Roles of Pseudomonas aeruginosa las and rhl Quorum-Sensing Systems in Control of Elastase and Rhamnolipid Biosynthesis Genes

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Two quorum-sensing systems (las and rhl) regulate virulence gene expression in Pseudomonas aeruginosa. The las system consists of a transcriptional activator, LasR, and LasI, which directs the synthesis of the autoinducer N-(3-oxododecanoyl) homoserine lactone (PAI-1). Induction of lasB encoding elastase and other virulence genes requires LasR and PAI-1. The rhl system consists of a putative transcriptional activator, RhlR, and RhlI, which directs the synthesis of N-butyryl homoserine lactone (PAI-2). Rhamnolipid production in P. aeruginosa has been reported to require both the rhl system and rhlAB encoding a rhamnosyltransferase. Here we report the generation of a ΔlasI mutant and both ΔlasI ΔrhlI and ΔlasR rhlR::Tn501 double mutants of strain PA01. Rhamnolipid production and elastolysis were reduced in the ΔlasI single mutant and abolished in the double-mutant strains. rhlAB mRNA was not detected in these strains at mid-logarithmic phase but was abundant in the parental strain. Further RNA analysis of the wild-type strain revealed that rhlA is organized as an operon. The rhlAB transcriptional start was mapped, and putative σ^54 and σ^70 promoters were identified upstream. To define components required for rhlAB expression, we developed a bioassay in Escherichia coli and demonstrated that PAI-2 and RhlR are required and sufficient for expression of rhlA. To characterize the putative interaction between PAI-2 and RhlR, we demonstrated that [^3H]PAI-2 binds to E. coli cells expressing RhlR and not to those expressing LasR. Finally, the specificity of the las and rhl systems was examined in E. coli bioassays. The las system was capable of mildly activating rhlA, and similarly, the rhl system partly activated lasB. However, these effects were much less than the activation of rhlA by the rhl system and lasB by the las system. The results presented here further characterize the roles of the rhl and las quorum-sensing systems in virulence gene expression.

Pseudomonas aeruginosa is an opportunistic human pathogen that infects immunocompromised individuals and people with cystic fibrosis (49, 60). Multiple cell-associated factors such as alginate, pili, and lipopolysaccharide are important in P. aeruginosa virulence. Additionally, many P. aeruginosa virulence factors, including toxins (exotoxin A and exoenzyme S), proteases (elastase, LasA protease, and alkaline protease), and hemolysins (phospholipase and rhamnolipid), are released from the bacterial cells. These factors have been found to contribute to the virulence of P. aeruginosa in animal models (3, 20, 21, 32), in vitro studies (2, 9, 26, 36), and clinical studies (23, 69). Expression of many P. aeruginosa virulence products is dependent on a particular environmental stimulus such as iron, nitrogen availability, temperature, or osmolarity, but in general, virulence factor expression does not occur until high cell density is achieved (2, 26). A mechanism through which P. aeruginosa regulates virulence gene expression in a cell density-dependent manner is known as quorum sensing.

Quorum sensing involves emission of an N-acyl homoserine lactone signal (an autoinducer [AI]) generated by a LuxI-type AI synthase. After reaching a threshold concentration, AI activates a LuxR-type transcriptional activator that induces specific genes (for a review, see reference 13). P. aeruginosa contains two separate quorum-sensing systems, the las and rhl systems. In las quorum sensing, the AI synthase, LasI, directs the synthesis of N-(3-oxododecanoyl) homoserine lactone (PAI-1) (46), which triggers the las-encoded transcriptional activator, LasR (15), to induce virulence genes such as lasB, lasA, apr, and toxA (14, 43, 65, 66). The las system also auto-regulates lasI, leading to the production of more PAI-1 (53). The importance of the las quorum-sensing system in P. aeruginosa virulence was recently demonstrated in vivo, where a ΔlasR mutant was shown to be avirulent in a mouse model of pneumonia (64).

The second P. aeruginosa quorum-sensing system, the rhl system, regulates production of rhamnolipid which has both hemolytic and biosurfactant properties (26, 29). Rhamnolipid is synthesized by the rhlAB-encoded rhamnosyltransferase (39). The four rhl genes required for rhamnolipid production are organized as a regulon with all genes transcribed in the same direction, 5^′-rhlABRI-3^′ (38–40). Components of the rhl quorum-sensing system include the rhl-encoded putative transcriptional activator, RhlR (38), and rhlI-encoded putative AI synthase, RhlI (40). A second P. aeruginosa autoinducer (PAI-2), N-butyryl homoserine lactone (47), was shown to restore rhamnolipid production in a P. aeruginosa rhlR mutant (40) and was later shown to require rhlI for its synthesis (68). Thus, it seemed likely that PAI-2 was the AI of rhamnolipid biosynthesis genes. Recent results showed that PAI-2 and RhlR enhanced expression of rhlD and rpoS in Escherichia coli (31). Clearly, rhlRD and rhlI are necessary for rhamnolipid biosynthesis in P. aeruginosa (38–40). Multicopy plasmids carrying rhlD and rhlI have shown a positive regulatory effect on rhlA expression in wild-type P. aeruginosa (40). This finding suggested rhlRD and rhlI are components required for autoinduction of the rhamnolipid biosynthesis genes rhlA and rhlD, but this has not been directly demonstrated.

In this report, we describe the generation of a ΔlasI mutant, a ΔlasI ΔrhlI double mutant, and a ΔlasR rhlR::Tn501 double
mutant of *P. aeruginosa* PAO1. Northern blot analysis of these mutants and the parent strain showed that the rhamnosyltransferase genes (*rhlAB*) were transcribed as a bicistronic RNA and that *rhlR* is transcribed independently of *rhlR*. In addition, we mapped the transcriptional start site of *rhlAB*. By constructing plasmids containing *rhlA* or *lasB* fusions with lacZ and a *rhlR* or *lasR* under control of the tac promoter (tacP), we develop an *rhl* AI bioassay in *E. coli* and demonstrate the specificity of the *las* and *rhl* systems in control of the elastase gene and rhamnolipid biosynthesis genes. Finally, using radiolabeled PAI-2, we provide evidence that PAI-2 binds to RhlR.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* stock cultures were maintained as follows. Late-log-phase cultures grown at 37°C with shaking in 50 ml of PTSB medium (5% peptone, 0.25% tryptic soy broth [pH 7] [41]) plus appropriate antibiotics were centrifuged at 6,000 × g for 10 min. Cells were resuspended in 1.5 ml of 10% (wt/vol) skim milk (Becton Dickinson Corp., Cockeysville, Md.) and stored at −70°C. Freshly isolated colonies from stock cultures were used for each *P. aeruginosa* experiment. *P. aeruginosa* was also grown in LB medium (51), on MacConkey agar (Difco Laboratories, Detroit, Mich.) containing gentamicin at 30 μg/ml, or on LBA (51) containing chloramphenicol at 30 μg/ml, streptomycin at 40 μg/ml, and tetracycline at 5 μg/ml. When required, the following antibiotics were used in the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; gentamicin, 50 μg/ml; and chloramphenicol at 30 μg/ml for *P. aeruginosa* cultures. Chromosomal DNA was prepared from *P. aeruginosa* cultures as described previously. DNA fragments were excised from agarose gels and purified by using the GentaClean protocol (Bio101 Corp., La Jolla, Calif.). Oligonucleotides were synthesized at the Core Nucleic Acid Laboratory at the University of Rochester. DNA sequencing was performed at the Sanger Center Institute (Cambridge, U.K.) (52) and the Molecular Biology Core Facility (St. Louis, Mo.) or Aldrich Chemical Corp. (Milwaukee, Wis.). Complex medium components were purchased from Difco. PAI-1 and PAI-2 were synthesized previously (45, 47).

