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The *Pseudomonas aeruginosa* Lectins PA-IL and PA-IIL Are Controlled by Quorum Sensing and by RpoS

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In *Pseudomonas aeruginosa*, many exoproduct virulence determinants are regulated via a hierarchical quorum-sensing cascade involving the transcriptional regulators LasR and RhlR and their cognate activators, *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL). In this paper, we demonstrate that the cytotoxic lectins PA-IL and PA-IIL are regulated via quorum sensing. Using immunoblot analysis, the production of both lectins was found to be directly dependent on the *rhl* locus while, in a *lasR* mutant, the onset of lectin synthesis was delayed but not abolished. The PA-IL structural gene, *lecA*, was cloned and sequenced. Transcript analysis indicated a monocistronic organization with a transcriptional start site 70 bp upstream of the *lecA* translational start codon. A *lux* box-type element together with RpoS (σ^S) consensus sequences was identified upstream of the putative promoter region. In *Escherichia coli*, expression of a *lecA*::*lux* reporter fusion was activated by RhlR/C4-HSL, but not by LasR/3O-C12-HSL, confirming direct regulation by RhlR/C4-HSL. Similarly, in *P. aeruginosa* PAO1, the expression of a chromosomal *lecA*::*lux* fusion was enhanced but not advanced by the addition of exogenous C4-HSL but not 3O-C12-HSL. Furthermore, mutation of *rpoS* abolished lectin synthesis in *P. aeruginosa*, demonstrating that both RpoS and RhlR/C4-HSL are required. Although the C4-HSL-dependent expression of the *lecA*::*lux* reporter in *E. coli* could be inhibited by the presence of 3O-C12-HSL, this did not occur in *P. aeruginosa*. This suggests that, in the homologous genetic background, 3O-C12-HSL does not function as a posttranslational regulator of the RhlR/C4-HSL-dependent activation of *lecA* expression.

Pseudomonas aeruginosa is an opportunistic human pathogen that produces a wide spectrum of exoproduct virulence determinants and secondary metabolites (33, 44, 53, 56). These include elastase, alkaline protease, LasA protease, exotoxin A, phospholipase C, exoenzyme S, hydrogen cyanide, and pyocyanin. *P. aeruginosa* also synthesizes two lectins termed PA-IL and PA-IIL (21, 26). These lectins appear to function as adhesins (66) as well as cytotoxins for respiratory epithelial cells (1, 2, 5). PA-IL, which exhibits specificity for the sugar galactose (21), is a tetrameric protein consisting of four 12.75-kDa subunits (21, 49). The gene encoding the PA-IL lectin gene has been isolated from *P. aeruginosa* ATCC 27853 (3). Subsequent sequence analysis identified an open reading frame (ORF) of 369 bp corresponding to the lectin structural gene, later termed *pa-1L* (3, 4) and here renamed *lecA* to conform with standard genetic nomenclature. PA-IIL is approximately 12 to 13 kDa (24) and exhibits a high specificity for fucose (19, 22, 26). PA-IL and PA-IIL in addition to mannose affinity have both been shown to interact with the ABO(H) and P blood group glycosphingolipid antigens which may contribute to the tissue infectivity and pathogenicity of *P. aeruginosa* (27). However, in contrast to many *Pseudomonas* virulence determinants, there is little information concerning lectin expression at the molecular level. Cell density and age of the culture are known to affect lectin synthesis, and the production of PA-IL and PA-IIL lectins and that of several other virulence factors have been reported to be coregulated (23, 25), suggesting the existence of common regulatory mechanisms.

The expression of multiple virulence and survival genes in *P. aeruginosa* is cell density dependent and relies on a cell-cell communication system termed "quorum sensing." This generic term is now commonly used to describe the phenomenon whereby the accumulation of a diffusible, low-molecular-weight signal molecule (sometimes referred to as a "pheromone" or "autoinducer") enables individual bacterial cells to sense when the minimal population unit or "quorum" of bacteria has been achieved for a concerted population response to be initiated (15). Quorum sensing is thus an example of multicellular behavior and modulates a variety of physiological processes including bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, plasmid conjugal transfer, and the production of virulence determinants in animal, fish, and plant pathogens (for reviews, see references 10, 15, 29, and 57).

