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Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*

(gene activation/*N*-acylhomoserine lactones/autoinduction/virulence)

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ABSTRACT **The opportunistic human pathogen *Pseudomonas aeruginosa* produces a variety of virulence factors, including exotoxin A, elastase, alkaline protease, alginate, phospholipases, and extracellular rhamnolipids. The previously characterized *rhlABR* gene cluster encodes a regulatory protein (RhlR) and a rhamnosyltransferase (RhlAB), both of which are required for rhamnolipid synthesis. Another gene, *rhlI*, has now been identified downstream of the *rhlABR* gene cluster. The putative RhlI protein shares significant sequence similarity with bacterial autoinducer synthetases of the LuxI type. A *P. aeruginosa rhlI* mutant strain carrying a disrupted *rhlI* gene was unable to produce rhamnolipids and lacked rhamnosyltransferase activity. Rhamnolipid synthesis was restored by introducing a wild-type *rhlI* gene into such strains or, alternatively, by adding either the cell-free spent supernatant from a *P. aeruginosa* wild-type strain or synthetic *N*-acylhomoserine lactones. Half-maximal induction of rhamnolipid synthesis in the *rhlI* mutant strain required 0.5 μM *N*-butyrylhomoserine lactone or 10 μM *N*-(3-oxohexanoyl)homoserine lactone. The *P. aeruginosa rhlA* promoter was active in the heterologous host *Pseudomonas putida* when both the *rhlR* and *rhlI* genes were present or when the *rhlR* gene alone was supplied together with synthetic *N*-acylhomoserine lactones. The RhlR–RhlI regulatory system was found to be essential for the production of elastase as well, and cross-communication between the RhlR–RhlI rhamnolipid regulatory system and the LasR–LasI elastase regulatory system was demonstrated.**

The use of small signaling molecules to monitor population density enables bacterial cells to rapidly adjust their metabolism in response to changing environmental conditions (1, 2). The LuxR–LuxI system mediates the cell-density dependent control of luminescence (*lux*) gene transcription in *Vibrio fischeri* (reviewed in ref. 2). LuxR is a transcriptional activator, and its activity depends on a diffusible substance, termed autoinducer, which is synthesized by cells containing a functional *luxI* gene. In *Pseudomonas aeruginosa*, the LasR–LasI system regulates the expression of the *lasB* gene, encoding elastase, and is assumed to serve as a means for cell-to-cell communication to allow the coordinated expression of virulence-associated genes (3, 4). The TraR–TraI system regulates the conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens* (5–7) and the production of exoenzymes and of the antibiotic carbapenem in *Erwinia carotovora* is regulated by the CarR–ExpI system (8–10).

All of the small diffusible autoinducers known to date belong to the class of *N*-acylated homoserine lactones (HSLs), harboring acyl substituents of various lengths (2, 11). A synthetic version of the *V. fischeri* *N*-(3-oxohexanoyl)-HSL autoinducer (VAI) restored the production of extracellular proteases in *E. carotovora* and *P. aeruginosa* mutants, thus providing evidence for the crossfunctionality of this compound (9). Early on, VAI was suggested to be widely involved in bacterial signaling (12). A novel strategy for the detection of VAI in culture fluids and for the isolation of *luxI* homologs, based on the activation of bioluminescence in *Escherichia coli* in the presence of *luxR* and *luxAB*, has revealed that VAI is produced in many different bacteria and that the corresponding VAI synthetases are equivalent (13). The interchangeability and specificity of the LuxR–LuxI and LasR–LasI regulatory systems have been demonstrated experimentally by using recombinant *E. coli* strains containing *lux* and *las* gene constructs (14). In these experiments, *lasB* gene expression was activated by the heterologous LuxR protein in the presence of synthetic VAI. Similarly, the *lux* genes were activated by LasR and the *P. aeruginosa* autoinducer (PAI), *N*-(3-oxododecanoyl)-HSL. Crossreactivity of the autoinducers with the heterologous activators was not observed.