**DNA techniques.** Plasmid DNA was purified and manipulated by using standard techniques (51). *E. coli* DH5α was used as a host strain for most molecular cloning. Restriction endonucleases, DNA-modifying enzymes, and T4 DNA ligase were purchased from Gibco/BRL (Gaithersburg, Md.). New England Biolabs (Beverly, Mass.). Plasmids were transformed (51) into *E. coli* and were electroporated (57) into *P. aeruginosa* as described previously. DNA fragments were excised from agarose gels and purified by using the GeneClean protocol (Bio101 Corp., La Jolla, Calif.). Oligonucleotides were synthesized at the Core Nucleic Acid Laboratory at the University of Rochester. DNA sequencing was performed at the Sanger Center Institute (Cambridge, U.K.) and the Molecular Biology Core Facility (St. Louis, Mo.) or Aldrich Chemical Corp. (Milwaukee, Wis.). Complex medium components were purchased from Difco. PAI-1 and PAI-2 were synthesized previously (45, 47).

**Construction of *P. aeruginosa* las and rhlA mutant strains.** To generate a *P. aeruginosa lasI* mutant, plasmid pHJ18 (46) was inserted as a 200-bp EcoRI fragment into pRK2013, carrying the lacI*::Tcr* cassette and eliminated approximately 40 kb but were incubated for a total of 82 to 88 h. At this time, cultures were centrifuged 16,000 × g for 5 min, and supernatants were passed through 0.22-μm-pore-size filters. Filters were extracted three times with 2 volumes of diethylpyrocarbonate. The pooled ether was washed with 0.05 M NaClO, and the ether phase was evaporated to dryness. The residue was dissolved in water. Rhamnose content in each sample was determined by duplicate orcinol assays compared to rhamnose standards (29, 37). Rhamnolipid was determined by the relation that 1.0 mg of rhamnose corresponds to 2.5 mg of cholesteryl (37).

**Elastolysis assay.** Elastolytic activity in *P. aeruginosa* culture fluids was determined by elastin Congo red (ECR) assays (2), with modifications. Briefly, cells from mid-log-phase cultures in PTSB were washed and resuspended in PTSB to an OD600 of 0.05. After 21 h at 37°C with shaking, culture supernatants were filtered (0.45-μm pore-size filter) and stored at −70°C. Triplicate 50-μl samples of culture filtrates were added to tubes containing 20 mg of ECR (Elastin Products Corp. Owingsville, Md.) and 1 ml of buffer (0.1 M Tris [pH 7.4], 50 mM CaCl2). Tubes were incubated 18 h at 37°C with rotation and then were placed on ice after 0.1 ml of 0.12 M EDTA was added. Insoluble ECR was removed by centrifugation, and the OD600 was measured. Absorption due to pigments produced by *P. aeruginosa* was corrected for by subtracting the OD600 of each sample that had been incubated in the absence of ECR.

**Complementation of mutations in *P. aeruginosa*.** The las deletion in *P. aeruginosa* PAO1-JP1 was complemented by lacp-las on pLASI-2. To complement both the *lasI* and *lasR* mutations in *P. aeruginosa* PAOI-2 (42), carrying both lacp-rhlII and lacp-las (each gene under control of a separate lacI) was used. To do this, rhlII was obtained on a 950-bp fragment of strain PAO1 DNA from pMB41 (from the *lasI* cluster 150 bp upstream of the *lasI* translational start to the *lasI* outer frame) cloned into pUC19, transforming a 1.4-kb EcoRI fragment which was ligated into the *lasI* deletion. This fragment was cloned into *P. aeruginosa* PAOI1 (wild type) and PDO100 (*Δ拉斯*) with the help of *E. coli* HB101 (pRK2013), using triparental matings as described by Deretic et al. (7). *E. coli* HB101 was used as the recipient and containing an approximately 20% (*n* = 250 Tc*°* exconjugants of strains PAO1 and PDO100, respectively) did not hybridize to either probe, indicating these exconjugants contained neither vector DNA nor the deleted portion of lasI. DNA from four of these Tc*°* exconjugants from strains PAO1 and PDO100 was analyzed by Southern blotting (described below).

In addition to the above-described mutants, we constructed a *P. aeruginosa lasI* mutant. Plasmid pMG319 has been used in the construction of strain PAO1-R1, the *P. aeruginosa* lasR*°* mutant (15). Here, pMG319 was mobilized from *E. coli* HB101 to *P. aeruginosa* PDO111 (*lasI*:Tcr501) by conjugation with the help of *E. coli* HB101(pRK2013). Tc*°* exconjugants were selected and screened for the absence of the vector sequence and the deleted portion of lasI as described above. DNA from four of these exconjugants was analyzed by Southern blotting.

**Southern blot analysis.** Chromosomal DNA was prepared from overnight cultures of *P. aeruginosa* exconjugants and parental strains grown in LB medium plus appropriate antibiotics with shaking as described by Silhavy et al. (56). After digestion with restriction endonucleases, DNA fragments were separated on 0.7% agarose gels, transferred to nylon membranes, and hybridized to 32P-labeled DNA probes (as described by Amersham). Southern analyses of Tc*°* exconjugants derived from strains PAO1, PDO100, and PDO111 are reported in Results.

**Rhamnolipid assay.** Rhamnolipid in *P. aeruginosa* culture fluids was detected as previously described (29), with modifications. Briefly, cells from mid-log-phase cultures grown in PTSB were washed and resuspended in modified FS medium to an optical density of 660 nm (OD660) of 0.15 and incubated at 37°C with shaking (lag phase). After 5 h, the cell density had reached approximately 40 h but were incubated for a total of 82 to 88 h. At this time, cultures were centrifuged 16,000 × g for 5 min, and supernatants were passed through 0.22-μm-pore-size filters. Filters were extracted three times with 2 volumes of diethylpyrocarbonate. The pooled ether was washed with 0.05 M NaClO, and the ether phase was evaporated to dryness. The residue was dissolved in water. Rhamnose content in each sample was determined by duplicate orcinol assays compared to rhamnose standards (29, 37). Rhamnolipid was determined by the relation that 1.0 mg of rhamnose corresponds to 2.5 mg of cholesteryl (37).

**Results.** Approximately 20% of exconjugants from strains PAO1 and PDO100 was analyzed by Southern blotting as described below.
### TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype and/or phenotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>P. aeruginosa</strong></td>
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<td>PAO1</td>
<td>Wild-type prototroph</td>
<td>This laboratory</td>
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<td>PAO-R1</td>
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<td>PAK</td>
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<td>PG201</td>
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<td>pBR322</td>
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<tr>
<td>pBluescript II SK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ori (ColE1) Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pRO1614</td>
<td>pBR322 carrying ori (P. aeruginosa) on 1.8-kb PvuI fragment</td>
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<td>pUC18 with a 1.8 kb PvuI fragment of pRO1614 containing ori (P. aeruginosa)</td>
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<td>pJPP1.7 with filled-in EcoRI site</td>
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<td>1.8-kb EcoRI fragment of strain PAO1 with ‘las’ on pluescript II KS</td>
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<td>pJMC31 with 3.0-kb KpnI-StyI fragment of pJPP1.7E (Δlasf), Te&lt;sup&gt;c&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>oriKdo mobRpl4 Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pRK2013</td>
<td>ori (ColE1) tra&lt;sup&gt;+&lt;/sup&gt; (RK2) Km&lt;sup&gt;r&lt;/sup&gt; helper plasmid for conjugation</td>
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<td>pMG319</td>
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<td>pBluescript II SK&lt;sup&gt;+&lt;/sup&gt; with rhlB on 825 bp BglI-BglII fragment from strain PG201 in both orientations</td>
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<td>pLAS1-2</td>
<td>lacp-las on pSW200</td>
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<td><strong>Plasmids used in protein expression</strong></td>
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<td>pEX1</td>
<td>pKK223-3 derivative (Pharmacia Corp., Uppsala, Sweden) containing lacP&lt;sup&gt;+&lt;/sup&gt; in PvuII site ori (ColE1), Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pEX1.8 carrying ori (P. aeruginosa) as a 1.8-kb PvuI fragment from pRO1614 in Syl site</td>
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<td>pKDT37</td>
<td>pEX1 containing lasR of strain PAO1 under tacp</td>
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<td>pECP59</td>
<td>pEX1.8 with lacp-lasR from pKDT37</td>
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of plII derived from pPAO-2. RNA molecular size standards were purchased from Gibco/BRL.