P. aeruginosa employs *N*-acylhomoserine lactones (AHLs) as quorum-sensing signal molecules and has evolved a sophisticated regulatory hierarchy linking quorum sensing to virulence and survival in the stationary phase (38, 52). Two separate quorum-sensing circuits (termed *las* and *rhl*), each of which possesses an AHL synthase (LasI or RhlI, respectively) and a sensor-regulator (LasR or RhlR, respectively), modulate gene transcription in response to increasing AHL concentrations (6, 17, 18, 37, 46, 47, 68). The AHLs that signal within the *las* and *rhl* systems are *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), respectively. Together, the two systems comprise a hierarchical cascade that coordinates the production of virulence factors and stationary-phase genes (via the alternative

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
P. aeruginosa		
PAO1	Wild type	Holloway collection
PANO67	NTG[a] mutant derived from PAO1	34
PAO1 *lecA*::*lux*	*lecA*::*luxCDABE* genomic reporter fusion in PAO1	This study
PAOR	*lasR* mutant derived from PAO1	38
PDO100	*rhlI* mutant derived from PAO1	6
PDO111	*rhlR* mutant derived from PAO1	6
PAO1 *rpoS* negative	*rpoS* mutant derived from PAO1	35
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	72
S17-1 λpir	*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn*7* λ*pir*	61
CC118 λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rps-1 rpoB argE*(Amp) *recA thi pro hsdRM*$^+$ RP4-2-Tc::Mu-Km::Tn*7* λ*pir*	32
XL1-Blue MR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
Plasmids		
pSB421	*Eco*RI fragment containing *luxCDABE* cassette and Km^r element cloned into pUC18*Not*	70
pUCP18	Like pUC18 but additional 1.8-kb stabilizing fragment for maintenance in *Pseudomonas* spp.	60
pKNG101	Suicide vector carrying the *sacBR* genes for sucrose sensitivity; Sm^r	36
pNQluxIII	*luxCDABE* genes cloned into pNQ705	This laboratory
pNQ705	Cm^r suicide plasmid derived from pGP704	45
Supercos1	Cosmid vector, pBR322-derived ori, Amp^r	Stratagene
pMW47.1	2-kb *Pst*I PAO1 DNA insert (*rhlRI*) in pUCP18	37
pMW471.2	1.3-kb *Pst*I-*Eco*RI fragment containing *rhlR* in pUCP18	37
p*lasR*	*lasR* locus cloned into pUCP18	This laboratory
pCF6b	32.6-kb PAO1 DNA insert containing the *lecA* gene in Supercos1	This study
pCF1	4.4-kb *Pst*I fragment derived from pCF6b containing the *lecA* gene in pUCP18	This study
pCF2	3.1-kb *Pst*I-*Hin*dIII fragment derived from pCF6b containing the *lecA* gene in pUCP18	This study
pCF3	600-bp *Eco*RI fragment derived from pCF6b containing the *lecA* gene in pUCP18	This study
pCol2	PCR fragments encoding the *lecA* gene region cloned into *Eco*RI and *Kpn*I sites upstream and downstream of *luxCDABE* Km^r in pSB421	This study
pCol4	~8-kb *Not*I fragment from pCol2 cloned into pKNG101	This study
pCol9	496-bp PCR fragment, encoding the *lecA* upstream region, cloned into the *Not*I site of pNQluxIII	This study

[a] NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

sigma factor, RpoS [38, 52]). Individually, each system's sensor-regulator modulates a regulon comprising an overlapping set of genes. However, the *las* system directly regulates the *rhl* system, thus providing overall coordination of quorum sensing and temporal gene expression in response to cell-to-cell communication (31, 67).

