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Synthesis of Multiple Exoproducts in *Pseudomonas aeruginosa* Is under the Control of RhlR-RhlI, Another Set of Regulators in Strain PAO1 with Homology to the Autoinducer-Responsive LuxR-LuxI Family

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Mutants of *Pseudomonas aeruginosa* PAO1 that were deficient in the ability to produce proteases that degrade casein were detected among the survivors of chemical mutagenesis. One such mutant (PDO31) showed reduced production of elastolytic activity, β-hemolytic activity, and pyocyanin. A 4.3-kb *Eco*RI fragment from a gene bank of PAO1 that complemented defects in PDO31 was found. Transposon mutagenesis and deletion derivatives of the clone were used in conjunction with complementation tests to determine the physical location of the gene of interest. Nucleotide sequence analysis revealed an open reading frame (*rhlR*) encoding a putative 27.6-kDa protein (RhlR) with homology to autoinducer-responsive regulators of quorum sensing systems such as LuxR of *Vibrio fischeri* and LasR of *P. aeruginosa*. Further sequence analysis downstream of *rhlR* revealed an independently transcribed gene (*rhlI*) that encodes a putative 22.2-kDa protein with homology to members of the family of autoinducer synthetases, such as LuxI of *V. fischeri* and LasI of *P. aeruginosa*. The *rhlRI* sequences were also recently reported by others (U. A. Ochsner and J. Reiser, Proc. Natl. Acad. Sci. USA 92: 6424–6428, 1995) as an autoinducer-mediated regulation mechanism for rhamnolipid biosurfactant synthesis in *P. aeruginosa* PG201. Mutants with defects in *rhlR* or *rhlI* were constructed in PAO1 by gene replacement, using clones modified by Tn*501* insertion. Compared with the wild type, the *rhlR* and *rhlI* mutants both showed defects in the production of elastase, LasA protease, rhamnolipid, and pyocyanin. Transcription from the gene for elastase, as measured with a *lasB-cat* fusion, demonstrated that production of elastase was subject to cell density-dependent gene activation in PAO1. However, transcription of *lasB-cat* in the *rhlI* mutant, which had lost the presumptive autoinducer synthetase (predicted to activate RhlR), showed low basal activity and had lost all cell density-dependent transcription of *lasB*. Thus, RhlR-RhlI represent the second autoinducer-responsive regulatory mechanism found in *P. aeruginosa* that controls expression of multiple virulence factor exoproducts, including elastase.

Pseudomonas aeruginosa has a well-deserved reputation as an important and ominous opportunistic bacterial pathogen in patients with underlying diseases (8, 27, 53). Patients with the autosomal recessive disease cystic fibrosis are particularly prone to chronic pulmonary infection with this organism. However, *P. aeruginosa* is also a significant etiologic agent in minimally compromised patients, such as those undergoing mechanical ventilation (6, 11, 54). Refined techniques for the diagnosis of ventilator-associated pneumonia have identified *P. aeruginosa* as an etiologic agent in 31 to 42% of cases, with attendant mortality rates of up to 87% (12, 39, 40). Assessments of directly attributable mortality due to *Pseudomonas* infection in ventilator-associated pneumonia have been reported as 38 to 42% when compared with matched control cases due to other etiologic organisms (9, 13).

An attribute that is widely regarded as a major contributor to the pathogenesis of *P. aeruginosa* is its ability to secrete numerous toxic compounds and degradative enzymes (10, 34). One of the most abundantly secreted proteins of *P. aeruginosa* is elastase, a protease with high activity and broad substrate specificity (42). Other exoproducts of *P. aeruginosa* also associated with pneumonia include LasA protease (3, 62), phospholipase C (34), exotoxin A (62), exoenzyme S (61), rhamnolipid (24, 36), pyocyanin (5, 60), and alginate (23).

Elastase and many other exoproducts of *P. aeruginosa* are not actively produced until the late logarithmic phase of growth, when the cell density is high (38). One mechanism by which bacteria respond to cell density is through an autoinducer-responsive transcriptional regulatory system. This involves a phenomenon often called quorum sensing, in which specific genes are activated at high population densities in response to chemical signals released by the bacterial cells into the growth medium (for a review, see reference 18). The *Vibrio fischeri* LuxR-LuxI pair may be the best understood example of a growing number of regulator-autoinducer systems (41). Here, LuxR forms a complex with the freely diffusible, low-molecular-weight *V. fischeri* autoinducer molecule (VAI) whose synthesis occurs through the autoinducer synthetase, LuxI. The LuxR-VAI complex forms only under conditions of high cell density because VAI diffuses away into the medium if the cell density is low. The formation of the LuxR-VAI complex is required for the efficient binding of LuxR to a positive regulatory site in the DNA upstream of the *luxI* operon (forming a positive feedback loop) and to an inhibitory DNA site upstream of the divergently transcribed *luxR* gene (forming a negative feedback loop). An additional binding site has been identified for the cyclic AMP (cAMP) receptor protein, and its binding has the opposite effects (41). Such a self-stimulating regulatory mechanism has the potential to generate a rapid and strong response to the appropriate stimulus

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype and/or properties[a]	Reference or source
P. aeruginosa		
PAO1	Wild-type prototroph	25
PDO31	*rhlR1* derivative of PAO1	This study
PDO100	*rhlI*Δ::Tn*501*-2 derivative of PAO1	This study
PDO111	*rhlR*::Tn*501*-11 derivative of PAO1	This study
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdRM recA13 supE44*	This laboratory
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
Plasmids		
pLAFR1	IncP1 *cos oriT* Tc^r	17
pLAFR3	pLAFR1 with an MCS	A. Darzins
pRK2013	ColE1 Tra^+(RK2) Km^r	14
RSF1010::Tn*501*	IncQ Hg^r	48
pMB1	pLAFR1 with 31 kb containing *rhlABRI′*	This study
pMB5	pLAFR1 with 4.3 kb containing *rhlABRI′*	This study
pMB6	pMB5 with *Bam*HI deletion	This study
pMB8	pMB5 with *Xho*I deletion	This study
pKSM5	pSelect with *lasB* Tc^r Ap^r	37
pBluescript II SK^+/KS^-	*ori* (ColE1) Ap^r	Stratagene
pMB13	pBluescript II SK^+ with 1.3 kb containing *rhlRI′*	This study
pCM1	*ori* (ColE1) *cat* (*Sal*I) Ap^r Tc^r Cm^r	Pharmacia
pCM3	*ori* (ColE1) *cat* (*Bam*HI) Ap^r Tc^r Cm^r	Pharmacia
pMB20	pLAFR3 containing pKSM5 with *lasB-cat* (*Sal*I)	This study
pMB26	pMB8 with *rhlR-cat* (*Bam*HI)	This study
pMB28	pLAFR3 with 6.5-kb *Pst*I fragment from PDO100 containing $rhlR^+$ *rhlI*Δ::Tn*501*-2	This study
pMB40	pLAFR3 with 2.9-kb *Pst*I fragment from PDO111 containing *rhlR*::Tn*501*-11 $rhlI^+$	This study
pMB41	pBluescript II KS^- with 2.2-kb *Bam*HI fragment from pMB40 containing *′rhlR* $rhlI^+$	This study

[a] Abbreviations not given in text: MCS, multiple cloning site; Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance.

by coordinating the response from an entire population of cells.