**Primer extension analysis.** Primer extension analysis was as described previously (1). Two oligonucleotides, P1 (5' GACGGCAACGGACGCACTTACAG-3') and P2 (5'-AGACCTTCGCGCAGCTTACAG-3'), complementary to nucleotides (nt) -89 to -110 and +17 to +5 relative to the rhlR start codon, respectively, were 5'-end labeled with the use of [γ-32P]ATP (3,000 Cpm/nmol, Dupont NEN) and 74 nucleotide kinase (Gibco Corp., 5'-la beled primers were annealed to P. aeruginosa RNA, and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.) was used to extend each primer. The resulting cDNAs were resolved on 8 M urea-8% polyacrylamide gels adjacent to a DNA sequence obtained by using the same oligonucleotide as used in each primer extension.

**Overexpression of RhlR.** We modified a tac expression vector to allow expression in E. coli or P. aeruginosa. Ends of a 1.8-kb PstI fragment of PRO1614 (containing a P. aeruginosa origin of replication) and SspI digested pEX1 (carrying tac and lacF) were made blunt by T4 DNA polymerase and ligated to form pEX1.8. rhlR was fused to tacP on EX1.8, and the spacing between the rhlR translational start site and Shine-Dalgarno region downstream of tacP was optimized. To do this, a DNA fragment containing the rhlR start was generated by PCR using Vent polymerase (New England Biolabs) in a Hybaid thermal reactor (National Labnet Corp., Woodbridge, N.J.) with the following reaction conditions: melting temperature, 95°C (1 min); annealing temperature, 60°C (1 min); and extension temperature, 72°C (1 min). 25 cycles. pSK25 containing rhlR from P. aeruginosa PG201 (which is 100% identical in amino acid sequence to RhlR from PA01) was used as a template for the primer rhlR-ORF (5’ATAGGCAATGAGGCAACGGAGC-3’) and the spike primer of pBluescript II SK’ (5’GTAATAACCCAGGCGCCATTACG-3’) to generate rhlR-ORF containing the start. The primer rhlR corresponds to a region of pBluescript II SK’ which is on the antisense strand of DNA relative to rhlR in pSK25. These primers were used to generate a 790-bp DNA fragment which contained rhlR under control of tacP. The tacP on pECP62 was confirmed by sequencing, which also revealed the expected 9-bp spacing between the Shine-Dalgarno region downstream of tacP and the translational start of rhlR. Protein expression was monitored by growing cultures of E. coli DH5α/pECP62 on IPTG (1 mM) to an OD600 of 0.5. Growth was continued for 2 h in the absence of 1 mM isopropyl-thio-galactopyranoside (IPTG). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell lysates, followed by Coomassie brilliant blue staining (51), revealed that an IPTG-inducible 26-kDa polypeptide was overexpressed in cells carrying pECP62 but not in those with pEX1.8.

**Bioassays for the rhl AI.** To do an rhl AI bioassay in E. coli, we constructed a plasmid carrying tacP-rhlR and an rhlA-lacZ reporter gene fusion. The rhlA-lacZ fusion on pCEP60, which contains 548 bp upstream of rhlA through the first 25 codons of rhlA in frame with lacZ, was released as a 4.70-kb XmnI fragment and ligated to the filled-in (by Klenow enzyme) SalI site upstream of tacP-rhlR on pJPP8, forming pECP61.5.

For rhl AI bioassays, overnight cultures of E. coli containing pECP61.5 were diluted to an OD600 of 0.08 and grown at 37°C with shaking to an OD600 of 0.3; 1 ml aliquots of culture were then grown for 90 min with IPTG (1 mM) in the presence or absence of AIs. β-Galactosidase activity was measured as described previously (34).

**Bioassays in P. aeruginosa.** Cells from overnight cultures of P. aeruginosa PAO-J2 containing pECP61.5 were washed and resuspended in PTSB medium to an OD600 of 0.05 and then grown at 37°C with shaking to an OD600 of 0.3. At this point, 1 ml aliquots of culture were transferred to tubes containing test samples. Growth was continued for 60 min, and cultures were placed on ice. Cells were washed in A medium, and β-galactosidase was assayed.

**Specificity of rhl and las autoinduction systems.** To examine the effects of the las autoinduction system on rhlR expression, we constructed a plasmid with tacP-lasR and an rhlA-lacZ fusion. Then we ligated a BamHI-HindIII fragment of pKDT37 containing tacP-lasR to pEX1.8 which had been digested with BamHI and HindIII. The resulting plasmid, pECP59, was digested with BstUII, and ends were filled in by Klenow enzyme and ligated to a 4.70-kb XmnI fragment from pECP60 containing the rhlA-lacZ fusion, resulting in pECP63. As a positive control plasmid for the effect of the las autoinduction system on lacl fusion, a lasB-lacZ fusion and tacP-lasR were cloned on the same pEX1.8 vector as follows. Ends of BstUII-digested pECP59 and the 3.75-kb EcoRI109I fragment of pKDT37 (which carries the lacl-ORF translational fusion of pTS400) were ligated in by Klenow enzyme and ligated to form pECP64. To study the effects of the rhl autoinduction system on lasB expression, plasmid pECP62.5 was constructed. To do this, ends of the 3.75-kb EcoRI109I fragment from pKDT37 (containing the lasB-lacZ translational fusion) were filled in, and the fragment was ligated into the filled-in SalI site of pJPP8. The resulting plasmid, pECP62.5, contained tacP-rhlR and lasB-lacZ.

Bioassays testing specificity of rhl and las autoinduction systems in E. coli DH5α containing either pECP62.5, pECP63, or pECP64 were performed as described above for the rhl AI bioassay using E. coli DH5α/pECP61.5.