Rhamnolipid biosynthesis in *P. aeruginosa* occurs during the late-exponential and stationary phases of growth, typically under conditions of nitrogen or iron limitation (15). Two genes (*rhlAB*) encoding a rhamnosyltransferase, RhlAB, which catalyzes the transfer of rhamnose from TDP-rhamnose to β-hydroxydecanoyl-β-hydroxydecanoate have recently been described (16) and a regulatory gene (*rhlR*) encoding a transcriptional activator, RhlR, was shown to positively regulate rhamnolipid biosynthesis (17). In this paper we present evidence that the RhlR regulatory protein acts in conjunction with the autoinducers *N*-butyryl-HSL and VAI.§

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions. The *P. aeruginosa* strains used have been described (16, 17). *Pseudomonas putida* KT2442 (18) was used as a heterologous host for gene expression studies. Plasmids pUO100, pUO103, and pUO106 contain the *rhlR*, *rhlI*, and *rhlRI* genes, respectively, inserted as *Eco*RI–*Hin*dIII fragments in the medium-copy-number, wide-host-range plasmid pJRD215 (19). Plasmid pUO61 contains an *rhlA'::'lacZ* fusion in the high-copy-number plasmid pPZ10 (16). For rhamnolipid production, a nitrogen-limited mineral salts medium (GS medium) with 2% glycerol as the carbon source was used (15). Spent supernatants from *P. aeruginosa* were prepared by centrifugation and filter sterilization of the culture fluid through a 0.45-μm membrane following cultivation for 5 days in GS medium at 37°C. Synthetic factor 2 and VAI were

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Abbreviations: HSL, homoserine lactone; PAI, *Pseudomonas aeruginosa* autoinducer; VAI, *Vibrio* autoinducer.

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§The *rhlI* sequence reported in this paper has been deposited in the GenBank data base (accession no. U11811).

obtained from E. P. Greenberg (University of Iowa), and PAI was provided by L. Passador (University of Rochester).

Genetic Techniques. The *P. aeruginosa* PG201::*'rhlI'* mutant strain and the PG201::*'lasR'* and PAO1::*'lasR'* strains were constructed by a plasmid integration approach (17). Details of the procedure will be made available upon request. A functional *lasR* gene was amplified by use of the primers 5'-GGACCAGGTGTGACTGGGTA-3' and 5'-ATGGCGAGAACCTGCCCTTC-3', resulting in a 1138-bp fragment which was subcloned into pCRII (Invitrogen) and transferred as an *Eco*RI fragment into pJRD215, to yield pUO125. Transformation of *Pseudomonas* strains and sequencing of DNA were carried as described (16).

Enzyme Assays and Rhamnolipid Quantitation. β-Galactosidase and rhamnosyltransferase activities and rhamnolipid concentrations were determined as described (16, 17). Elastase was detected in nutrient agar plates containing elastin (20) and quantitated by the elastin-Congo red assay (21).

RESULTS

Isolation and Characterization of the *rhlI* Gene. A 5.8-kb *Eco*RI–*Hin*dIII fragment spanning the *rhl* gene cluster (Fig. 1*A*) was subcloned to yield pUO58, and the DNA sequence of both strands of a 1.4-kb *Bgl* II–*Sal* I subfragment containing the region downstream of the *rhlABR* genes was determined. A 603-bp open reading frame was identified between the *rhlABR* gene cluster and the *pheC* gene, which maps at 47 min on the *P. aeruginosa* chromosome (Fig. 1*A*). The DNA sequence of the corresponding *rhlI* gene and the deduced primary structure of the putative RhlI protein are shown in Fig. 1*B*. The putative 22-kDa RhlI protein shares significant sequence similarity with several of the HSL autoinducer synthetases (Fig. 2). The overall identities are 31% with *V. fischeri* LuxI (22), 29% with *P. aeruginosa* LasI (4), 29% with *E. carotovora* ExpI (8), and 21% with *A. tumefaciens* TraI (5).