LasR-LasI form a set of autoinducer-responsive transcriptional regulators that were recently discovered in *P. aeruginosa* and show homology to LuxR-LuxI of *V. fischeri* (19, 50). LasR appears to be a global regulator that is involved in the transcriptional control of several genes, including elastase (19), LasA protease (57), and alkaline protease (20). Such control by LasR requires LasI, which synthesizes an autoinducer, termed PAI (50), consisting of 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-dodecanamide [or *N*-(3-oxododecanoyl)homoserine lactone] (51). The LasR-PAI system in *P. aeruginosa* has been described as a mechanism for cell-cell communication in response to cell density to allow coordinate expression of virulence-associated genes for concentrating the attack upon the host (50).

Recent studies by Pearson et al. (52) provided the first evidence that a second *N*-acylhomoserine lactone signal is produced by *P. aeruginosa* PAO1 and is also involved in the transcriptional control of elastase. This finding suggests that the quorum sensing system in this organism is complex. Here we describe the isolation of a mutant of *P. aeruginosa* PAO1 with a defect in elastase production, and its complementing clone was shown to encode a second autoinducer-responsive transcriptional regulatory system in *P. aeruginosa* PAO1 called RhlR-RhlI. RhlR was recently reported as a transcriptional regulator of rhamnolipid biosurfactant synthesis (44). During our studies, the RhlR-RhlI pair was also reported by Ochsner and Reiser (45) as an autoinducer-mediated regulatory system controlling the synthesis of several exoproducts in *P. aeruginosa* PG201. We constructed mutants carrying mutations in both genes for RhlR and RhlI, and their pleiotropic phenotypes confirmed the role of this pair in the global regulation of extracellular virulence products. Studies on the regulation of the gene encoding elastase (*lasB*) demonstrated the requirement of this system for its growth phase-dependent transcriptional activation. Thus, *P. aeruginosa* possesses at least two autoinduction systems to regulate the expression of its numerous secreted virulence factors in a cell density-dependent manner.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1. Bacteria were cultured in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl [pH 7.5]), peptone-tryptic soy broth (PTSB; 5% peptone, 0.25% tryptic soy broth), peptone-glycerol broth (PB; 1% peptone, 1% glycerol), pyocyanin production broth (PPB; 2% peptone, 0.14% magnesium chloride, 1% potassium sulfate, 2% glycerol), or a minimal medium (58). Media were solidified with 1.5% Bacto Agar (Difco). Unless specified otherwise, antibiotics were used at the following concentrations (per milliliter): ampicillin, 100 μg for *Escherichia coli*; carbenicillin, 300 μg for *P. aeruginosa*; kanamycin, 30 μg for *E. coli*; tetracycline, 20 μg for *E. coli* or 100 μg for *P. aeruginosa*; and mercuric chloride, 15 μg for both *E. coli* and *P. aeruginosa*. Casein-agar plates contained 1.5% skim milk (Difco) and 0.8% nutrient broth (Difco). Elastin-agar plates contained 0.5% elastin (Sigma) and 0.8% nutrient broth (Difco). The reproducibility of results was enhanced by the use of distilled, deionized water in all media and agar plates.

Isolation of mutants with defects in proteolytic enzyme production. *P. aeruginosa* PAO1 (26) has become a standard reference strain for studies on pathogenesis, genetics, and gene mapping, and it expresses most of the virulence factors commonly associated with *P. aeruginosa* pneumonia (see above). Strain PAO1 was subjected to chemical mutagenesis as described previously (21). Briefly, 2 ml of PAO1 culture in the late logarithmic phase of growth was mixed with 50 μl of *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (2.5 mg/ml) and incubated for 10 min. Cells were washed, inoculated into L broth, and incubated overnight. Cultures were diluted and spread onto L-agar plates, and the isolated colonies were patched onto casein-containing agar. Those colonies exhibiting decreased proteolytic activity were identified by small zones of pericolony clearing following incubation at 37°C. Mutants were then tested for deficiencies in the ability to degrade elastin particles embedded in elastin-agar plates.

DNA manipulations. Most routine DNA manipulations and plasmid extrac-

tions were performed according to standard methods (35). We used a gene bank containing partially digested *Eco*RI fragments of genomic PAO1 DNA and prepared in the IncP1 cosmid vector pLAFR1 (17) as described by Goldberg and Ohman (21). Triparental matings were used to mobilize recombinant plasmids from *E. coli* to *P. aeruginosa* as previously described (21). DNA in pBluescript II SK^+ was sequenced as previously described (16). The sequence data were assembled and analyzed with the LaserGene software package for Macintosh (DNA-Star).

Immunoblot analysis. Protein samples were suspended in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1 mg of bromphenol blue per ml, 5% 2-mercaptoethanol [pH 6.8] and were loaded onto a 12.5% polyacrylamide gel for electrophoresis (32). Immunoblotting was performed with affinity-purified, polyclonal rabbit antielastase antibodies as previously described (37).