In the present paper, we demonstrate that (i) the production of both PA-IL and PA-IIL is regulated via quorum sensing, (ii) the expression of the *lecA* gene is directly dependent on both RhlR/C4-HSL and RpoS, and (iii) the addition of exogenous 3O-C12-HSL to *P. aeruginosa* does not advance or inhibit RhlR/C4-HSL-driven *lecA* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 and XL1-Blue MR were used for cloning experiments. *E. coli* CC118 λpir was used for the construction of pCol2, pCol4, and pCol9, and *E. coli* S17-1 λpir was used for reporter gene studies and conjugation experiments. Bacteria were grown at 37°C in Luria-Bertani (LB) medium or on LB agar plates (58). For studying growth-phase-dependent lectin production, strains were grown at 37°C for 24 h in 250 ml of LB medium with shaking at 200 rpm. Samples were taken approximately every 2 h over the first 16 h and finally at 24 h. Where indicated, C4-HSL, C6-HSL, or 3O-C12-HSL was added to the growth medium prior to inoculation, at concentrations ranging from 0 to 100 μM. Standard methods were used for the preparation of competent cells and for plasmid electroporation into *E. coli* and *P. aeruginosa* (58, 62). Conjugal transfer was performed as described by Kaniga et al. (36). Where required, kanamycin, chloramphenicol, and ampicillin were added at 25, 34, and 50 μg/ml, respectively, for *E. coli*. For *P. aeruginosa*, kanamycin, streptomycin, and carbenicillin were added at 250, 150, and 300 μg/ml, respectively.

DNA manipulation. DNA was manipulated by standard methods (58). Restriction enzymes (Promega UK Ltd.) were used according to the manufacturer's instructions. Agarose gel electrophoresis and Southern blot transfer were performed essentially as described by Sambrook et al. (58). DNA probes were labeled with digoxigenin and detected using the DIG Luminescent Detection Kit supplied by Boehringer Mannheim. The IsoQuick kit (ORCA Research Inc.) was employed for the isolation of chromosomal DNA of *P. aeruginosa*. For isolation of plasmid DNA from *E. coli*, the Qiagen Mini and Midi kits (Qiagen Ltd.) were used.

RNA isolation and Northern blot analysis. Total RNA of *P. aeruginosa* was isolated according to the hot phenol-chloroform procedure described by Oelmüller et al. (48) with the modifications described by Gerischer and Dürre (20). For Northern blots, RNA was separated in denaturing formaldehyde gels and transferred to Hybond N$^+$ nylon membranes (Amersham Pharmacia Biotech Ltd.) as described by Sambrook et al. (58). Probes were labeled with [α-^{33}P]ATP using the Random Primers Labeling kit (Life Technologies Inc.). The probe was generated by PCR using the primers lecA.1F (ATATATCGGAGATCAATCA TGGCTTGG) and lecA.1R (CGTTCAGACCGAAGCGTGTTGAAGC). The DNA template was pCF1. Hybridizations and washings were performed as previously described (20). Fragment sizes were estimated by comparison with the 0.16- to 1.77-kb RNA ladder from Life Technologies Inc.

Primer extension analysis. Primer extension analysis was carried out as described by Gerischer and Dürre (20) except that SuperScript reverse transcriptase (Life Technologies Inc.) was used. The oligonucleotide used was RNA2 (ACCTGCCCTGCTTCGTTATTAG).

S1 nuclease analysis. For S1 nuclease analysis, single-stranded DNA was synthesized and labeled with α-^{35}S-dATP using a T7 sequencing kit from Amersham Pharmacia Biotech Ltd. The sequencing instructions of the manufacturer were modified as follows. After denaturation of plasmid DNA and annealing of

the respective oligonucleotide (RNA2), 3 μl of labeling mix, 1 μl of α-^{35}S-dATP (10 μCi), and 2 μl of T7 polymerase (1.5 U/ml) were added and incubated at 37°C for 3 min. After addition of 1 μl of deoxyribonucleoside triphosphates (800 μM each) and incubation at 37°C for 15 min, the reaction was stopped by phenol-chloroform extraction, and subjected to ethanol precipitation. The DNA pellet was suspended in 20 μl of H_2O and stored at −20°C. Labeled DNA (0.25 μg) and RNA (10 to 40 μg) were mixed, dried, and incubated at 85°C for 10 min after the addition of 15 μl of hybridization buffer (40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 7.0], 1 mM EDTA, and 400 mM NaCl in 80% [vol/vol] formamide). Hybridization was performed at 40°C for 4 h. RNA and single-stranded DNA were removed by the addition of 300 μl of S1 mapping buffer (40 mM sodium acetate [pH 5.0], 250 mM NaCl, 1 mM $ZnCl_2$, 20 U of S1 nuclease per ml, and 20 μg of denatured salmon sperm DNA per ml) and incubated at 30°C for 30 min. The reaction was stopped by phenol-chloroform extraction after the addition of 6 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS), 30 μl of 0.2 M EDTA (pH 8.0), and 30 μl of 3 M sodium acetate (pH 8.0) followed by ethanol precipitation and suspension of the precipitate in 5 μl of H_2O.