**Synthesis of biologically active 3HPAI-2.** Tritium-labeled PAI-2 (3HPAI-2) was synthesized by using procedures similar to those described for synthesis of tritium-labeled V. fischeri luclux AI-1, [3H]-[3-oxoheanoyl] homoserine lactone (‘H-VAI-1) (27), and [3H]-PAI-2 (45). First, N-crotonoyl-l-homoserine lactone, an alkene analog of PAI-2, was synthesized by coupling sodium crotonate and l-homoserine lactone hydrochloride by the method of Eberhardt et al. (11) and was purified by reverse-phase high-performance liquid chromatography (HPLC) using water and methanol (C8 column, 1 by 25 cm; Beckman Corp., Fullerton, Calif.). Analysis by 1H nuclear magnetic resonance (NMR) spectroscopy (in [D$_2$]O and in [D$_3$]HCl) on a 300-MHz Bruker instrument at the Department of Chemistry, University of Rochester) indicated the purified product was N-crotonoyl homoserine lactone. The N-crotonoyl homoserine lactone was radiola beled by reduction using tritium gas in the presence of a Pd catalyst by Dupont NEN. Tritium NMR spectroscopy of the synthesis product (3HPAI-2) was performed on a 300-MHz Bruker instrument by Dupont NEN (proton decoupled in [D$_3$]HCl). The 1H NMR spectrum of the synthetic 3HPAI-2 contained only three peaks (2.20, 1.65, and 0.95 ppm), which correspond to tritium at positions 2, 3, and 4, respectively, on the acyl side chain of PAI-2 in a random manner. This result indicated that half of the synthetic 3HPAI-2 contained tritium in the methylenes at positions 2 and 3 and half contained tritium in the methylenes at positions 3 and 4, as could be expected for catalytic reduction of α,β unsaturated groups on acyl chains (57).

When 3HPAI-2 was applied to C$_8$ reverse-phase HPLC, a single radioactive peak that was the sole peak of PAI-2 activity [assayed in E. coli DH5α (pECP61.5)] was eluted with the same retention time as unlabeled PAI-2. In addition, when 3HPAI-2 was applied to the same HPLC together with excess unlabeled PAI-2 as the carrier, as described for the characterization of ‘H-VAI-1 (27), a single peak of radioactivity coeluted with PAI-2 activity (data not shown). Specific activity of 3HPAI-2 was determined in the rhl AI bioassay in E. coli to be 100 Ci/mmol, and bioactivity of 3HPAI-2 was indistinguishable from that of unlabeled PAI-2 (data not shown). We stored 3HPAI-2 in ethyl acetate at -20°C.

**3HPAI-2 binding assays.** The binding assay was based on the method of Hanzelka and Greenberg (19) for binding of 3HPAI-1 to E. coli expressing the V. fischeri LuxR. Overnight cultures of E. coli DH5α containing either pJPP8, pECP59, or pEX1.8 were diluted in LB medium to an OD600 of 0.05 and incubated at 37°C with shaking until an OD600 of 0.5 was reached. At this point, IPTG was added to a final concentration of 1 mM and growth was continued for an additional 2 h. Next, 1 ml of culture with 60 µl of 3HPAI-2 in water (solutions of 3HPAI-2 in ethyl acetate were evaporated in tubes under a stream of N$_2$ gas, and 3HPAI-2 was dissolved in water). Samples were incubated for 30 min at 25°C. After 5 min on ice, samples were centrifuged (16,000 × g for 15 min at 4°C), and the cells were washed twice in resuspension in 1 ml of ice-cold phosphate-buffered saline (pH 7) and by centrifugation as before. After this, cells were centrifuged for 10 min to remove all supernatant fluid. Finally, cells were resuspended in 30 µl of phosphate-buffered saline, and radioactivity in the cell suspension was determined by standard scintillation counting procedures. All binding experiments were performed four times.
RESULTS

Construction and characterization of quorum-sensing mutants of strain PAO1. Previous reports indicated that the two *P. aeruginosa* quorum-sensing systems, las and rhl, regulate each other to some extent (4, 31, 40, 47, 48). To determine the quorum-sensing systems’ effects on each other and how they control virulence genes, a *P. aeruginosa* lasI mutant was desired. Previous attempts to obtain this mutant had been unsuccessful (47, 53). We speculated that these attempts failed for technical reasons and tried again. Figure 1A shows a schematic diagram showing lasR and lasI genes on the *P. aeruginosa* PAO1 chromosome and relevant restriction sites for PstI (P) and SphI (Sp). Also shown is the region of pJPP4 that contains *P. aeruginosa* DNA with a Tc^r cartridge replacing part of lasI (described in Materials and Methods). The thick black line represents the probe used for the Southern blot in panel B. (B) Autoradiograph of a Southern blot of chromosomal DNA isolated from four Tc^r P. aeruginosa exconjugants (lanes A to D) and from wild-type *P. aeruginosa* (lane PAO1), digested with PstI and SphI as indicated. Uncut plasmid pJPP4 was run as a control. DNA was electroblotted on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized to an 813-nt P^32-labeled DNA probe as indicated in panel A. Hybridization to a 2.0-kb PstI fragment from the exconjugants indicated that the PstI site in the 3′ end of lasI was lost. Hybridization to a 2.0-kb SphI fragment indicated that the SphI site in the Tc^r cartridge was gained.

![Figure 1](http://jb.asm.org/)

**FIG. 1.** Construction of a *P. aeruginosa* ΔlasI mutant strain. (A) Schematic diagram showing lasR and lasI genes on the *P. aeruginosa* PAO1 chromosome and relevant restriction sites for PstI (P) and SphI (Sp). Also shown is the region of pJPP4 that contains *P. aeruginosa* DNA with a Tc^r cartridge replacing part of lasI (described in Materials and Methods). The thick black line represents the probe used for the Southern blot in panel B. (B) Autoradiograph of a Southern blot of chromosomal DNA isolated from four Tc^r P. aeruginosa exconjugants (lanes A to D) and from wild-type *P. aeruginosa* (lane PAO1), digested with PstI and SphI as indicated. Uncut plasmid pJPP4 was run as a control. DNA was electroblotted on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized to an 813-nt P^32-labeled DNA probe as indicated in panel A. Hybridization to a 2.0-kb PstI fragment from the exconjugants indicated that the PstI site in the 3′ end of lasI was lost. Hybridization to a 2.0-kb SphI fragment indicated that the SphI site in the Tc^r cartridge was gained.

placed in the same manner as in strain PAO-JP1 (data not shown). This resulted in a ΔlasI ΔrhlI double mutant that was named strain PAO-JP2.

Since lasI has been shown to direct the synthesis of PAI-1 (46), we tested ethyl acetate extracts of culture fluids from the *P. aeruginosa* mutant strains PAO-JP1 and PAO-JP2 for PAI-1. As expected, culture fluid extracts from both strains did not contain PAI-1, whereas culture fluid extracts of the parental strains PAO1 and PDO100, respectively, contained PAI-1 (data not shown). This finding demonstrated that lasI was required in *P. aeruginosa* to direct the synthesis of PAI-1. It further shows that rhlI is not required for synthesis of PAI-1 and that the rhl quorum-sensing system does not regulate lasI expression.

In addition to the above-described mutants, we also wanted to generate a lasR rhlR double mutant. To do this, we mobilized a previously constructed suicide plasmid containing ΔlasR::Tc^r (15) into strain PDO111, a rhlR::Tn501 mutant of PAO1 (4). Southern blot analysis of ΔlasI P. aeruginosa PDO111 exconjugants resulted in an autoradiograph (data not shown) virtually identical to that reported for strain PAO-R1, the ΔlasR mutant (15). This result indicated that lasR had been deleted from strain PDO111, and this lasR rhlR double-mutant strain was named PAO-JP3.