Tn*501* plasmid mutagenesis and gene replacement in *P. aeruginosa*. Recombinant plasmids were subjected to Tn*501* mutagenesis as previously described (48). The effects of the Tn*501* insertions on complementation of exoproduct deficiencies were tested in mutant PDO31, and sites of insertions were mapped by restriction analysis, taking advantage of the terminal *Eco*RI sites of Tn*501* (8.3 kb). Cloned DNAs with Tn*501* insertions were exchanged for chromosomal sequences in *P. aeruginosa* PAO1 by homologous recombination, using a transduction method and phage F116L as previously described (48) except that 10 mM $MgCl_2$ and 10 mM $CaCl_2$ were added to all media to maximize phage adsorption to cells. Potential chromosomal mutants were selected for mercuric ion resistance (Hg^r) encoded by Tn*501* and then screened for tetracycline sensitivity (Tc^s) to show loss of the vector. Gene replacements were tested via DNA-DNA hybridization analysis: a 0.8-kb *Eco*RI-*Bam*HI *rhlR′-rhlI′* fragment of pMB8 (^{32}P labeled) was used as a probe of the PDO100 chromosome, and a 1.9-kb *Bam*HI *′rhlB-rhlR′* fragment of pMB5 (digoxigenin labeled) was used as a probe of the PDO111 chromosome.

Assays of proteolytic and elastolytic activities. Standardized culture conditions were used to evaluate proteolytic activities in culture supernatants of *P. aeruginosa* strains. Overnight cultures were used to inoculate (1:100) fresh growth medium; these cultures were grown to mid-logarithmic phase (optical density at 600 nm [OD_{600}] of 0.6) and then used to inoculate (1:100) 10 ml of fresh medium, which was incubated at 37°C with vigorous aeration. Maximal elastase accumulated in the extracellular medium of PTSB cultures by ~20 h. Dilutions of samples were assayed for proteolytic activities to establish the linear range of the reactions. Casein-hydrolyzing proteolytic activity was determined as previously described (29) with azocasein (Sigma) as the substrate, and units of proteolytic activity were expressed as the increase in A_{400} per milligram of total protein. Elastolytic activity was determined as previously described (46) with elastin Congo red (Sigma) as the substrate, and units of elastolytic activity were expressed as the increase in A_{495} per milligram of total protein. Protein concentration was determined in duplicate by the Bradford method. LasA protease activity was examined in supernatants from 18-h standardized cultures grown in L broth. LasA was measured, using its staphylolytic activity, by determining the rate of lysis of a standard suspension of killed *Staphylococcus aureus* in hypotonic phosphate-buffered saline (PBS) as previously described (30).

Assays of rhamnolipid, pyocyanin, and exotoxin A. Minor modifications of a procedure previously described (36) were used for the assay of rhamnolipid. Cultures in peptone-glycerol broth (20 ml) were incubated at 37°C for 48 h with vigorous aeration, and culture supernatants were filtered (0.45-μm-pore-size filters). Heat-labile β-hemolysin (e.g., phospholipase C) activity in the samples was destroyed by autoclaving (121°C for 20 min), a treatment to which rhamnolipid-mediated hemolytic activity is resistant. Serial twofold dilutions of the samples in PBS (pH 7.4) were mixed with equal volumes of a 1% (vol/vol) suspension of human blood in PBS (pH 7.4) and incubated at 37°C for 1 h. Units of hemolytic activity were defined as the inverse of the highest dilution showing complete hemolysis. Pyocyanin was quantitated spectrophotometrically in chloroform extracts of supernatants from standardized 24-h PPB cultures (37°C with vigorous aeration) as described previously (7). PPB or agar plates incorporating PPB were used in cross-feeding experiments. An estimate of exotoxin A secretion was obtained with a modified Ouchterlony double-diffusion technique using rabbit anti-toxin A (47).

***cat* operon fusions.** The promoterless *cat* gene, encoding chloramphenicol acetyltransferase (CAT), has often been used as a quantitative indicator of promoter activity in *P. aeruginosa* (22, 63, 64). A *lasB-cat* reporter plasmid (with a low copy number) was constructed to place *cat* transcription under the control of the promoter of *lasB* (encoding *P. aeruginosa* elastase in *P. aeruginosa*). The intragenic *Sal*I fragment of *lasB* in pKSM5 (37) was replaced with a promoterless *cat* (*Sal*I) cartridge from pCM1 (Pharmacia). In other clones, *cat* was used to determine the direction of transcription by inserting the cartridge into the same restriction site in both orientations. *P. aeruginosa* strains carrying plasmids with *cat* inserts were grown under standardized conditions for elastase production. Cells were collected by centrifugation and lysed by sonication, and CAT antigen levels were determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) for CAT (5 Prime → 3 Prime). Values were expressed as nanograms of CAT per milligram of total protein.

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been submitted to the GenBank/EMBL data banks and assigned accession number X40458.

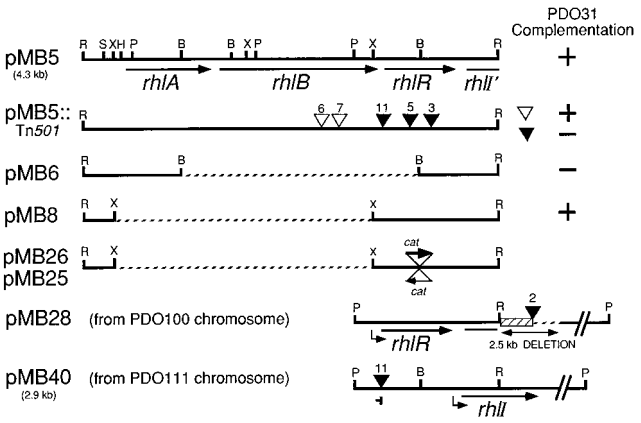

FIG. 1. Restriction maps of *P. aeruginosa* DNA in plasmids used in this study. Arrows indicate the locations of ORFs for *rhlA*, *rhlB*, *rhlR*, and *rhlI*. Dotted segments represent deletions, and hatched areas indicate residual vector DNA of vector origin. Complementation was of both the elastolytic and pyocyaninogenic defects in *rhlR1* mutant strain PDO31. Inverted triangles show Tn*501* insertion sites; filled triangles indicate that complementation was blocked. The transcriptional orientations of *cat* insertions are indicated with arrows. PDO31 is a nitrosoguanidine-generated mutant exhibiting pleiotropic deficits in production of elastase, pyocyanin, and β-hemolysins. PDO100 is an *rhlR*$^+$ *rhlI*Δ::Tn*501*-2 mutant of PAO1 with a 2.5-kb deletion that includes the 3′ portion of *rhlI*. PDO111 is an *rhlR*::Tn*501*-11 insertion mutant of PAO1. pMB40 contained additional *Pst*I fragments not shown. Restriction endonuclease sites: B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I.

