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Regulation of Quorum Sensing by RpoS in *Pseudomonas aeruginosa*

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The LasR-LasI and RhlR-RhlI quorum-sensing systems are global regulators of gene expression in the opportunistic pathogen *Pseudomonas aeruginosa*. Previous studies suggest that the RhlR-RhlI system activates expression of *rpoS*. We constructed merodiploid strains of *P. aeruginosa* containing the native *rpoS* gene and an *rpoS-lacZ* fusion. Studies of *lacZ* transcription in these strains indicated that *rpoS* was not regulated by RhlR-RhlI. We also generated an *rpoS* null mutant. This *rpoS* mutant showed elevated levels of *rhlI* (but not *rhlR*) transcription, elevated levels of the RhlI-generated acylhomoserine lactone quorum-sensing signal, and elevated levels of RhlR-RhlI-regulated gene transcription. These findings indicate that there is a relationship between RpoS and quorum sensing, but rather than the RhlR-RhlI system influencing the expression of *rpoS*, it appears that RpoS regulates *rhlI*.

Two acylhomoserine lactone (acyl-HSL) quorum-sensing signal systems are involved in transcriptional regulation of many genes in *Pseudomonas aeruginosa*. The two systems are the LasR-LasI and RhlR-RhlI (also termed the Vsm) regulatory circuits. LasI is required for synthesis of the signal *N*-(3-oxododecanoyl)-HSL ($3OC_{12}$-HSL), and LasR is a transcriptional activator that responds to 3O-C_{12}-HSL. The RhlI enzyme is responsible for synthesis of *N*-butyryl-HSL (C_4-HSL), and RhlR is a transcriptional activator that responds to C_4-HSL. The two quorum-sensing systems do not act independently of one another, but a hierarchy exists with LasR required for the activation of *rhlR* and to some extent *rhlI* (for reviews of quorum sensing see references 7, 8, and 24). Quorum sensing has been reported to control the expression of genes, including those coding for extracellular enzymes, secondary metabolites (e.g., pyocyanin and hydrogen cyanide), toxins, genes of unknown function, and the *rpoS* gene, which codes for a homolog of an *Escherichia coli* stationary-phase σ factor (30).

In *E. coli*, RpoS competes with the housekeeping sigma factor (σ70) for RNA polymerase, and RpoS enhances expression of more than 35 genes, many of which are involved in the general stress response (14, 18). *P. aeruginosa rpoS* has been cloned and sequenced (30). As with *E. coli*, the *rpoS* gene in *P. aeruginosa* is induced as cultures enter stationary phase (6). A *P. aeruginosa rpoS* null mutant shows a defect in the general stress response and a defect in the regulation of exoproducts, including pyocyanin and exotoxin A (12, 29).

The *rhl* quorum-sensing system has been reported to activate transcription of *rpoS* in *P. aeruginosa* (15). As an extension of an investigation of quorum-sensing-regulated genes in *P. aeruginosa* (31), we constructed an *rpoS* mutant. This mutant overproduced the RhlR-RhlI-controlled secondary metabolite pyocyanin. This finding prompted our study of the relationship between the RhlR-RhlI system and RpoS. The studies described here with the $RpoS^-$ mutant and with *P. aeruginosa* strains carrying an *rpoS-lacZ* allele led to the conclusion that RhlR-I does not regulate RpoS expression but that RpoS does influence the transcription of *rhlI*.

The bacterial strains and plasmids used in this study are described in Table 1. Unless otherwise specified, cultures were grown in Luria-Bertani (LB) broth or on LB agar (26) as described previously (28). For pyocyanin measurements, *P. aeruginosa* was grown in *Pseudomonas* broth. DNA manipulations involved standard methods (2), and DNA was sequenced at the University of Iowa DNA Core Facility. Southern blotting was performed using standard methods (2) with PCR-generated digoxigenin-11-dUTP-labeled DNA probes. Transformation of *P. aeruginosa* was done as follows. Cells from a mid-logarithmic-phase culture (optical density at 600 nm [OD_{600}], 0.6 to 0.8) were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and suspended in cold 150 mM $MgCl_2$ for 30 min. After another centrifugation, the cells were suspended for 10 min in 100 μl of cold 150 mM $MgCl_2$ with 0.5 to 1 μg of plasmid DNA. The cells were heat shocked at 37°C for 3 min and then incubated on ice for 5 min, after which 1 ml of LB broth was added. This suspension was incubated with shaking at 37°C for 30 min and then cells were plated on selective agar.