We began our characterization of the mutants by examining how major targets of quorum sensing (such as elastase and rhamnolipid) were affected. We analyzed cell-free culture fluids of *P. aeruginosa* PAO-JP1 (lasI), PDO100 (rhlI), PAO-JP2 (lasI rhlI), and PAO1 (wild type) for elastolytic activity in ECR assays and for rhamnolipid in orcinol assays (Table 2). Both elastolysis and rhamnolipid levels were significantly lower in strains PAO-JP1 and PDO100 than in the wild-type strain PAO1 (each strain contained the control vector pSW200 [Table 2]). Elastolytic activity and rhamnolipid production were completely restored when strain PDO100 contained pJPP45 (rhlI^rhlI^rhlI) or was grown in the presence of PAO-JP1 (Table 2). Both phenotypes were also complemented when strain PAO-JP1 contained pLASI-2 (lasI^rhlI^rhlI^rhlI) (Table 2). Interestingly, addition of PAI-1 to cultures of strain PAO-JP1 (pSW200) fully restored elastolysis but only partially restored rhamnolipid (Table 2). This finding suggests that either (i) the PAI-1 concentration tested was not optimal for rhamnolipid production or (ii) other AIs known to be less abundantly synthesized by the lasI gene product (46) may contribute to rhamnolipid biosynthesis.

Neither rhamnolipid nor elastolysis was detectable in culture fluids of strain PAO-JP2 (lasI^rhlI^rhlI^rhlI) (Table 2). Full complementation of rhamnolipid and elastolysis occurred when PAI-1 and PAI-2 were added together to cultures of strain PAO-JP2 (pSW200) and when strain PAO-JP2 contained pJPP42 (lasI^rhlI^rhlI^rhlI) (Table 2). Elastolysis and rhamnolipid were only partially restored when strain PAO-JP2 contained either pJPP45 (rhlI^rhlI^rhlI^rhlI) or pLASI-2 (lasI^rhlI^rhlI^rhlI) (Table 2). These results confirmed our findings with the lasI and rhlI single mutants [strains PAO-JP1 (pSW200) and PDO100(pSW200)], which indicated that both lasI and rhlI are required for wild-type levels of elastolysis and rhamnolipid.

Addition of either PAI-1 or PAI-2, at the concentrations tested, to strain PAO-JP2(pSW200) resulted in partial restoration of elastolysis (Table 2). Adding PAI-2 to this strain, however, essentially restored wild-type levels of rhamnolipid, whereas PAI-1 alone had no effect on rhamnolipid production (Table 2). Elsewhere we have shown PAI-1 alone had no effect on rhamnosyltransferase gene expression (rhlA-lacZ) in strain PAO-JP2(pECp60) (48). There, we showed that PAI-2 only partially restored rhlA expression and that both PAI-1 and PAI-2 were needed for maximal expression (48). However, the...
levels of AIs that we tested in those studies were 10-fold less than those tested here. In addition, the experimental conditions in that study used early-stationary-phase cells grown in rich PTSB medium to measure rhlA-lacZ gene expression (48), whereas here rhamnolipid, which accumulates in culture fluids, was measured from late-stationary-phase cultures in GS minimal medium. Thus, different culture conditions and measurements likely reflect the observations that PAI-2 partially complemented rhlA expression production in the lasI rhlI double mutant [strain PAO-JP2(pEPc60)] (48) but almost fully complemented rhamnolipid in PAO-JP2(pSW200) (Table 2).

Northern analysis of rhlA, rhlB, and rhlR. Because lasI was found to be required for P. aeruginosa wild-type levels of rhamnolipid production (Table 2), and elsewhere we showed PAI-1 is involved in rhlA expression (48), we examined the effect of lasI on transcription of rhamnolipid biosynthesis genes by Northern blot analysis of P. aeruginosa RNA. Previous studies indirectly suggested that rhlA and rhlB may be corepressed from a putative promoter upstream of rhlA (39). However, this observation was based on complementation analysis of rhamnolipid production by P. aeruginosa rhlAB transposon mutants and on expression of rhlA', rhlB', and rhlAB' translational fusions with lacZ in P. aeruginosa (39). We probed P. aeruginosa RNA from mid-log-phase cells (OD600 of 0.9) grown in GS medium with 32P-labeled internal fragments of rhlA, rhlB, rhlR (Fig. 2A), and pilA (positive control). The rhlA probe consistently yielded a strong 1.15-kb band in addition to a less intense 2.4-kb band when hybridized to RNA from either strain PAO1 (Fig. 2B, lane 5) or strain PG201 (data not shown), but the rhlB probe did not hybridize to RNA from any of the mutant strains (Fig. 2C, lanes 1 to 4). This finding indicated that las and lasR are also required for expression of rhlB. Interestingly, a 2.4-kb transcript was detected in Northern analysis of strain PAO1 or PG201 RNA with either the rhlA or rhlB probe. However, unlike the rhlA probe, which also hybridized to a 1.15-kb transcript (Fig. 2B, lane 5), the rhlB probe hybridized only to a 2.4-kb transcript (Fig. 2C, lane 5, and data not shown for strain PG201). Possibly a transcriptional terminator between rhlA and rhlB could account for the 1.15-kb transcript detected by rhlA probes. The 2.4-kb transcript detected in P. aeruginosa wild-type RNA by using either the rhlA or rhlB probe is in agreement with the hypothesis that rhlA and rhlB are organized as an operon (39).

We next wanted to determine if the putative rhlAB transcript was independent from that of rhlR, which is found immediately downstream of rhlB. We probed for P. aeruginosa rhlR message and observed a strong 0.85-kb band with RNA from strains PAO-R1 and PAO1 (Fig. 2D, lanes 1 and 5, respectively) that was consistently less intense in strains PAO-JP2 and PAO-JP1 (Fig. 2D, lanes 3 and 4, respectively) and totally absent as expected in the rhlR lasR mutant, PAO-JP3 (Fig. 2D, lane 2). Thus, the 0.85-kb size of the rhlR transcript detected in our Northern blot hybridizations was consistent with that predicted by previous deletion analyses (4, 38). The fact that the rhlR probe gave a 0.85-kb band and not a 2.4-kb band confirmed that rhlR was not cotranscribed with the 2.4-kb rhlAB mRNA.

Thus, the results in Fig. 2 prove that the 720-bp rhlR transcript is transcribed as a monocistronic message from its own promoter and that rhlA and rhlB are transcribed as a 2.4-kb bicistronic RNA. Our results also indicate that a second rhlA message (1.15 kb) is produced.

As a control to show that RNA from each strain was intact and loaded equally, we used an internal fragment of P. aeruginosa pilA, which encodes the structural subunit of pilin, as a probe. The pilA probe hybridized to a 0.7-kb RNA from strain PAO1 and its derivatives as expected, indicating that RNA

<table>
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<th>Strain</th>
<th>Chromosomal genotype</th>
<th>Gene(s) on plasmid</th>
<th>AI addition</th>
<th>Elastolysis ($A_{495}$)</th>
<th>Rhamnolipid (mg/ml)</th>
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* Where indicated, cultures were grown in the presence of each AI at 12 μM.

$^*$ Elastase activities of culture fluids from 21-h cultures in PTSB medium were measured in ECR assays as described in Materials and Methods. Values are average $A_{495}$ of at least three experiments (each in triplicate) ± standard deviation. Numbers are averages of two or three experiments (each in duplicate) ± standard deviation. ND, not determined.
from all strains was present in equal amounts (Fig. 2E and reference 14).

Transcriptional start site mapping of rhlB mRNA. To further study rhlAB regulation, the transcriptional start site of rhlA was mapped by primer extension with the use of two separate oligonucleotide primers (described in Materials and Methods). Primer extensions with both primers showed two rhlA transcriptional start sites. In Fig. 3, we present the results obtained with the use of primer P1 and total RNA from P. aeruginosa grown in GS or LB medium. Major and minor extension products obtained with RNA from cells grown in GS medium are indicated by the upper and lower arrowheads, respectively, in lane GS (Fig. 3A). These products suggest that rhlA transcription starts at nt -228 (major start) or -183 (minor start) relative to the rhlA translational start codon. These results were identical to those obtained with primer P2 (data not shown). No extended products were visible when RNA from strains grown in LB medium was tested (Fig. 3A). However, longer exposure showed a very light band in lane LB at the same position as the upper arrowhead (data not shown).