RESULTS

Isolation of a mutant deficient in several virulence-associated exoproducts. Strain PAO1 was subjected to chemical mutagenesis, and isolated colonies from surviving bacteria were screened for deficits in extracellular proteolytic activity. Twelve isolates exhibiting small zones of pericolony clearing when grown on casein-containing agar at 37°C were obtained. Among these, three also failed to degrade elastin particles embedded in agar plates. One mutant, called PDO31, was selected for further investigation because of its striking elastolytic defect. Further investigation revealed that PDO31 was also defective in the production of pyocyanin (blue pigment) and hemolysin activity (on blood-agar), suggesting that the gene mutated was normally involved in the production of several exoproducts associated with pathogenesis.

Isolation of a clone complementing defects in PDO31. To obtain a clone which complemented the elastolytic defect of PDO31, members of a gene bank containing *Eco*RI fragments of the PAO1 chromosome in a broad-host-range vector were mobilized into PDO31 with selection for Tc^r. Transconjugates were screened for restoration of elastolytic activity on elastin-agar plates, which led to the isolation of pMB1, a complementing clone containing three *Eco*RI fragments (totaling 31 kb). Subcloning led to the identification of pMB5, a complementing clone containing a single *Eco*RI fragment of 4.3 kb in pLAFR1, and a partial restriction map was developed (Fig. 1). Transposon mutagenesis was used to obtain pMB5::Tn*501* derivatives, and the sites of insertions were correlated with failure to complement the elastolytic defect of PDO31. This analysis showed that the gene of interest was located near the right side of the clone as drawn in Fig. 1. Deletion of internal *Bam*HI fragments from pMB5 (pMB6) resulted in the loss of complementing ability, but a clone deleted of *Xho*I fragments (pMB8) remained active. These results with deletion derivatives were consistent with those obtained from the Tn*501* insertion analysis.

DNA sequence analysis reveals a *luxR* homolog called *rhlR*. The nucleotide sequence of the 1.3-kb *Xho*I-*Eco*RI fragment

containing the gene of interest was determined, and potential open reading frames (ORFs) were found in both directions. To determine which was the correct reading frame, a promoterless *cat* gene was inserted into the *Bam*HI site of pMB8 in both orientations, thus forming pMB25 and pMB26 (Fig. 1). PAO1 (pMB25) and PAO1(pMB26) were grown with high aeration in selective PTSB medium, a condition that permits high-level production of elastase. Specific CAT antigen levels in cell sonic extracts were determined, and samples from PAO1 carrying pMB26 had about 10-fold more CAT than those with pMB25 (data not shown), suggesting that the correct ORF was from left to right as drawn in Fig. 1. This ORF consists of 726 nucleotides, with a predicted translation product of 241 amino acids (27,580 Da) having a pI of 7.01 and charge of 0.01 at pH 7.0.

In a preliminary report (4), we described this gene complementing the elastolytic defect in PDO31 as *lasM* (elastase modulator), encoding a putative transcriptional regulator. A database search for similar sequences revealed that Ochsner et al. (44) had recently reported the same gene under the designation of *rhlR* (for rhamnolipid regulatory gene) but from a different strain of *P. aeruginosa*. We detected only six nucleotide differences between the two sequences (data not shown), and none changed the predicted amino acid sequence. Although named for its effect on rhamnolipid biosynthesis, we adopted the name *rhlR* for this global regulator to avoid any confusion. The *rhlR* product (RhlR) shares significant homology with known transcriptional regulatory proteins, and such similarities were also observed by Ochsner et al. (44): *E. coli* UvrC (40% identity), *P. aeruginosa* LasR (31% identity), *Rhizobium leguminosarum* RhiR (29% identity), and *V. fischeri* LuxR (23% identity).

DNA sequence analysis downstream of *rhlR* reveals *rhlI*, a *luxI* homolog. The striking similarity of RhlR to members of the family of autoinducer-responsive (quorum sensing) transcriptional regulators (e.g., LuxR and LasR) suggested that a cognate gene for autoinducer formation might be located adjacent to *rhlR*. Immediately upstream of *rhlR* are *rhlA* and *rhlB*, which are involved in the synthesis of rhamnolipid (43), and our sequence analysis confirmed the presence of the *rhlB* gene at this location in PAO1 (Fig. 1). To identify a potential autoinducer synthetase gene downstream of *rhlR*, a clone that contained additional DNA was needed. This was obtained by using a strategy to clone a genomic *Pst*I fragment that contained *rhlR* and thus sequences downstream of the terminal *Eco*RI site of pMB5. Genomic DNA of strain PDO111, an *rhlR*::Tn*501*-11 insertion mutant (described below), was digested with *Pst*I because it does not cut Tn*501*, and *Pst*I fragments were ligated into vector pLAFR3. This DNA was introduced into *E. coli*, and a clone containing *rhlR*::Tn*501*-11 was obtained by selecting for the Hg^r marker, thus producing pMB40 (Fig. 1). From this, a 2.1-kb *Bam*HI-*Pst*I *′rhlR* fragment was cloned (pMB41) and subjected to a sequence analysis. Approximately 1,600 bp were added to the 3′ end of the sequence described above containing *rhlR*. Two overlapping ORFs of 636 and 591 bp were observed downstream of *rhlR* and transcribed in the same direction. The 591-bp ORF, termed *rhlI*, was presumed to be correct because it demonstrated the anticipated homology to known autoinducer synthetases, including LuxI. The sequence of the *rhlI* gene predicted a protein of 22,202 Da (196 amino acids with a charge of −3.69 at pH 7.0 and an isoelectric point of 5.442). An alignment of RhlI to known autoinducer synthetases (data not shown) indicated its similarity to *Pseudomonas aureofaciens* PhzI (36% identity), *V. fischeri* LuxI (30% identity), *P. aeruginosa* LasI (25% identity), and *Erwinia carotovora* ExpI (20% identity).

Construction of *rhlR*::Tn*501* and *rhlI*Δ mutants via gene replacement. To construct a defined *rhlR* mutant, the *rhlR*::Tn*501*-11 derivative of pMB5 (Fig. 1) was chosen for gene replacement in PAO1. Fragments of pMB5::Tn*501*-11 were transduced into PAO1 with selection for Hg^r encoded by Tn*501*, and colonies were screened for Tc^s, indicating loss of the plasmid. One *rhlR* mutant was named PDO111, and its replacement of the chromosomal *rhlR* gene with the mutated allele was confirmed by DNA-DNA hybridization (data not shown). In preliminary tests, PDO111 demonstrated the expected defect in elastase production.