To construct a *P. aeruginosa rpoS* mutant, we amplified a 2.8-kb *P. aeruginosa* chromosomal DNA fragment containing the *rpoS* gene by using the Expand long-template PCR system (Boehringer Mannheim). The *rpoS* fragment was cloned to form pRPOS-1 by using an Original TA cloning kit (Invitrogen, Carlsbad, Calif.). This plasmid was digested with *Hin*cII and ligated to a PCR-generated 1.1-kb *aacC1* (encoding gentamicin acetyltransferase) fragment from pBBR1MCS-5 to generate pRPOS-Gm. A pRPOS-Gm *Eco*RI fragment containing the inactivated *rpoS* gene was cloned into *Eco*RI-digested pSUP102 to generate pMW102, which contains the *aacC1* gene 266 bp downstream of the translational start codon of *rpoS*. The *rpoS* in pMW102 is flanked by 1.5 kb of *P. aeruginosa* DNA upstream and 1.3 kb downstream. With *E. coli* HB101(pRK2013) as a helper, we used triparental mating to mobilize pMW102 into *P. aeruginosa* PAO1 (5). We selected gentamicin-resistant colonies and identified a tetracycline-sensitive mutant, PAO-MW20. This mutant contained *rpoS*::*aacC1* in place of *rpoS*, as shown by a Southern analysis with *aacC1* and *rpoS* probes.

For complementation of PAO-MW20, a 1.2-kb chromosomal DNA fragment containing the *rpoS* gene from *P. aerugi-*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
P. aeruginosa		
PAO1	Wild type	10
PAO-MW20	*rpoS* mutant of PAO1, Gm^r	This study
PAO-R1	Δ*lasR* derivative of PAO1, Tc^r	9
PAO-MW1	Δ*lasI* Δ*rhlI* derivative of PAO1, Hg^r Tc^r	31
PDO111	*rhlR*::Tn*501* derivative of PAO1, Hg^r	3
E. coli		
DH5α	F^- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	26
S17-1	*thi pro hsdR recA RP4-2* (Tet::Mu) (Km::Tn*7*)	28
Plasmids		
pCR2.1	TA cloning vector, Ap^r	Invitrogen
pEX1.8	Broad-host-range expression vector, Ap^r	23
pSUP102	pACYC184 carrying mobRP4, Cm^r Tc^r	27
pRK2013	tra^+ helper plasmid for triparental matings, Km^r	5
pHRP315	Ω Sm^r/Sp^r cassette, Ap^r	20
pHRP309	Promoterless *lacZ*, Gm^r	20
pTL61T	*lacZ* transcriptional fusion vector, Ap^r	17
pBBR1MCS-5	Broad-host-range vector, Gm^r	13
pRPOS-1	pCR2.1 containing a 2.8-kb *rpoS* fragment	This study
pRPOS-Gm	pRPOS-1 with *aacC1* interrupting the *rpoS* gene, Gm^r	This study
pMW102	*rpoS*::*aacC1* on pSUP102, mobilizable *rpoS* knockout plasmid, Gm^r	This study
pMW105	pEX1.8 containing p*tac-rpoS*, Ap^r	This study
pMW301	Broad-host-range *hcnAB-lacZ* reporter, Ap^r Cb^r	This study
pMW303	Broad-host-range *phzABC-lacZ* reporter, Ap^r Cb^r	This study
pMW304	Broad-host-range *rhlR-lacZ* reporter, Gm^r Sp^r	This study
pMW305	Broad-host-range *rhlI-lacZ* reporter, Gm^r Sp^r	This study
pTn5-Gm	pUTmini-Tn*5* containing *aacC1*, Gm^r	This study
pJBA24	Cloning vector for creating reporter fusions on Tn*5*, Ap^r	1
pMRP24-RPOS	pJBA24 containing *rpoS* promoter, Ap^r	This study
pMW24-RPOS	*rpoS* promoter-*lacZ* fusion vector, Ap^r	This study
pUTminiTn5-Gm	Delivery plasmid for mini-Tn*5*-Gm, Ap^r Gm^r	This study
pTn5-RPOS	pUTmini-Tn*5* carrying *rpoS-lacZ* from pMW24-RpoS	This study

