZ* expression. Whether this theory is correct remains to be proven, but it is apparent that *rhlI* regulation is quite complex and probably involves multiple layers of control, perhaps working at different stages of growth (see below).

We also learned that PQS and $C_4$-HSL cooperatively induce *lasB*. Previous reports have shown that *lasB* transcription is positively regulated by all three *P. aeruginosa* intercellular signals (15, 17, 22). In addition, we knew that the induction of *lasB* by PQS required a functional *rhl* quorum-sensing system (22). Therefore, we examined the effect of adding both PQS and $C_4$-HSL to strains PAO-R1 and PDO100 containing pTS400 (Table 3). In both strains, PQS and $C_4$-HSL had a cooperative effect on the induction of *lasB*. In strain PAO-R1 (pTS400) the two signals had a synergistic effect on *lasB* induction, and in strain PDO100(pTS400) the signals' effect was additive. The reason for this difference is not clear, but in either case, *lasB′-lacZ* was induced to a greater level in the presence of both signals than with either individual signal. This indicates that the induction of *lasB* requires a complex chain of events that we are continuing to learn about.

Finally, our analysis of PQS production indicated that this signal was produced much later in the growth cycle than a typical quorum-sensing signal. The concentration of PQS in culture medium was negligible in the late log phase of growth and was at a maximum late in the stationary phase of growth (Fig. 2). PQS was most abundant between 30 and 42 h of growth and had decreased after 48 h of growth (Fig. 2). This indicated that strain PAO-JP2(pECP39) either made less PQS after 48 h of growth or produced a factor capable of degrading PQS at that time. Nevertheless, it is apparent that PQS is not involved in sensing cell density, because the signal is produced at a time after cell density has become stable. This leads one to ponder an interesting question. Given that $C_4$-HSL is produced during the log phase of growth and PQS is not produced until late in the stationary phase of growth, then what is the purpose of PQS inducing *rhlI*? We speculate that PQS may induce *rhlI* in order to further upregulate the *rhl* quorum-sensing system. This could be beneficial to the organism, since the *rhl* quorum-sensing system has been shown to control the production of elastase, alkaline protease, and the biosurfactant rhamnolipid (2, 8, 12, 17). The production of proteases at a later stage of growth could help increase the availability of nutrients during infections because of the tissue-destroying capabilites of these enzymes. At the same time, rhamnolipid helps cells to utilize long-chain fatty acids as sources of carbon (11), which would benefit bacteria that have depleted available nutrients. These theories lead us to conclude that *P. aeruginosa* may use PQS as a signal to induce genes controlled by the *rhl* quorum-sensing system in order to respond to stress encountered by late-stationary-phase cultures.

As more is learned about the quorum-sensing hierarchy of *P. aeruginosa*, it becomes clear that this is an extremely complex signal transduction pathway. To help clarify the circuitry of these interrelated systems, we have provided a schematic diagram that summarizes our present level of understanding (Fig. 3). While the effects of PQS are intriguing, we are only beginning to understand how this intercellular signal fits in the grand scheme of *P. aeruginosa* quorum sensing. The ability of PQS to induce an important virulence factor gene (*lasB*) and genes for quorum-sensing components (*rhlI*, *rhlR*, and *lasR*) indicates that understanding its role in quorum sensing will be a key to determining its effect on virulence.

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