A mutant altered in the *rhlI* gene (*rhlI*Δ) was also desired, and this was obtained through the following fortuitous events. During the transposon mutagenesis procedure described above, we obtained pMB5::Tn*501*-2, which had lost the ability to complement the pyocyanin and elastolytic defects in PDO31. However, it was ultimately recognized that the site of insertion in pMB5::Tn*501*-2 was actually in vector sequences near *rhlR*. Others have also observed that a transposon insertion in plasmid vector sequences can affect the ability of the cloned DNA to complement a chromosomal defect (e.g., exoenzyme S synthesis in *P. aeruginosa*; [15]), possibly by inhibiting local supercoil relaxation and reducing transcription. Plasmid pMB5::Tn*501*-2 had been used in the gene replacement procedure to transfer the Tn*501*-2 mutation to the chromosome, and a mutant called PDO100, which had Tn*501*-2 (Hg^r), had lost the vector-encoded marker (Tc^s), and also exhibited a defect in expression of elastase activity, was obtained. The DNA-DNA hybridization analysis, using a 0.8-kb *Bam*HI-*Eco*RI *′rhlR* fragment (Fig. 1) as a probe, produced a pattern of hybridization suggesting that no changes had occurred in this DNA (data not shown). Because Tn*501*-2 was in the vector, the gene replacement event in PDO100 could not have occurred by double homologous recombination, since *P. aeruginosa* DNA was on only one side of the transposon. This finding suggested that a single homologous recombination was later disproportionately resolved via a heterologous recombination event with the chromosome (possibly through a transposon-mediated process). To further analyze the nature of the chromosomal defect, *Pst*I fragments of PDO100 genomic DNA were cloned into pLAFR3, using the Tn*501*-2-encoded Hg^r to select transformants containing the sequences of interest and thus form pMB28 (Fig. 1). The DNA flanking Tn*501*-2 in pMB28 was sequenced, and *rhlR* was found to be intact. However, further sequence analysis and restriction mapping showed that PDO100 had an ~2.5-kb deletion of adjacent chromosomal DNA that was replaced by a fragment (~300 bp) of the vector. This chromosomal deletion began at the *Eco*RI site in *rhlI* and included the next downstream gene, which others have reported to be *pheC*, a gene involved in L-phenylalanine biosynthesis (43). Interestingly, deletion of the *pheC* gene does not confer phenylalanine auxotrophy because of the presence of two pathways for phenylalanine biosynthesis in *P. aeruginosa* (65).

***rhlR* and *rhlI* have separate promoters.** Our complementation of PDO31 with *rhlR* clones modified by upstream transposon insertions (e.g., Tn*501*-7) and upstream deletions (pMB8) indicated that *rhlR* has its own promoter (Fig. 1). The close proximity of *rhlR* and *rhlI*, and their transcription in the same direction, might suggest the possibility of an *rhlR-rhlI* operon. If this were the case, then the *rhlR*::Tn*501* mutation would be polar on *rhlI*. However, *rhlR* and *rhlI* appear to be independently transcribed. As already observed (44), the sequence between *rhlR* and *rhlI* contains a potential *rho*-inde-

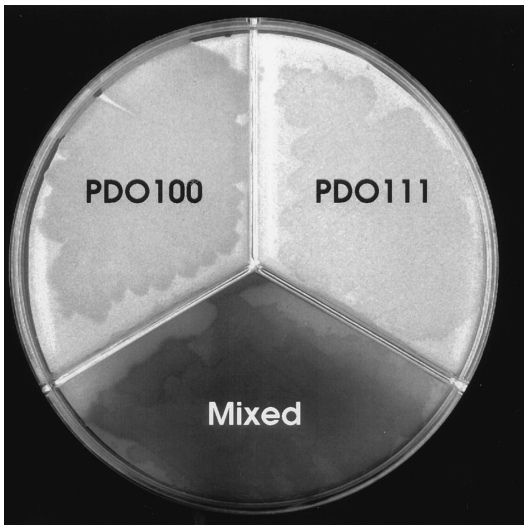

FIG. 2. Solid-medium demonstration of cross-feeding in *P. aeruginosa* with a *P. aeruginosa rhlI*-dependent autoinducer(s). A single tricompartment petri dish containing solidified PPB in all compartments is heavily inoculated in two compartments, each with 24-h PPB cultures of a *P. aeruginosa* PAO1 *rhlR*$^+$ *rhlI*Δ::Tn*501*-2 mutant (PDO100) or an *rhlR*::Tn*501*-11 insertion mutant of PAO1 (PDO111), resulting in the double streaking of one compartment. Incubation is at 37°C for 12 to 36 h (a 24-h incubation is depicted). Pyocyanin pigment production is evident only in the mixed compartment.

pendent terminator. In addition, 5 bp downstream of *rhlR* is an 11-bp (AGGGCGCGCCG) sequence separated by 2 bp from its inverted repeat, and this could form a transcriptional terminator or possibly a regulatory site. This combination of two potential terminators militates against the *rhlR* promoter being a significant contributor to *rhlI* transcription. Direct evidence for independent transcription was provided through our complementation analysis. Plasmid pMB40 (Fig. 1), containing *rhlR*::Tn*501* and *rhlI* (from the chromosome of PDO111), was capable of complementing defects in pyocyanin and elastase production in the *rhlI*Δ mutant strain, PDO100. Thus, the *rhlR*::Tn*501* mutation was not polar on *rhlI* expression.

We also examined the effects of the mutations on the synthesis of the putative autoinducer produced by the *rhlI* gene product and the response to autoinducer. Figure 2 illustrates that neither the *rhlR* mutant (PDO111) nor the *rhlI* mutant (PDO100) produced the blue pigment pyocyanin. However, when they were mixed on the same agar medium and allowed to incubate, pigment production was readily restored, apparently as a result of cross-feeding of autoinducer from PDO111 to PDO100. The *rhlI* mutant (PDO100) can also be complemented by spent culture supernatants from either the wild type or the *rhlR* mutant (data not shown). These observations suggest that the phenotype of PDO111 is RhlR$^-$ RhlI$^+$ and that the phenotype of PDO100 is RhlR$^+$ RhlI$^-$.