nosa was created by PCR amplification using the Expand long-template PCR system, digested with *Eco*RI and *Hin*dIII, and ligated to *Eco*RI-*Hin*dIII-digested pEX1.8 to generate the p*tac-rpoS* plasmid pMW105. Mutations in *rpoS* were complemented by transformation of *P. aeruginosa* with pMW105.

For the construction of *rhlI-lacZ* and *rhlR-lacZ* transcriptional fusions, a two-step cloning procedure with pHRP315 and pHRP309 was used (20). A 588-bp PCR product beginning 435 bp upstream of the *rhlR* translational start site and a 445-bp PCR product beginning 250 bp upstream of the *rhlI* transcriptional start site were used as promoter fragments for making *lacZ* fusions. Each promoter fragment was directionally cloned downstream of the spectinomycin resistance cassette of pHRP315. The promoter fragments were then cloned upstream of the *'lacZ* gene of pHRP309 to form the *rhlI-lacZ* and *rhlR-lacZ* transcriptional fusion plasmids pMW304 and pMW305. The *hcnAB-lacZ* and *phzABC-lacZ* fusion plasmids pMW301 and pMW303 were constructed as follows. Expand long-template PCR was used to amplify the chromosomal *hcnAB-lacZ* and *phzABC-lacZ* transcriptional fusions in *P. aeruginosa* qsc128 and qsc131 (31). The *hcnAB-lacZ* fragment begins 619 bp upstream of the qsc128 *lacZ* insertion, and the *phzABC-lacZ* fragment begins 886 bp upstream of the qsc131 *lacZ* insertion. The two PCR fragments were cloned into *Sal*I-digested, end-polished pEX1.8 to form pMW301 (*hcnAB-lacZ*) and pMW303 (*phzABC-lacZ*). To minimize read-through from plasmid promoters, orientations were selected that placed the transcriptional terminator of pEX1.8 upstream of the promoter regions of the *phz* and *hcn* fusions. The addition of $3OC_{12}$-HSL and C_4-HSL to the *P. aeruginosa* $LasI^-$ $RhlI^-$ mutant, PAO-MW1, containing pMW301 or pMW303 resulted in a >100-fold increase in β-galactosidase. This confirmed the presence of the quorum-sensing-controlled promoters on pMW301 and pMW303.

We constructed *rpoS-lacZ* reporters as follows. A 240-bp PCR-generated DNA fragment beginning 201 bp upstream of the *rpoS* transcriptional start site was ligated to *Kpn*I-*Xba*I-digested pJBA24, forming pMRP24-RPOS. This *rpoS* plasmid was digested with *Sph*I and *Hin*dIII and ligated to a 3.2-kb PCR-generated *lacZ* fragment from pTL61T to form pMW24-RPOS. *Not*I-digested pMW24-RPOS was ligated to *Not*I-digested pUTminiTn5-Gm to yield pTn5-RPOS. This plasmid is a mobilizable vector containing an *rpoS-lacZ* transcriptional fusion and a gentamicin resistance marker within mini-Tn*5*. The *rpoS-lacZ* fusion is flanked by transcriptional stops to minimize read-through from chromosomal promoters and is in the opposite orientation to the gentamicin resistance marker within the transposon. Introduction of pTn5-RpoS into *P. aeruginosa* was done by mating with *E. coli* S17-1 as described above. Five transconjugants that contained single Tn*5* insertions at locations other than the chromosomal *rpoS* gene (as determined by a Southern blot analysis) that showed growth rates in LB broth that were indistinguishable from that of the wild-type strain (PAO1), and that showed a wild-type pyocyanin production phenotype (see below) were used for expression studies.