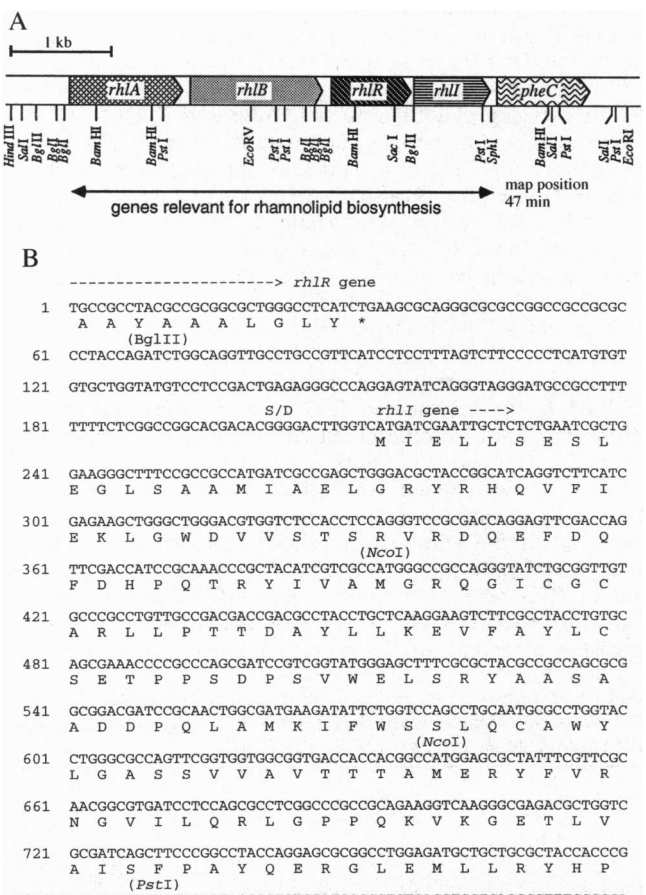

FIG. 1. (*A*) Map of the *P. aeruginosa* PG201 *rhlABRI* gene cluster involved in rhamnolipid biosynthesis and its regulation. (*B*) Nucleotide sequence of the *rhlI* gene, which is located between the *rhlR* and *pheC* genes. A putative Shine–Dalgarno (S/D) sequence and the relevant restriction sites are shown above the sequence.

Characterization of a Mutant Strain Affected in the *rhlI* Gene. Rhamnolipid production and rhamnosyltransferase activity were absent in a *P. aeruginosa rhlI* mutant strain which was constructed by disrupting the *rhlI* gene. Various synthetic *N*-acyl-HSL compounds were examined for their stimulatory effects on rhamnolipid synthesis in this strain. The newly discovered *N*-butyryl-HSL (factor 2) from *P. aeruginosa* (23), VAI, and PAI were added to exponentially growing cultures (OD_{600}, 0.15) of the *rhlI* mutant strain, at final concentrations ranging from 10 nM to 30 μM, and the rhamnolipid concentrations of the stationary-phase subcultures (OD_{600}, 5.5 ± 0.5) were determined (Fig. 3*A*). Factor 2 was the most efficient inducer of rhamnolipid synthesis, with 100 nM resulting in partial induction and 1 μM being sufficient for full induction. VAI at ≥10 μM was also capable of inducing rhamnolipid production. PAI did not trigger rhamnolipid formation, even at a concentration of 30 μM. The addition of 1% (vol/vol) supernatant of the PG201 wild-type strain previously grown for 5 days under the same conditions stimulated rhamnolipid synthesis in the *rhlI* mutant strain, and the addition of 10% supernatant resulted in full induction (Fig. 3*B*). A qualitatively identical induction pattern was observed when the supernatant from the rhamnolipid-deficient UO299 Tn*5*-GM mutant strain, which is affected in the rhamnosyltransferase gene (*rhlA*), was used. Interestingly, supernatant from an *rhlR* mutant culture did not contain any inducing activity, indicating that the *rhlR* gene itself is required for autoinducer synthesis, presumably through transcriptional activation of the *rhlI* gene. This finding is consistent with the fact that all other autoinducer synthetases studied to date are positively regulated by their cognate regulators in conjunction with the appropriate