Characterization of exoproduct production by *rhlR* and *rhlI* mutants. PDO111 and PDO100 were characterized for a variety of extracellular activities relative to the wild type, PAO1. Optimal culture conditions for exoproduct production and measurement were used to evaluate extracellular levels of elastolytic activity, LasA protease activity, and total casein-degrading proteolytic activity. Rhamnolipid-mediated β-hemolysis (heat stabile) and pyocyanin were also quantitated in supernatants. An estimate of relative exotoxin A production was obtained by immunoprecipitation. The results of these tests are shown in Table 2 and summarized as follows. The *rhlR* mutant (PDO111) and *rhlI* mutant (PDO100) both showed reduced production of elastolytic activity (6- and 5-fold, respectively), LasA protease activity (6- and 2.5-fold, respectively), and casein-degrading activity (4- and 2-fold, respectively). Inactivation of either gene abolished our ability to detect either rhamnolipid or pyocyanin in the culture medium. The *rhlR* mutant (PDO111) and *rhlI* mutant (PDO100) still produced exotoxin A under the conditions tested, although the amount from PDO100 may have been somewhat reduced.

Mutation in *rhlI* affects elastase synthesis and not the elastase secretory apparatus. The pleiotropic deficiency in exoproduct production observed in the *rhlR* and *rhlI* mutants might be explained by a general defect in the secretory apparatus. If this were the case, then intracellular accumulation of exoproducts would be expected. To test this, an antielastase immunoblot analysis was performed on supernatants and cell sonic extracts of PAO1 and PDO100. Both strains were grown under standardized conditions in PTSB, and the cell and supernatant fractions were collected. Using an immunoblot analysis, we readily observed elastase in the supernatant and cell lysates of the wild-type strain. However, only trace amounts were detected in either fraction of the mutant PDO100 (data not shown). Thus, these results suggest that *rhlI* regulates the synthesis of elastase rather than the apparatus for the secretion of elastase.

RhlR and RhlI regulate the transcription of *lasB* encoding elastase. We next determined the effect of mutation in *rhlR* or *rhlI* on the transcription of *lasB*. Plasmid pMB20 (Fig. 3) was constructed as a low-copy-number *lasB-cat* gene fusion to report *lasB* promoter activity, and it was transferred to PAO1, PDO100, and PDO111. These strains were grown for 20 h in selective PTSB to promote elastase production, and CAT was quantitated in cell sonic extracts. The values obtained (Table 3) demonstrated that mutations in *rhlR* and *rhlI* reduced *lasB* promoter activity by about 400- and 100-fold, respectively. This effect on *lasB-cat* transcription was more dramatic than the effect seen on cumulative elastolytic activity (i.e., ~5-fold reduction; Table 2) in the supernatant over the 20-h period of growth. This may be due to autodegradation of elastase when it is present in a high concentration (e.g., at stationary phase) and would result in an underestimate of the total elastase activity released. Thus, both *rhlR* and *rhlI* positively regulate the transcription of *lasB*, with *rhlR* playing a greater role. Since *rhlR* and *rhlI* appear to be separately transcribed, these data also suggest both genes are required for maximal transcription of the *lasB* gene. However, the pleiotropic phenotype dis-

TABLE 2. Effect of mutation in *rhlR* or *rhlI* on exoproduct synthesis in *P. aeruginosa*

Exoproduct	% of wild-type activity or quantity[a]	
	RhlR$^-$ RhlI$^+$	RhlR$^+$ RhlI$^-$
Elastase	17.5	19.6
LasA protease	15.0	38.0
Casein-degrading proteases	27.2	46.3
Rhamnolipid	0	0
Pyocyanin	0	0
Exotoxin A	+	+

[a] Strains examined were PAO1 (*rhlR*$^+$ *rhlI*$^+$), PDO111 (*rhlR*), and PDO100 (*rhlI*Δ). All values shown are means of determinations from triplicate standardized cultures and were compared with the mean values from triplicate cultures of PAO1, all of which were grown, processed, and assayed concurrently. Values shown as 0 indicate below detectable limits. See Materials and Methods for media, culture conditions, and assay methods used. Typical values for PAO1 are as follows: elastase, 7.5 U/mg of protein; casein-degrading proteases, 102.5 U/mg of protein; LasA protease, 332.1 U/mg of protein; rhamnolipid, 8 U; pyocyanin, 0.458 μg/ml; toxin A, + (indicating an antibody-antigen precipitant band).

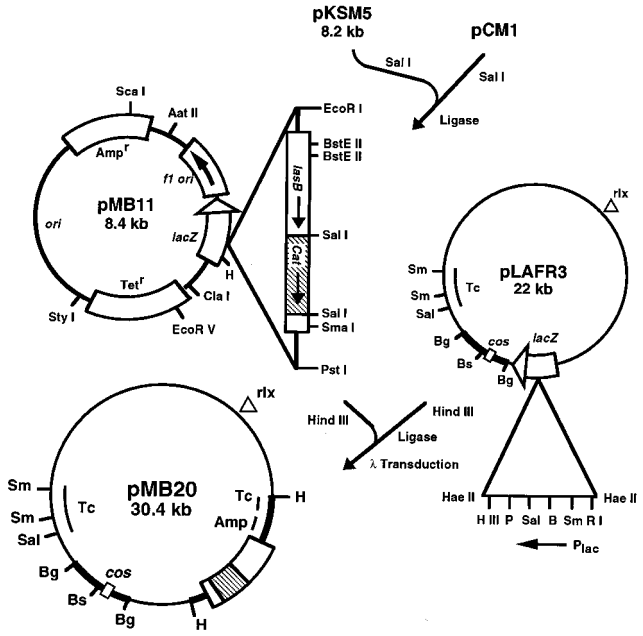

FIG. 3. Construction of a *lasB-cat* transcriptional reporter plasmid. The 0.6-kb intragenic *Sal*I fragment of *lasB* in pKSM5 was replaced with a 0.8-kb promoterless *cat* cartridge from pCM1 in the orientation to place the *cat* gene under the control of the *lasB* promoter. The resulting 8.4-kb plasmid (pMB11) was linearized by *Hin*dIII digestion and ligated into the unique *Hin*dIII site of pLAFR3. The resultant 30.4-kb broad-host-range plasmid (low copy number in *P. aeruginosa*), pMB20, contained a *lasB-cat* operon fusion and conferred ampicillin-carbenicillin (Amp) and tetracycline (Tc) resistances. *P. aeruginosa* strains containing pMB20 were grown under standardized conditions that promote elastase production, and cell sonic extracts were tested for nanograms of CAT per milligram of total protein, using an ELISA for CAT. Restriction endonuclease sites: Sm, *Sma*I; Sal, *Sal*I; Bg, *Bgl*II; Bs, *Bst*EII; H and H III, *Hin*dIII; P, *Pst*I; RI, *Eco*RI.

played in Table 2 supports a role for this regulatory complex in the maximal transcription of several other extracellular virulence factor-encoding genes as well.