As shown in lane GS in Fig. 3A, a single major extension product was produced and no products larger than this were detected. A minor extension product mapped to nt -183 up-

stream of the rhlA translational start may represent a second transcriptional start site of rhlA. Alternatively, this could be due to a premature transcriptional stop caused by a compression contained in the DNA sequence at this position upstream of rhlA. Sequence analysis of the region immediately upstream from the potential minor start site did not reveal any obvious promoter or regulatory sequences. Therefore, we believe that the minor primer extension product is likely an artifact. If the DNA upstream from the major rhlA transcriptional start site revealed both a putative σ^54-type promoter at nt -27 to -11 (Fig. 3B) and a putative σ^52-type promoter (TTGACA-N_7-TATAAT) at nt -32 to -6 (TTACCG-N_7-TAAAAA). Site-directed mutagenesis will be required to confirm the functional identity of the rhlA promoter.

Interestingly, some genes controlled by σ^54 are regulated by environmental signals such as nitrogen availability. We observed much less rhlA mRNA in cells grown in nitrogen-rich LB medium than in those grown in the nitrogen-limited GS medium (Fig. 3A), consistent with the predictions of earlier studies that found both rhlA-lacZ expression and rhamnolipid production to be dramatically increased when P. aeruginosa is grown in GS medium (39). To begin to determine if rhlA is a σ^54-controlled gene, we examined rhlA-lacZ expression from pECP60 contained in a P. aeruginosa rpoN (σ^54) mutant, strain PAK-N1, and its wild-type parent, strain PAK. Under previously described growth conditions (48), in the presence and absence of rpoN, β-galactosidase activities were 224 ± 15 and 13.9 ± 8.7 Miller units, respectively. Thus, rhlA-lacZ expression decreased 15-fold in the absence of σ^54. In addition, we probed RNA from strains PAK and PAK-N1 with an internal BamHI fragment of rhlA and detected the same faint 2.4-kb band and more intense 1.15-kb band from strain PAK as we observed from strains PAO1 and PG201, but no hybridization occurred with RNA from strain PAK-N1 (data not shown).

Together, the Northern analysis and rhlA-lacZ expression in the presence or absence of rpoN indicated that σ^54 plays some role in the expression of rhlA, but further study is needed to determine if σ^54 directly controls rhlA.

Quorum-sensing control of rhlA. In P. aeruginosa, expression of rhlA has been shown to require rhrR, and the production of rhamnolipids was shown to depend on rhlR and either rhlI or PAI-2 (38, 40). To identify components of P. aeruginosa that are both necessary and sufficient for expression of the rhlA gene, we developed a bioassay for quorum-sensing control of rhlA in E. coli DH5α by cloning a plasmid containing an rhlA-lacZ fusion and tacp-rhlR (pECP61.5) required both rhlR and at least 100 nM PAI-2 (Fig. 4A). Half-maximal activation of the rhlA promoter occurred with approximately 2.5 μM PAI-2 (38, 40). To identify components of P. aeruginosa that are both necessary and sufficient for expression of the rhlA gene, we developed a bioassay for quorum-sensing control of rhlA in E. coli DH5α by cloning a plasmid containing an rhlA-lacZ fusion and tacp-rhlR (pECP61.5). Expression of rhlA-lacZ in E. coli DH5α (pECP61.5) required both RhlR and at least 100 nM PAI-2 (Fig. 4A). Half-maximal activation of the rhlA promoter occurred with approximately 2.5 μM PAI-2 (Fig. 4A). In contrast, PAI-1 had almost no effect on rhlA expression at concentrations below 10 μM and only a slight effect at 50 μM (data not shown). From these data, we conclude that PAI-2 is the AI required for expression of the P. aeruginosa rhlAB operon.

To confirm these results for E. coli, we developed a bioassay for the rhl AI in P. aeruginosa. In the P. aeruginosa lasI rhlI double-mutant strain PAO-JP2 (pECP61.5), half-maximal rhlA activation occurred at a PAI-2 concentration 10-fold lower than that in E. coli (Fig. 4B). Half-maximal activation in strain PAO-JP2 occurred with approximately 250 nM PAI-2 (Fig. 4B). Others have shown 250 nM PAI-2 restored rhamnolipid production in a P. aeruginosa rhlI mutant (40). The results in Fig. 4 demonstrated that PAI-2 was the AI required for ex-
expression of the *P. aeruginosa* rhlAB operon and suggest that, like LuxR and LasR, which interact with VAI-1 (19) and PAI-1 (45), respectively, PAI-2 most likely interacts with RhlR to induce *rhlA* expression.

PAI-2 binds *E. coli* expressing *rhlR*. To characterize the interactions between PAI-2 and its putative target, RhlR, we synthesized $^3$HPAI-2 and used it to develop a binding assay to study the putative interaction between PAI-2 and RhlR. Previous results of studies using *E. coli* have shown that PAI-2 does not activate LasR to induce *lasB* expression (47) and does not block binding of $^3$HPAI-1 to *E. coli* expressing LasR (45). Figure 5A shows that $^3$HPAI-2 specifically binds *E. coli* expressing RhlR but not to *E. coli* expressing LasR. Binding of $^3$HVAI-1 to *E. coli* expressing LuxR has been shown to be enhanced about 10-fold when the cells contain pGroESL which has *lacp-groESL* for increased expression of the *E. coli* chaperonins GroES and GroEL (19). Figure 5A demonstrates that binding of $^3$HPAI-2 to cells with RhlR was enhanced fourfold in the presence of pGroESL. Nonspecific binding was 9% of total binding and was not affected by pGroESL. Half-maximal binding of $^3$HPAI-2 to cells expressing both RhlR and GroESL occurred with approximately 0.5 $\mu$M $^3$HPAI-2 (Fig. 5B). This is the same level of PAI-2 as needed for half-maximal induction of rhamnolipid in a *P. aeruginosa* rhl mutant (40). These results strongly support the notion that PAI-2 binds RhlR and that chaperonins encoded by *groESL* may facilitate proper folding of RhlR.

**Specificities of the las and rhl quorum-sensing systems.** Since *rhlAB* mRNA, rhamnolipid, and *rhlA*-lacZ were not expressed in *P. aeruginosa* PAO-JP2 (*lasI rhlI*) (Fig. 2, Table 2, and reference 48, respectively), we measured the effects of the *las* and *rhl* quorum-sensing systems on our *rhlA*-lacZ and *lasB*-lacZ reporters in *E. coli*. To do this, we constructed a series of plasmids carrying tacp-lasR or tacp-rhlR and either a *rhlA*-lacZ or *lasB*-lacZ translational fusion. We monitored $\beta$-galactosidase activities of *E. coli* containing the plasmids to measure the expression of the *rhlA*-lacZ and *lasB*-lacZ fusions. Addition of PAI-1 caused LasR (expressed from pECP63) to activate *rhlA*-lacZ 10-fold, but this was much less than the 320-fold activation of *rhlA*-lacZ by PAI-2 and RhlR (expressed from pECP61.5) (Table 3). As shown in Table 3, *E. coli* containing either pECP61.5 or pECP63 gave extremely low basal expression (approximately 1.7 Miller units) of *rhlA*-lacZ with no addition of AIs.