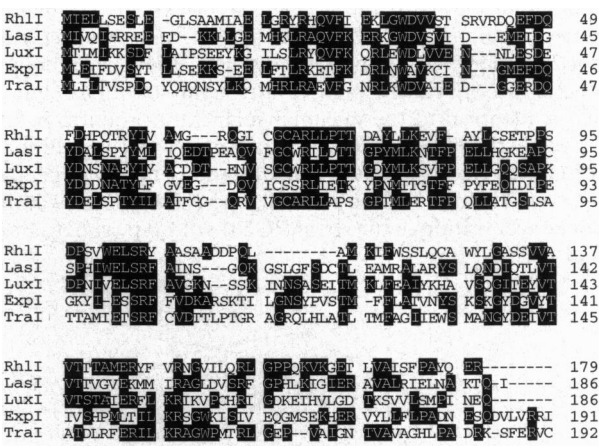

FIG. 2. Primary structure alignment of the RhlI protein with other HSL autoinducer synthetases. Consensus amino acid residues are indicated as white letters on a black background. Numbers refer to amino acid residues.

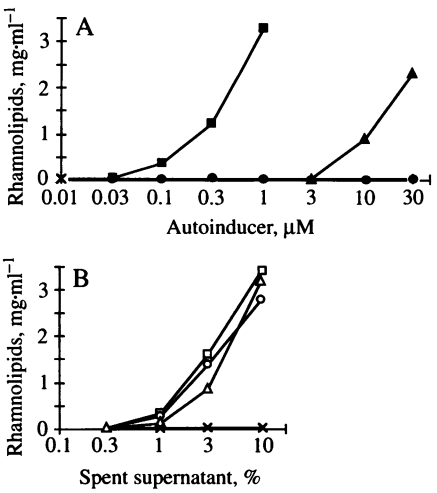

FIG. 3. Induction of rhamnolipid production by synthetic *N*-acyl-HSLs or spent culture supernatants. (*A*) Synthetic *N*-acyl HSLs, including factor 2 (■), VAI (▲), and PAI (●). (*B*) Spent culture supernatants from the PG201 wild-type strain (□), the UO299 *rhlA* mutant strain (○), an *rhlR* mutant strain (×), and a *lasR* mutant strain (△). The percentage values refer to the relative proportion of supernatant added.

autoinducer. Supernatant from a *lasR* mutant strain exhibited rhamnolipid inducing activity comparable to that of the PG201 wild-type strain. Since *lasI* transcription and PAI production are absent in *lasR* mutant strains (4), the rhamnolipid autoinducer appears to be different from PAI.

Effect of Autoinducer Concentration on Rhamnolipid Formation. Fig. 4 shows rhamnolipid production by the *rhlI* mutant strain in response to the addition of factor 2 during the early-exponential growth phase. Rhamnolipid production started within 3 hr after induction, but the maximal volumetric rhamnolipid synthesis rate occurred some 20–36 hr after induction. The maximal volumetric surfactant productivities in response to various concentrations of spent PG201 supernatant or synthetic factor 2 were subsequently calculated from the rhamnolipid concentrations measured in samples taken 20, 24, 30, and 36 hr after induction. The dose–response curves obtained in this way showed a linear increase in the maximal rhamnolipid synthesis rates (Fig. 5). Maximal stimulation occurred with ≥8% PG201 supernatant and ≥1 μM with factor 2. Under the given conditions, the maximal rhamnolipid synthesis rate reached a plateau at roughly 60 $\mu g \cdot ml^{-1} \cdot hr^{-1}$, which is identical to the maximal surfactant production rate observed in the PG201 wild-type strain under these conditions. As estimated from Fig. 5, the concentration of factor 2 needed for half-maximal induction was ≈0.5 μM. Hence, the autoinducer concentration in the spent PG201 wild-type supernatant was at least 10 μM.

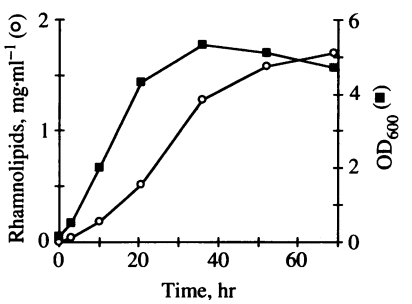

FIG. 4. Growth and rhamnolipid production of an *rhlI* mutant strain in the presence of factor 2. GS minimal medium was used and 0.6 μM factor 2 was added during the early-exponential growth phase.