To analyze *lacZ* expression in reporter constructs, cultures were inoculated to an initial OD_{600} of 0.001 to 0.005, and

β-galactosidase was measured at various time points as outlined by Miller (19). To monitor levels of acyl-HSLs, *P. aeruginosa* was grown in LB broth buffered with morpholinepropanesulfonic acid (MOPS) (250 mM, pH 7.0). Cells were removed from 5-ml samples by centrifugation at 6,000 × *g* for 15 min. The cell-free culture fluid was extracted three times with 5 ml of acidified ethyl acetate, and the extract was dried under a stream of nitrogen gas. The residue was dissolved in 500 μl of acidified ethyl acetate, and acyl-HSL levels were assessed by means of bioassays (21, 22) with synthetic $3OC_{12}$-HSL and C_4-HSL as standards (Quorum Sciences, Inc., Coralville, Iowa). Pyocyanin was extracted from *P. aeruginosa* PAO1 and PAO-MW20 grown in *Pseudomonas* broth at 37°C, with shaking (starting OD_{600}, 0.005), with chloroform. Pyocyanin in the chloroform extracts was measured spectrophotometrically at 520 nm according to the method of Essar et al. (4).

A recent report described a subset of quorum-sensing-controlled genes in *P. aeruginosa* that required acyl-HSLs for full activation, but even in the presence of acyl-HSLs, activation did not occur until the onset of stationary phase (31). Because previous investigations had indicated that the RhlR-RhlI quorum-sensing system activates *rpoS* (15), we hypothesized that the responses of genes that required both quorum sensing and stationary phase might be mediated by *rpoS*. To better understand the interactions between *rpoS* and quorum sensing in *P. aeruginosa* PAO1, an $RpoS^-$ mutant was constructed through allelic exchange of the wild-type *rpoS* with a plasmid-borne *rpoS*::*aacC1* mutation. Cultures of the $RpoS^-$ mutant grown in LB broth or agar were obviously dark blue, whereas the parent strain, PAO1, was not. Because pyocyanin is a blue pigment, we measured the pyocyanin levels in cultures. Pyocyanin was detected earlier and accumulated to higher levels in the *rpoS* mutant than in the wild type (Fig. 1). In late-logarithmic-phase cultures, the $RpoS^-$ mutant produced approximately 10-fold more pyocyanin than did the wild type, PAO1. Complementation of the *rpoS* mutation in PAO-MW20 with pMW105 resulted in pyocyanin levels similar to those found in the parent (Fig. 1). These experiments indicate that *rpoS* negatively regulates the production or secretion of pyocyanin. Our results are consistent with a previous report of increased pyocyanin levels (twofold) in a *P. aeruginosa rpoS* mutant compared to the parent strain (29), although the closer examination of pyocyanin synthesis reported here shows that in logarithmic-phase cultures repression of pyocyanin synthesis by RpoS can be much greater than twofold.