The *rhl* quorum-sensing system has been reported to affect *lasB* expression. In particular, both *lasB*-cat activity and elastolysis were shown to be reduced in *P. aeruginosa* rhlR and rhlI mutants (4). To determine if RhlR can activate *lasB*-lacZ in *E. coli*, pECP62.5 was constructed. Expression of *lasB*-lacZ increased sixfold when *E. coli* expressing RhlR (pECP62.5) was grown with 10 $\mu$M PAI-2 (Table 3). This result indicated the *rhl* quorum-sensing system is capable of partially activating *lasB*. As a control for LasR-mediated *lasB*-lacZ expression, we constructed pECP64. PAI-1 (50 nM) and LasR (expressed...
from pECP64) fully activated lasB−lacZ 20-fold (Table 3), as expected (46). Interestingly, high concentrations of PAI-1 (10 μM) caused both lasB expression and rhlA expression to decrease in E. coli expressing LasR (pECP64 and pECP63, respectively [Table 3]). These negative effects appear similar to results for E. coli expressing the V. fischeri LuxR (from tacp-luxR), where low levels of VAI-1 activated luxR expression (from a luxRp-luxAB reporter fusion) whereas high levels of VAI-1 repressed luxRp expression (54). Basal levels of β-galactosidase activity in E. coli containing lasB−lacZ in the presence of either RhlR (pECP62.5) or LasR (pECP64) were not identical (33 and 87 Miller units, respectively). The results in Table 3 demonstrate that the rhl quorum-sensing system can recognize and slightly activate lasB and that the las quorum-sensing system can recognize and slightly activate rhlA. These data also indicate the great specificity of each transcriptional activator protein and its cognate AI (RhlR plus PAI-1 and LasR plus PAI-2 do not activate either reporter). Clearly the rhl system has a much greater effect on rhlA than on lasB, and similarly, the las system preferentially activates lasB over rhlA.

DISCUSSION

Here we report the generation of three mutants important for the study of P. aeruginosa quorum sensing. Despite failures during previous attempts to construct a lasI mutant strain (47, 53), we were able to generate a lasI mutant and lasI rhlI double mutant. In addition, we created a lasR rhlR double mutant of P. aeruginosa. Thus, with mutations in each of the two P. aeruginosa quorum-sensing systems, we were equipped to examine the effects of the two systems on each other as well as to better define the regulation of the major target of the rhl system itself, the rhamnolipid biosynthesis operon rhlAB.

In this report, we showed that both P. aeruginosa rhamnolipid production and rhlA expression are controlled by both the rhl and las quorum-sensing systems. Particularly, we found that production of elastolytic activity and rhamnolipid were reduced in the P. aeruginosa lasI and rhlI mutant strains (PAO-JP1 and PDO100, respectively). Brint and Ohman have previously reported a decrease in elastolysis and the total loss of rhamnolipid activity (hemolysis) in strain PDO100 (4). This discrepancy between the studies most likely reflects differences

FIG. 4. Bioassays for the rhl AI in E. coli and P. aeruginosa. (A) Dose response of E. coli DH5α(pECP61.5) to increasing amounts of PAI-2. Expression of rhlA in the presence of RhlR and increasing concentrations of PAI-2 was monitored with β-galactosidase activity assays. β-Galactosidase levels in cultures that received no addition of PAI-2 were 1.7 ± 0.3 Miller units. (B) rhl AI bioassay in P. aeruginosa PAO-JP2. The expression of the rhlA−lacZ reporter in strain PAO-JP2(pECP61.5) in the presence of RhlR and increasing concentrations of PAI-2 was monitored as in panel A. β-Galactosidase levels from cultures that received no addition were 100 ± 8 Miller units.

FIG. 5. Evidence for PAI-2 binding to RhlR. (A) Binding of 3HPAI-2 to E. coli overexpressing RhlR or LasR. Shown is radioactivity remaining with E. coli DH5α (in the presence and absence of pGroESL) carrying the vector control (pEX1.8) or expressing LasR (pECP59) or RhlR (pJPP8) after incubation with 250 nM 3HPAI-2. (B) Dose response for binding of 3HPAI-2 to E. coli DH5α expressing RhlR. Shown is radioactivity remaining with cells containing pGroESL and either pJPP8 (●) or pEX1.8 (○) after incubation with increasing concentrations of 3HPAI-2. Assays were performed as described in Materials and Methods.
TABLE 3. Specificities of P. aeruginosa las and rhl quorum-sensing components in E. coli

<table>
<thead>
<tr>
<th>AI added</th>
<th>rhlA-lacZ expression in the presence of:</th>
<th>lasB-lacZ expression in the presence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RhlR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LasR&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>1.7 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>PAI-2 (50 nM)</td>
<td>4.7 ± 0.7</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>PAI-2 (10 μM)</td>
<td>610 ± 26</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>PAI-1 (50 nM)</td>
<td>1.6 ± 0.1</td>
<td>20 ± 0.6</td>
</tr>
<tr>
<td>PAI-1 (10 μM)</td>
<td>9.1 ± 1.1</td>
<td>12.1 ± 13.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are averages of four β-galactosidase assays in Miller units ± 1 standard deviation for each. Assays were performed as described in Materials and Methods.

<sup>b</sup> E. coli DH5<sup>a</sup> containing pECP61.5, carrying a rhlA-lacZ reporter fusion and tacp-rhlR.
<sup>c</sup> E. coli DH5<sup>a</sup> containing pECP63, carrying a rhlA-lacZ reporter fusion and tacp-lasR.
<sup>d</sup> E. coli DH5<sup>a</sup> containing pECP62.5, carrying a lasB-lacZ reporter fusion and tacp-rhlR.
<sup>e</sup> E. coli DH5<sup>a</sup> containing pECP64, carrying a lasB-lacZ reporter fusion and tacp-lasR.

in growth media (peptone-glycerol broth in reference 4; GS medium here) and assay methods (they used an erythrocyte hemolysis assay for rhamnolipid activity; we used the orcinol assay for the presence of rhamnolipid).

Rhamnolipid and elastolysis were totally absent in the P. aeruginosa lasI rhlI double mutant (strain PAO-JP2), which indicated that production of these virulence factors requires both quorum-sensing systems. Both elastolysis and rhamnolipid were fully complemented in strains PAO-JP1, PDO100, and PAO-JP2 by the addition of the appropriate wild-type genes on plasmids (Table 2). Exogenously added PAI-1 and PAI-2 complemented elastolysis as expected (Table 2), but only PAI-2 complemented rhamnolipid production (Table 2). Exogenously added PAI-1 partially complemented rhamnolipid production in the lasI mutant (strain PAO-JP1) and had no effect on rhamnolipid in the lasI rhlI double mutant (strain PAO-JP2) (Table 2). We have shown that PAI-1 has no effect on rhlA expression in the P. aeruginosa lasI rhlI double mutant (48), and in Table 3 we observed only a minor effect of PAI-1 double mutant on rhlA expression and reconstitute the components necessary for expression of rhlA in E. coli. Development of a bioassay allowed us to demonstrate that RhlR and PAI-2 are required and sufficient for induction of the rhamnolipid biosynthesis operon in E. coli (Fig. 4A). Interestingly, 10-fold less PAI-2 was necessary to induce the same reporter in our P. aeruginosa lasI rhlI double mutant (Fig. 4B). This increased sensitivity of the reporter to PAI-2 in P. aeruginosa may be due to better uptake of PAI-2 through P. aeruginosa membranes. To date, only one study has examined AI transport through bacterial cells. HVAI-1 was found to penetrate both V. fischeri and E. coli by diffusion (28). Future work using 3HPAI-2 will elucidate the mechanism of PAI-2 transport into bacterial cells. HVAI-1 was found to penetrate both V. fischeri and E. coli by diffusion (28). Future work using 3HPAI-2 will elucidate the mechanism of PAI-2 transport into bacterial cells.