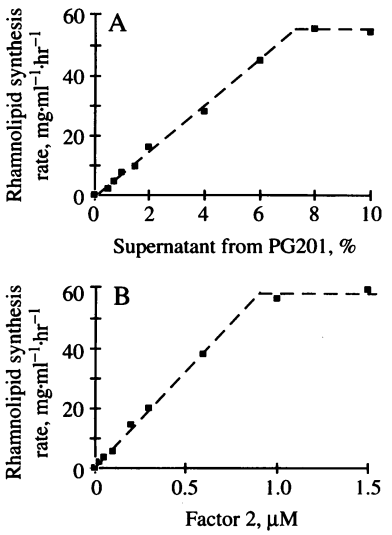

FIG. 5. Maximal volumetric rhamnolipid production rate observed during the cultivation of an *rhlI* mutant strain in the presence of spent culture supernatant from the PG201 wild-type strain (*A*) or factor 2 (*B*).

Control of the *rhlA* Promoter by the RhlR–RhlI Regulatory System. The activity of the *rhlA* promoter was studied by using plasmid pUO61, carrying an *rhlA*::*lacZ* fusion, in combination with plasmids containing *rhlR*, *rhlI*, or *rhlRI*. In *P. aeruginosa* PG201 carrying pUO61, referred to here as PG201[61], the addition of spent culture supernatant from PG201 or of synthetic factor 2 led to a significant increase in β-galactosidase, and this effect was also observed when multiple copies of *rhlR* and/or *rhlI* were present (Table 1). The same two-plasmid system was used to study the autoinducer-dependent *rhlA* promoter activation in the heterologous host *P. putida* KT2442, which lacks the *rhl* gene cluster (U.A.O., unpublished results). In *P. putida* KT2442[61]/pUO100, containing the *rhlA*::*lacZ* fusion and multiple copies of the *rhlR* gene, the *rhlA* promoter was active upon addition of spent PG201 supernatant or of VAI or factor 2, whereas PAI had no effect (Table 2). Furthermore, *rhlA* promoter activity was high when both *rhlR* and *rhlI* were present in multiple copies.

Effect of the Cloned RhlR–RhlI Regulatory System on Rhamnolipid and Elastase Formation. We reported previously that an *rhlR* mutant strain showed pleiotropic effects on elastase and pyocyanin synthesis (17). Since elastase production in *P. aeruginosa* requires the LasR–LasI autoinducer regulatory system (4, 11, 14), the *rhlR* and *rhlI* genes appear to represent a second autoinducer regulatory system which is

Table 1. Stimulation of *rhlA* promoter activity in response to the addition of autoinducer or to the presence of multiple copies of the *rhlR* and *rhlI* genes in *P. aeruginosa* PG201[61]

Addition of supernatant or of gene(s) in trans	β-Galactosidase activity, Miller units		
	LB medium	GS minimal medium	
		Expon.	Stat.
None	40	680	7,700
PG201 supernatant (2%)	70	4200	12,600
Synthetic factor 2 (0.5 μM)	160	4800	14,000
rhlR	1600	3500	23,000
rhlI	260	2200	18,000
rhlR, rhlI	1150	5600	15,000

β-Galactosidase activity was determined after 20 hr of cultivation in LB medium and after 20 hr [exponential phase (Expon.)] and 120 hr [stationary phase (Stat.)] of cultivation in GS minimal medium.

Table 2. Activation of the *rhlA* promoter in the heterologous host *P. putida* KT2442[61]/pUO100, carrying multiple copies of the *rhlR* gene, by autoinducers or the cloned *rhlI* gene

Gene(s) in trans	Autoinducer	β-Galactosidase activity, Miller units	
		LB medium	M9 medium
rhlR	None	<10	<10
rhlR	PG201 supernatant (3%)	90	1100
rhlR	PAI (1 μM)	<10	<10
rhlR	VAI (1 μM)	50	520
rhlR	Factor 2 (1 μM)	310	160
rhlR, rhlI	None	2850	9800

β-Galactosidase activity was determined after 20 hr of cultivation in LB medium or in M9 medium containing 0.5% glucose. Cultures in LB medium were grown to an OD_{600} of 3.0–3.5. In M9 medium the OD_{600} reached was 0.3–0.5.