***rhlI* is required for cell density-dependent activation of *lasB* transcription.** A typical feature of autoinducer-responsive transcriptional regulator systems (e.g., LuxR-VAI) is a response to increased cell density. Several reports have demonstrated that maximal elastase accumulation in the culture medium of *P. aeruginosa* occurs during the late logarithmic to early stationary phases, when the cell density is high (38, 46, 49). We used the *lasB-cat* transcriptional reporting plasmid pMB20 (Fig. 4)

TABLE 3. Effect of mutation in *rhlR* or *rhlI* on *lasB-cat* transcription in *P. aeruginosa*[a]

Strain	Relevant genotype	CAT antigen level[b] (ng of CAT/mg of protein)
PAO1(pMB20)	*rhlR*$^+$ *rhlI*$^+$ *lasB-cat*	90,551
PDO111(pMB20)	*rhlR rhlI*$^+$ *lasB-cat*	195
PDO100(pMB20)	*rhlR*$^+$ *rhlI lasB-cat*	821
PAO1(pLAFR3)	*rhlR*$^+$ *rhlI*$^+$ (vector control)	<20

[a] Samples of cells were obtained via centrifugation from standardized 20-h cultures in PTSB containing 100 μg of tetracycline per ml to ensure that each cell contained pMB20 or vector plasmid.

[b] In cell sonic extracts of wild-type and mutant *P. aeruginosa* containing a transcriptional reporter plasmid with *lasB-cat*. Values represent one of several experiments which produced comparable results. All values are the means of at least duplicate determinations by ELISA and were standardized to the amount of protein in each sonic extract.

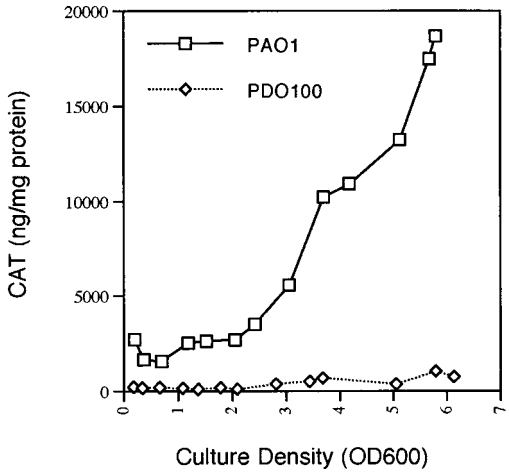

FIG. 4. Effect of *rhlI* mutation on cell density-dependent transcription of the elastase structural gene as determined with a *lasB-cat* fusion on pMB20. Shown are the results of standardized cultures of PAO1 (wild type) and its derivative PDO100 (*rhlI*Δ) in PTSB containing tetracycline growing at 37°C with maximum aeration. Samples were taken at the intervals shown for measurements of culture density (OD_{600}, on dilutions when necessary), CAT in cell sonic extracts as determined by ELISA, and total protein in the extracts. Similar results were obtained when either tetracycline or carbenicillin was used to maintain the reporter plasmid in these strains.

to evaluate *lasB* transcription as a function of cell density and to evaluate the effect of *rhlI* mutation. Standardized PTSB cultures of PAO1 and PDO100 carrying pMB20 were periodically sampled to measure increasing culture density (OD_{600}) and intracellular CAT accumulation due to *lasB-cat*. The resulting values for CAT antigen were plotted as a function of cellular density of the cultures, which also compensated for the somewhat slower growth of PDO100 than of the wild type. The results (Fig. 4) showed that specific *lasB* promoter-directed CAT was relatively constant in the wild-type strain until the culture density reached an OD_{600} of about 2.0 (approximately 2×10^9 cells per ml) and then increased about 4-fold at an OD_{600} of 4.0 and 7-fold at an OD_{600} of 6.0. In contrast, the specific intracellular CAT levels were markedly lower in all of the *rhlI* mutant samples than in wild-type samples. The basal level of *lasB-cat*-directed CAT in early samples was about 10-fold lower in the *rhlI* mutant. Also, no culture density-dependent increase in *lasB-cat*-directed CAT was seen; in contrast, wild-type samples above an OD_{600} of 2.0 showed dramatic increases. Thus, the *rhlI* gene product appears to be required for cell density-dependent transcription of the elastase structural gene in *P. aeruginosa.*

DISCUSSION

To identify a regulator of protease production in *P. aeruginosa*, we used a strategy that employed the isolation of an exoprotease-defective mutant (PDO31), although characterization of the mutant showed it to have pleiotropic defects. This was followed by genetic complementation with a wild-type PAO1 gene bank, sequence analysis, construction of defined mutants, and analysis of a protease gene fusion. The sequence analysis of our clones encoding the putative regulator revealed the *rhlR-rhlI* gene pair, which encoded proteins with homology to LuxR-LuxI in *V. fischeri*. Ochsner et al. (44) had reported the *rhlR* gene as being required for the synthesis of biosurfactant glycolipids called rhamnolipids, which confer heat-stable hemolytic activity, but also noted that an *rhlR* mutant strain

showed pleiotropic effects on elastase and pyocyanin synthesis. Since *rhlR* is located adjacent to genes (*rhlAB*) for rhamnolipid biosynthesis, its direct role in *rhlAB* expression was presumed likely. The possibility remained that the pleiotropic defects seen in the production of other exoproducts (e.g., elastase) could be indirect as a result of some membrane perturbation due to the loss of these glycolipids. However, we found that our *rhlI* mutant did not accumulate elastase within the cell, which would be the expected phenotype of a transport defect. Mutation in *rhlR* or *rhlI* also caused defects in the production of other extracellular virulence factors, including LasA protease and the redox-active pigment pyocyanin, suggesting that their transcription is also under RhlR-RhlI control in *P. aeruginosa*. Thus, RhlR-RhlI apparently represent a global regulatory system and a new addition to the burgeoning family of autoinducer-responsive regulators in gram-negative bacteria that sense their own cell density.