Northern blot analysis indicated the rhlA and rhlB genes were expressed as a bicistronic 2.4-kb rhlAB transcript and as a smaller 1.15-kb rhlA transcript. The smaller rhlA RNA may be due to an unknown transcriptional termination and antitermination mechanism or may be a product of RNase processing of the rhlAB transcript. Our Northern blots also proved rhlR was transcribed independently of rhlA and rhlB. Previous deletion analyses of the rhlR upstream region (4, 38) and earlier observations in P. aeruginosa, using both insertion mutants of rhlA and rhlB as well as rhlA<sup>c</sup>, rhlB<sup>c</sup>, and rhlB<sup>c</sup> fusions with lacZ (39), further support our evidence that rhlAB is transcribed as a bicistronic mRNA and that rhlR is transcribed from its own promoter. Elsewhere, we and others used rhlR-lacZ transcriptional fusions to show that rhlR expression is increased fivefold over its basal level by the las quorum-sensing system (31, 48). Based on our Northern analysis of rhlR mRNA presented here, however, it is clear that the las system is not required for basal levels of rhlR expression (Fig. 2D). As suggested before (31, 48), activation of rhlR by the las system seems to be part of the quorum-sensing hierarchy where the las system exerts control over the rhl system. Our Northern analysis supports this conclusion but suggests that basal levels of rhlR expression are dependent on mechanisms other than the las quorum-sensing system.

A major rhlA transcriptional start site was identified at nt −228 upstream of the translational start of rhlA (upper arrowhead in Fig. 3A). The 2.4-kb rhlAB and 1.15-kb rhlA mRNAs detected by Northern blotting (Fig. 2B and C) are likely produced from the major rhlA transcriptional start site, but further transcript mapping will be needed to prove this.

By mapping the major transcriptional start of P. aeruginosa rhlA, we were able to construct an appropriate reporter for rhlA activity and reconstitute the components necessary for expression of rhlA in E. coli. Development of a bioassay allowed us to demonstrate that RhlR and PAI-2 are required and sufficient for induction of the rhamnolipid biosynthesis operon in E. coli (Fig. 4A). Interestingly, 10-fold less PAI-2 was necessary to induce the same reporter in our P. aeruginosa lasI rhlI double mutant (Fig. 4B). This increased sensitivity of the reporter to PAI-2 in P. aeruginosa may be due to better uptake of PAI-2 through P. aeruginosa membranes. To date, only one study has examined AI transport through bacterial cells.

Another possible explanation for decreased AI sensitivity in P. aeruginosa PAI-2, we have demonstrated that 3HPAI-2 specifically binds E. coli expressing RhlR but not LasR (Fig. 5). Elsewhere we have shown that PAI-1 blocks binding of 3HPAI-2 to E. coli.

The fact that lasI on pLASI-2 partially restored rhamnolipid when carried by strain PAO-JP2, whereas PAI-1 did not, suggested that other AIs may be responsible. In addition to directing PAI-1 synthesis, the lasI gene product makes small amounts of other AIs (46) such as VAI-1 (10). These other AIs may be responsible for the partial complementation of rhamnolipid that we observed by pLASI-2. VAI-1 clearly does not activate LasR (17, 45, 46). Thus, we speculate that the small amount of VAI-1 that is likely to be made by the P. aeruginosa lasI product may be capable of inducing rhamnolipid biosynthesis via RhlR. However, the fact that PAI-2 (i) is much more abundantly produced (>200-fold) than VAI-1 by P. aeruginosa (46, 47), (ii) induces rhamnolipid biosynthesis at more than 20-fold-lower concentrations than VAI-1 (40), and (iii) alone almost fully restored rhamnolipid production by our lasI rhlI double mutant (strain PAO-JP2(pSW200) (Table 2)) emphasizes its role as the major autoinducer of rhamnolipid production.

Based on our Northern analysis of rhlR mRNA presented here, however, it is clear that the las system is not required for basal levels of rhlR expression (Fig. 2D). As suggested before (31, 48), activation of rhlR by the las system seems to be part of the quorum-sensing hierarchy where the las system exerts control over the rhl system. Our Northern analysis supports this conclusion but suggests that basal levels of rhlR expression are dependent on mechanisms other than the las quorum-sensing system.
expressing RhlR and inhibits PAI-2 activity in the rhlA bioassay (48). Others have observed inhibition of LuxR and VAI-1 induction of lux genes by a second V. fischeri AI (N-octanoyl homoserine lactone) produced by the ainS gene product (30). Both are examples of posttranslational control by two quorum-sensing systems of the same bacterium.

Our study supports the notion that PAI-2 binds specifically to the RhlR protein. Previous studies by others (5, 19) provide strong evidence for an AI-binding region on LuxR, but these results were also obtained in E. coli expressing ALuxR proteins and not with purified proteins. Future work with purified RhlR will be essential to directly prove that PAI-2 binds RhlR and to determine the position and number of PAI-2 binding sites.

Quorum-sensing and α factors. The results presented here have demonstrated quorum-sensing control of the rhamnolipid biosynthesis operon rhlAB. As expected for genes controlled by quorum sensing, we found a lux-type element (17) centered at −40 bp upstream of the major rhlAB transcriptional start. This putative cis-acting promoter element shows identity to the lux and tta boxes and contains 13 of 20 nt identical to the OP1 las box upstream of the P. aeruginosa elastase gene, lasB (reference 50 and Fig. 3B). Further inspection of the DNA upstream of the rhlAB transcriptional start has revealed two possible promoter motifs (α54 and α70). Identification of the promoter and other cis-acting elements involved in rhlAB transcription will require site-directed mutagenesis of this region. Protein purification and DNA footprinting studies will be necessary to determine if either RhlR or LasR binds to the putative lux box.

To date, quorum sensing has not been implicated in regulation of genes also controlled by α54-type promoters. Transcription from these promoters requires the binding of an activator protein to an upstream activation sequence (UAS), usually >100 bp upstream of the transcriptional start site. This binding is followed by looping of the intervening DNA and direct activator contact with RNA polymerase-α54 holoenzyme (which can bind the promoter but is unable to form an open promoter complex until contact is made with the activator protein bound to the UAS), resulting in transcription (33). Most of these in vitro studies have focused on two such activators, NtrC and NifA, which are found in a number of gram-negative bacteria (6). In P. aeruginosa, PilR was shown to footprint a 40-bp UAS consisting of three repeating 5′-N2(C/G)TGTC-3′ sequences in a tandem array from nt 120 to 80 upstream of the α54-controlled pilA transcriptional start (25). This type of UAS, however, is not present in the 300 bp upstream of the rhlA transcriptional start.

In contrast to extensive in vitro studies of α54 and its activators, much less is known about AI-LuxR-type transcriptional activation (13). Based on recent in vitro transcription assays and DNA footprinting using RNA polymerase-α70 holoenzyme and a purified AI-independent domain of LuxR to the V. fischeri lux promoter region (61, 62) as well as mutational analyses of the lux, tra, and las boxes (13, 50), quorum-sensing regulation of target genes has been shown to involve DNA elements that are overlapping or near the promoters of these genes. The −35 region of the putative α70 promoter at nt −32 upstream of the rhlAB transcriptional start overlaps the las box (Fig. 3B), as would be expected based on those previous studies. Determination of exactly how the las and rhl quorum-sensing systems directly interact with the rhlA and lasB promoters awaits future studies.

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