involved in elastase production. Rhamnolipid and elastase formation in the *P. aeruginosa* PG201 and PAO1 wild-type strains and in the *rhlR, rhlI*, and *lasR* mutant strains containing plasmid-borne copies of the corresponding wild-type genes were investigated (Table 3). Surfactants and elastase were absent in the *rhlR* or *rhlI* mutant strains, but both activities were at least partially restored upon addition of the corresponding wild-type genes. Mutant strains affected in the *lasR* gene lacked elastase but produced rhamnolipids at levels comparable to those of the wild-type strains. These findings suggest that both the RhlR–RhlI and LasR–LasI regulatory systems are required for elastase formation and that only RhlR–RhlI affects rhamnolipid production. Interestingly, an *rhlR* mutant harboring multiple copies of the *lasR* gene produced elastase at a 4-fold higher level than the PG201 strain, and rhamnolipids were also detectable, albeit at levels of only about 4% of those found in the PG201 wild-type strain. Similarly, *lasR* mutants derived from either the PG201 or the PAO1 strain produced significant amounts of elastase when multiple *rhlR* gene copies were present. These findings suggest that the *rhlR* and *lasR* genes are, to some extent, interchangeable and that crossregulation occurs when these genes are present in multiple copies.

Table 3. Rhamnolipid production and elastase activity in *P. aeruginosa* wild-type and mutant strains harboring multiple copies of *rhlR, rhlI*, or *lasR*

Strain	Gene(s) on plasmid	Rhamnolipids, $mg \cdot ml^{-1}$	Elastase, unit(s)
PAO1/pJRD215	—	2.20	1.70
PG201/pJRD215	—	1.60	0.39
PG201/pUO100	*rhlR*	2.02	1.05
PG201/pUO103	*rhlI*	2.13	0.26
PG201/pUO106	*rhlR, rhlI*	2.67	0.68
PG201::*'rhlI'*/pJRD215	—	<0.005	<0.03
PG201::*'rhlI'*/pUO103	*rhlI*	0.91	0.06
PG201::*'rhlR'*/pJRD215	—	<0.005	<0.03
PG201::*'rhlR'*/pUO100	*rhlR*	0.98	0.75
PG201::*'rhlR'*/pUO125	*lasR*	0.06	1.67
PG201::*'lasR'*/pJRD215	—	1.57	<0.03
PG201::*'lasR'*/pUO100	*rhlR*	1.82	0.22
PG201::*'lasR'*/pUO125	*lasR*	1.50	1.48
PAO1::*'lasR'*/pJRD215	—	1.92	<0.03
PAO1::*'lasR'*/pUO100	*rhlR*	2.04	0.60
PAO1::*'lasR'*/pUO125	*lasR*	1.85	1.26

Rhamnolipid concentrations were determined after 100 hr of cultivation in GS minimal medium; mean values of duplicate orcinol assays are shown. Elastase activities were measured after 18 hr of cultivation in LB medium; 1 unit of elastase activity corresponds to 1 A_{495} $unit \cdot ml^{-1} \cdot hr^{-1}$ in the elastin-Congo red assay.

DISCUSSION

The results presented here provide strong evidence that the regulation of rhamnolipid production in *P. aeruginosa* is mediated by the RhlR–RhlI system involving an autoinducer. A tentative model for the regulation of rhamnolipid production is shown in Fig. 6. According to this model, the RhlR regulatory protein is activated by binding its cognate autoinducers, factor 2 and/or VAI, which are produced by the RhlI autoinducer synthetase. The *rhlI* gene itself is under the control of the RhlR regulator. The binding of activated RhlR protein to target sites upstream of the *rhlA* promoter enhances transcription of the *rhlAB* operon, which encodes rhamnosyltransferase, the key enzyme of rhamnolipid biosynthesis.