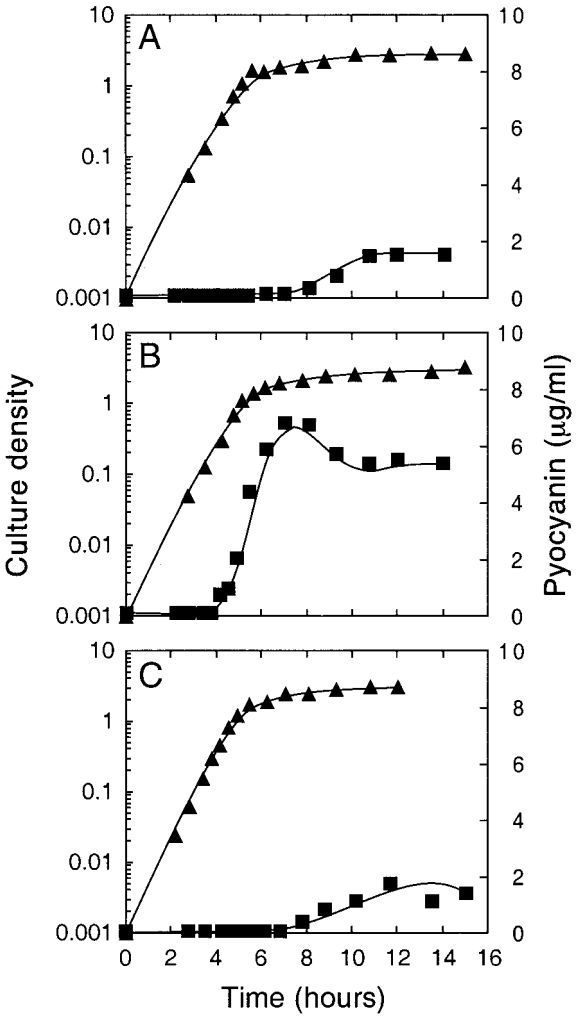

FIG. 1. Influence of RpoS on pyocyanin production. Growth was measured as OD_{600} (▲) and pyocyanin levels (■) are expressed as a function of time in *P. aeruginosa* PAO1 (A), the *rpoS* mutant strain PAO-MW20 (B), and PAO-MW20 with the p*tac-rpoS* plasmid pMW105 (C). For the latter (C), isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 100 μM.

The production of pyocyanin is induced by the *rhl* quorum-sensing system in *P. aeruginosa* (16). Furthermore, the *phzAB CDEFG* operon is required for pyocyanin production, and transcription of a *phzABCDEFG* operon-*lacZ* chromosomal fusion is increased more than 700-fold by the addition of $3OC_{12}$-HSL and C_4-HSL to a $LasI^-$ $RhlI^-$ mutant (31). Thus, increased pyocyanin production in $RpoS^-$ mutants might be the result of enhanced transcription of *rhlR* or *rhlI*. To test this hypothesis, we measured β-galactosidase activity in *P. aeruginosa* carrying an *rhlR-lacZ* reporter on pMW304 or an *rhlI-lacZ* reporter on pMW305. Levels of β-galactosidase synthesis directed by the *rhlR* reporter on pMW304 were similar in the $RpoS^-$ mutant PAO-MW20 and the parent strain, PAO1 (Fig. 2A). However, β-galactosidase synthesis directed by the *rhlI* reporter on pMW305 occurred earlier during growth and reached a higher level in PAO-MW20 than it did in the parent (Fig. 2B). These experiments indicate that RpoS represses the transcription of *rhlI* (probably indirectly) but not of *rhlR* early in growth.

The repression of *rhlI* by RpoS appears to be relatively mild, but small changes in *rhlI* expression may have a significant impact on C_4-HSL levels in early logarithmic phase, where C_4-HSL levels are normally low. Thus, we monitored acyl-HSL synthesis during culture growth (Table 2). As assessed with an Rhl bioassay (22), C_4-HSL levels in the *rpoS* mutant were elevated compared to those of the parent. As assessed with a Las bioassay (21), levels of $3OC_{12}$-HSL were unaffected by the RpoS mutation. In early-logarithmic-phase cultures of the $RpoS^-$ mutant, we detected sufficient levels of C_4-HSL for induction of Rhl quorum-sensing-regulated genes, but in early-logarithmic-phase wild-type cultures there was no detectable C_4-HSL. This suggests that the increase in pyocyanin production that results from an RpoS mutation is due to increased levels of C_4-HSL, which are the result of enhanced transcription of *rhlI*.

To establish that the increased pyocyanin levels in *P. aeruginosa* PAO-MW20 were due to increased transcription of the quorum-sensing-regulated *phz* genes, we compared *phzC-lacZ* transcription in the *rpoS* mutant to that in the parent (Fig. 3). Induction of *phzC-lacZ* occurred earlier in the *rpoS* mutant and coincided with the increased levels of C_4-HSL (Fig. 2). To determine if other genes that are regulated by quorum sensing