Recently, Oshsner and Reiser (45) also identified the RhlI component as an autoinducer synthetase of the LuxI type. They disrupted the *rhlI* gene in *P. aeruginosa* and showed a defect in rhamnolipid production that could be restored by exogenous autoinducer (either cell-free spent supernatant from a wild-type strain or synthetic *N*-acylhomoserine lactones). Our analysis of RhlR-RhlI control in *P. aeruginosa* has been more focused on elastase gene (*lasB*) regulation. The regulation of elastase in PAO1, using a *lasB-cat* transcriptional fusion, clearly showed cell density-dependent activation of this gene. Consistent with this hypothesis, the inactivation of *rhlI* encoding the putative autoinducer synthetase completely inactivated this response to cell density. Cross-feeding experiments showed that exoproducts from an *rhlR* mutant contained inducing activity (e.g., autoinducer) that could restore exoproduct production (e.g., pyocyanin) in an *rhlI* mutant.

RhlR and RhlI are similar to LasR and LasI, another pair of regulator-autoinducer synthetase proteins in *P. aeruginosa* that are involved in the production of multiple exoproducts. LasR has been shown to be a positive transcriptional regulator of the genes encoding elastase, LasA protease, and alkaline protease, with enhancement of toxin A expression (19, 20, 57). RhlR controls the production of elastase, LasA protease, rhamnolipid, pyocyanin, and possibly other factors. Thus, there is considerable overlap in the control activities of RhlR and LasR, but they are distinct control elements. The RhlR protein (27.6 kDa) shows only 31% identity with the LasR protein (26.6 kDa), and the RhlI protein (22.2 kDa) shows 25% identity with the LasI protein (22.8 kDa). Also, the *rhlRI* and *lasRI* genes are widely separated, mapping to 47 min (45) and ~12 min (55), respectively, on the 75-min map of the *P. aeruginosa* chromosome. Thus, these are separate regulatory systems, although both appear to act as global regulators of virulence factor transcription in *P. aeruginosa*.

The apparent overlap in form and function between the RhlR-RhlI and LasR-LasI systems suggests that they may interact in some way. The studies by Passador et al. (50), using *E. coli* containing a *lasB-lacZ* reporter system and *lasR* expressed under an exogenous promoter, showed that culture filtrates containing PAI were sufficient to activate *lasB* expression in this heterologous host, suggesting that no other *P. aeruginosa*-derived factors are required. *E. coli* containing *lasI* produces biologically active PAI, which was determined to be *N*-(3-oxododecanoyl)homoserine lactone [or 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-dodecanamide] (51). However, Pearson et al. (52) recently showed that PAI could not activate *lasB-lacZ* expression in *P. aeruginosa*, but rather a second *N*-acylhomoserine lactone signal identified as *N*-butyrylhomoserine lactone (or *N*-[tetrahydro-2-oxo-3-furanyl)butanamide] was required. Yet when *lasR* was expressed under the control of an exogenous promoter in *P. aeruginosa*, PAI could activate *lasB-lacZ* expression in the native host, suggesting that this second factor may be involved in *lasR* control (52). Interestingly, VAI, *N*-(3-oxohexanoyl)homoserine lactone [or *N*-(β-ketocaproyl)homoserine lactone], has been reported to be produced by *P. aeruginosa* (1). Also, certain elastase-deficient *P. aeruginosa* mutants could be restored to exoenzyme production by the addition of exogenous VAI from *V. fischeri* (28). Our studies on the RhlR-RhlI system also indicate that another level of *lasB* regulation exists that involves autoinduction, although we did not address the position of RhlR-RhlI in the potential hierarchy of regulators. However, the recent studies of Ochsner and Reiser (45) show cross-regulation between the RhlR and LasR regulatory systems and provide evidence that the RhlR regulatory protein acts in conjunction with the autoinducers *N*-butyrylhomoserine lactone and VAI.

It is curious that *P. aeruginosa* has at least two autoinducer-responsive systems that both regulate exoenzyme biosynthesis, including elastase. The presence of dual autoinducer-responsive regulators suggests that different stimuli or target genes may be involved. However, this situation is not unique. *V. fischeri* synthesizes a second *N*-acylhomoserine lactone (31), and more than one autoinducer has been proposed for the complex quorum sensing system of *Vibrio harveyi* (2). Two autoinducer synthetase genes (*carI* and *expI*) have been found in *Erwinia carotovora* (56). It is also noteworthy that RhlI is more homologous to PhzI from *P. aureofaciens* (36% identity) than to LasI from *P. aeruginosa* (25% identity). PhzI (accession number L33724) is an autoinducer synthetase required for the efficient production of various phenazine antibiotics in *P. aureofaciens*. Pyocyanin is an analogous phenazine compound, unique to *P. aeruginosa*, and its expression is under RhlI control.

The presence of autoinduction systems is becoming increasingly more recognized in gram-negative bacteria (18, 56). One approach to identify autoinducer synthetase genes from enteric bacteria used an assay of functional homology, and this showed that proteins with as little as 25% identity to *V. fischeri* LuxI can be biologically equivalent (56). With the notable exception of the LuxR-VAI system, the mechanisms by which they function have yet to be fully elucidated. However, all appear to operate in the cell density-dependent transcription of target genes. The role of a cAMP receptor protein in the regulation of the *luxR-luxI* regulon in *Vibrio* sp. was mentioned above (for a review, see reference 41). West et al. (59) recently identified a gene from *P. aeruginosa* called *vfr* (for virulence factor regulator) which encodes a homolog (67% identical, 91% similar) of the cAMP receptor protein of *E. coli*. Their studies also showed that Vfr is involved in the expression of proteases and transcription of exotoxin A in *P. aeruginosa*. If the *rhlR-rhlI* pair is mechanistically analogous to the *luxR-luxI* system, then a cAMP receptor protein-like protein could be involved in its regulation. It is plausible that Vfr interacts with the *rhlR-rhlI* regulatory gene complex as part of its effects on virulence factor expression in *P. aeruginosa*.

Quorum sensing systems for coordinate regulation of several virulence factors may explain in part the extraordinary success of *P. aeruginosa* as a nosocomial pneumonia pathogen. For example, in alveolar abscesses which develop during pneumonia, *P. aeruginosa* grows to high cell densities. Autoinduction systems would be expected to sense these conditions, resulting in the high-level production of toxic and degradative products. Also, the presence of a dual autoinduction system for cell density expression of virulence factors may provide the sensory

input from the environment necessary for a concerted attack upon the host.

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ADDENDUM

Latifi et al. (33) have recently reported the identification of *rhlR-rhlI*, obtained through complementation of an elastase-negative mutant of PAO1, as evidence for multiple families of quorum-sensing modulons interactively regulating gene expression in *P. aeruginosa.*

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