The sequence-homology studies suggest that the RhlR–RhlI pair belongs to the LuxR–LuxI family of quorum-sensing regulatory systems (2). In *P. aeruginosa*, the well-characterized LasR–LasI autoinducer regulatory system involving PAI is responsible for *lasB* gene activation (3, 4, 11, 14). Another autoinducer, VAI, originally detected in *V. fischeri*, was also found in spent culture supernatants of *P. aeruginosa* (12). The addition of VAI to certain elastase-deficient mutant strains restored elastolytic activity, and thus this compound was initially thought to act in conjunction with LasR (9). Recently, a third *N*-acyl-HSL, factor 2, has been purified from *P. aeruginosa* culture fluids (23). It remains to be investigated whether factor 2 and/or VAI is synthesized by the RhlI protein, but our findings suggest that the RhlR regulator can bind both types of autoinducers. LuxR has been shown to bind a variety of autoinducer analogs and was suggested to contain more than one autoinducer binding site. Thus, several different types of autoinducers may act simultaneously on the same regulatory protein, thereby affecting its DNA-binding activity (14). However, neither LuxR nor LasR has appreciable activity with the heterologous *Vibrio* or *Pseudomonas* autoinducers (14).

Crossregulation between the RhlR and LasR regulators was observed. Multiple copies of the *lasR* gene in an *rhlR* mutant led to the production of low but detectable levels of rhamnolipids. In contrast, a *lasR* mutant harboring multiple *rhlR* gene copies produced high levels of elastase. These findings suggest that the LasR–LasI and RhlR–RhlI systems can at least partially substitute for each other. The RhlR protein may enhance PAI production, leading to enhanced expression of

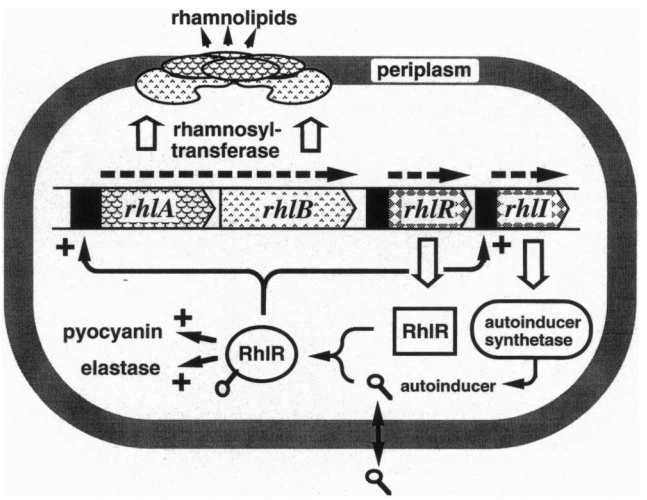

FIG. 6. Model for the regulation of rhamnolipid production in *P. aeruginosa*.

the elastase structural gene, *lasB*. Alternatively, the RhlR–RhlI system may be involved in the regulation of the *lasA* gene, which encodes a second elastase that has been reported to affect the observed high overall elastolytic activity in *P. aeruginosa* (24). Multiple copies of the *lasR* gene resulted in only a limited rhamnolipid production in an *rhlR* mutant strain, possibly indicating that multiple copies of the *lasR* gene may lead to the preferential activation of the promoter of the heterologous *rhlI* autoinducer synthetase gene, rather than to the activation of the *rhlA* gene promoter. This view is supported by a recent study (25) which suggests an autoinducer regulatory hierarchy: LasR and low PAI concentrations were found to activate primarily *lasI* gene expression in a regulatory loop; with the accumulation of PAI, the secondary activation of virulence-product genes such as *lasB* was found to occur. However, the heterologous LuxR–VAI complex had no effect on *lasI* gene expression (25).

P. aeruginosa provides a further example of a microorganism in which two related autoinduction regulatory systems occur. Multiple *N*-acyl-HSLs, produced by two distinct autoinducer synthetases, have recently also been reported to occur in the marine symbiotic bacterium *V. fischeri* (26). It will be interesting to determine how the two unexpectedly complex autoinducer-dependent regulatory systems of *P. aeruginosa* influence the expression of their target genes and how they interact with each other.

This paper is dedicated to Armin Fiechter on the occasion of his 70th birthday. We thank Dr. E. P. Greenberg and Dr. L. Passador for kindly providing us with synthetic factor 2, VAI, and PAI and for stimulating discussions concerning autoinduction.

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