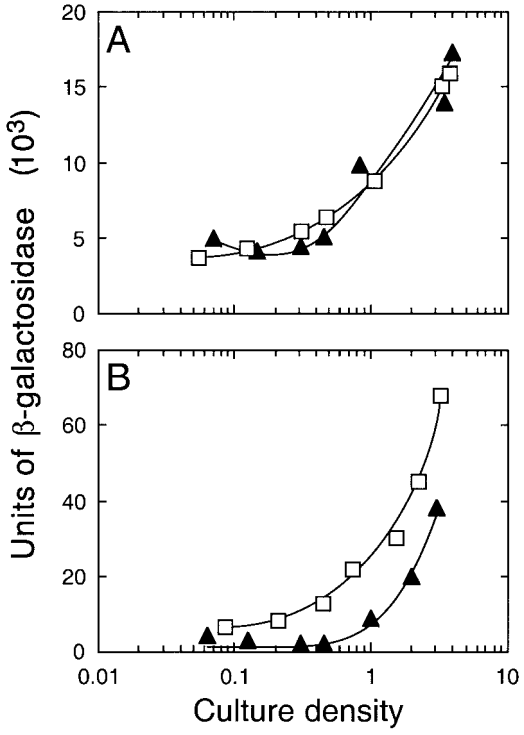

FIG. 2. Influence of *rpoS* on *rhlR* and *rhlI* transcription in *P. aeruginosa*. (A) *P. aeruginosa* containing *rhlR-lacZ* plasmid pMW304; (B) *P. aeruginosa* containing *rhlI-lacZ* plasmid pMW305. Levels of β-galactosidase in the wild-type PAO1 (▲) and the *rpoS* mutant MW20 (□) are shown. Culture density is measured as OD_{600}.

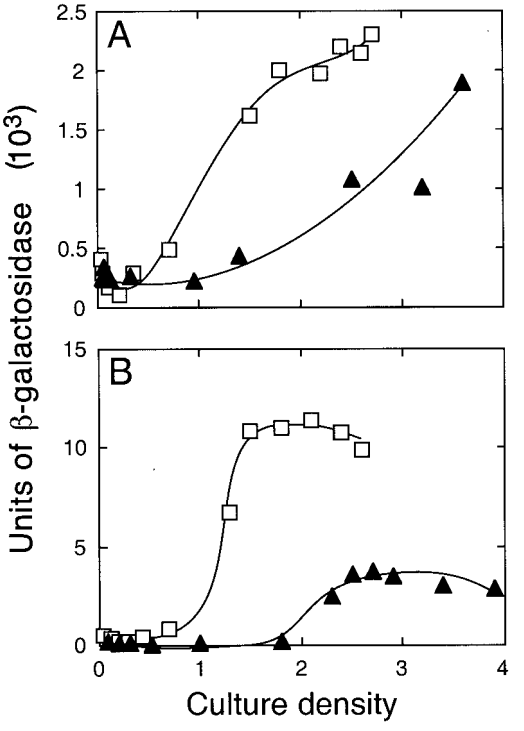

FIG. 3. Expression of *hcnAB-lacZ* (pMW301) (A) and *phzABC-lacZ* (pMW303) (B) in *P. aeruginosa* PAO1 (▲) and PAO-MW20 (□).

are affected by RpoS, we monitored expression of an *hcnAB-lacZ* transcriptional fusion. The *hcn* operon is involved in the production of the secondary metabolite hydrogen cyanide, and it is activated by the *rhl* quorum-sensing system (31). As with *phzC-lacZ*, transcription of *hcnB-lacZ* occurred earlier and was increased in the $RpoS^-$ mutant, compared to the parent (Fig. 3).

Expression of *rpoS* in *P. aeruginosa* has been shown by RNA analysis and immunodetection of the RpoS protein to be growth phase regulated (6, 30). As in *E. coli*, transcription of *rpoS* and the amount of RpoS in cells increases at the onset of stationary phase. It has also been reported that *P. aeruginosa rpoS* is activated by RhlR-RhlI (15). This suggests that there is a regulatory loop in which RhlR-RhlI activates *rpoS* and the *rpoS* product, in turn, represses *rhlI*. To begin an investigation of this possible loop, we constructed *rpoS-lacZ* merodiploids in the *P. aeruginosa* parent (PAO1), in a $LasR^-$ mutant (PAO-R1), in an $RhlR^-$ mutant (PDO111), and in a $LasI^-$ $RhlI^-$ mutant (PAO-MW1), as described above. As expected, in the parent, expression of the *rpoS-lacZ* fusion was growth phase dependent. However, contrary to published reports (15), the pattern of expression in the quorum-sensing mutants was indistinguishable from the pattern in the wild type. Furthermore, the addition of $3OC_{12}$-HSL and C_4-HSL to the $LasI^-$ $RhlI^-$ mutant did not affect *rpoS* expression (Fig. 4). These results indicate that the RhlR-RhlI quorum-sensing system does not regulate *rpoS* transcription under the conditions of our experiment.

Our results indicate that RpoS functions to repress *rhlI*, and this repression manifests itself in early logarithmic phase. Expression of RpoS-controlled genes during exponential growth

TABLE 2. Acyl-HSL concentrations in cultures of a *P. aeruginosa* $RpoS^-$ mutant (PAO-MW20) and the wild type, PAO1

Culture density (OD_{600})	$3OC_{12}$-HSL (μM)[a]		C_4-HSL (μM)[a]	
	PAO1	MW20	PAO1	MW20
0.25	0.5 ± 0.2	0.7 ± 0.1	<0.1	0.4 ± 0.1
1.5	3.1 ± 0.4	2.8 ± 0.3	0.5 ± 0.1	1.9 ± 0.1
3.0	4.4 ± 0.7	4.0 ± 0.6	3.9 ± 0.3	8.4 ± 0.7
4.0	3.8 ± 0.4	3.5 ± 0.5	12.8 ± 0.7	19.9 ± 1.1

[a] Values are means ± 1 standard deviation.

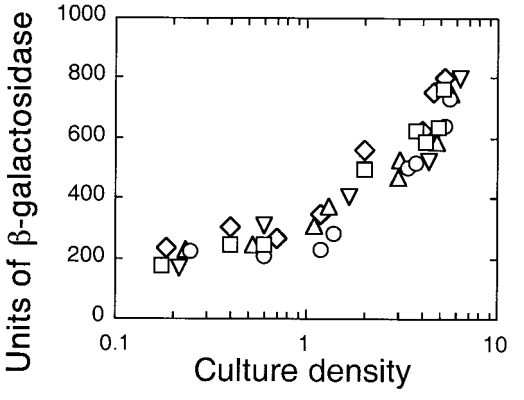

FIG. 4. Transcription of chromosomal *rpoS*::*lacZ* in *P. aeruginosa* PAO1 (△), the $LasR^-$ mutant PAO-R1 (□), the $RhlR^-$ mutant PDO111 (▽), the $LasI^-$ $RhlI^-$ double mutant PAO-MW1 (○), and PAO-MW1 in medium containing $3OC_{12}$-HSL (2 μM) and C_4-HSL (5 μM) (◇). Five *rpoS-lacZ* transconjugants were analyzed, and the results were indistinguishable from those shown.

is known to occur in *E. coli*, where RpoS-dependent expression of *xthA* (25) and *katG* (11) occurs in the early logarithmic phase of growth. In the case described here, RpoS functions in early-logarithmic-phase repression of *rhlI* transcription. It is not an unreasonable hypothesis that RpoS functions to repress all early C_4-HSL-regulated genes. Suh et al. (29) reported that an *rpoS* mutant caused more tissue damage in a rat chronic-lung-infection model than did the wild type and suggested that this could have resulted from elevated levels of pyocyanin in the mutant. Although our data are not inconsistent with this suggestion, it is possible that other genes regulated by C_4-HSL might encode virulence factors that contribute to lung damage. In fact, hydrogen cyanide production could easily contribute to tissue damage.

In summary, we have investigated the influence of quorum sensing on *rpoS* expression in *P. aeruginosa*. Our data indicate that *rpoS* transcription is not regulated by quorum sensing. This is in contrast to a previous report that RhlR-RhlI is required for the growth phase-dependent expression of *rpoS* (15). In our study, quorum-sensing mutants as well as the wild type showed induction of the *rpoS-lacZ* reporter upon entry into stationary phase. Also, the addition of acyl-HSL signals to a quorum-sensing signal production mutant carrying *rpoS-lacZ* did not affect expression of β-galactosidase (Fig. 4). The differences between our results and those reported previously could be explained by the copy number of the reporters (single copy in our study versus multicopy), differences in *P. aeruginosa* strains, or experimental conditions. Regardless of the explanation for the conflicting results, we report a novel relationship between *rpoS* and quorum sensing in *P. aeruginosa* involving the repression of *rhlI* transcription by *rpoS*.

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