PCR. PCR amplifications were performed in 100-μl volumes as described previously (71).

DNA sequencing and sequence analysis. Automated nonradioactive sequencing reactions were carried out using the BigDye terminator cycle sequencing kit in conjunction with a 373A automated sequencer (Perkin-Elmer Applied Biosystems). For radioactive sequencing, the T7 sequencing kit from Amersham Pharmacia Biotech Ltd. was used. Sequence analysis and database searches were performed with the Genetics Computer Group (Madison, Wis.) software packages and the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/). For sequence comparisons, the program Gap (complete protein sequences) or BestFit (for truncated protein sequences) was used. Genes encoding tRNAs were identified using the tRNAscan-SE program (http://www.genetics.wustl.edu/eddy/tRNAscan-SE/) (39).

Synthesis of AHLs. C4-HSL, C6-HSL, and 3O-C12-HSL were synthesized essentially as described previously by Chhabra et al. (8). AHLs were dissolved in acetonitrile before being added to growth medium.

Detection of lectins by Western blotting. *P. aeruginosa* cells grown in LB medium as described above were suspended to an optical density at 600 nm (OD_{600}) of 1.0 in SDS-polyacrylamide gel electrophoresis sample buffer and lysed by sonication. Prior to electrophoresis, samples were boiled and loaded onto SDS–15% polyacrylamide gels and either stained with Coomassie brilliant blue or electrophoretically transferred to nitrocellulose. PA-IL and PA-IIL were detected on immunoblots using the respective monospecific polyclonal antibody (13) followed by an anti-rabbit immunoglobulin G-horseradish peroxidase conjugate and developed with diaminobenzidine and H_2O_2 as described by Harlow and Lane (30).

Cloning of the *lecA* gene. Based on the structural gene coding for PA-IL cloned from *P. aeruginosa* ATCC 27853 (3, 4), the oligonucleotide primers lecA.1F and lecA.1R were designed (for sequence, see "RNA isolation and Northern blot analysis") and used to amplify a 599-bp fragment from *P. aeruginosa* PAO1 by PCR. This fragment was labeled with digoxigenin and used to probe a PAO1 cosmid library constructed in the Supercos cosmid vector (Stratagene). Four positive cosmids were identified and designated pCF5a, pCF9a, pCF6b, and pCF10a. Southern blot analysis of the four cosmids revealed that the probe hybridized with a 4.4-kb *Pst*I fragment, a 7-kb *Hin*dIII fragment, and a 0.6-kb *Eco*RI fragment in each of the four clones. The probe also hybridized to equivalent-size restriction fragments from chromosomal DNA, digested with the same restriction enzymes, confirming that the four cosmid clones are derived from *P. aeruginosa* PAO1 (data not shown). Cosmid pCF6b was chosen for subcloning, and subsequently, a 4.4-kb *Pst*I fragment, a 3.1-kb *Hin*dIII-*Pst*I fragment, and a 0.6-kb *Eco*RI fragment were cloned separately into the vector pUCP18 (60), resulting in plasmids pCF1, pCF2, and pCF3, respectively.

Construction of *lecA*::*luxCDABE* reporter fusion. For construction of the *E. coli*-based *lecA*::*luxCDABE* reporter plasmid, pCol9, a DNA fragment containing the region upstream of *lecA* was amplified by PCR, incorporating *Not*I restriction sites, using the primers Col21 (CCGGTTCGACCCCGGCTGCGGC CGCCATTGTGTTTCCTGGCGTTCAGC) and Col22 (CGACGATGGTAAT GACAGCGGCCGCATTCTAGATAATCGACGTTACC). The resulting 496-bp promoter fragment was cloned in the correct orientation into the unique *Not*I site in pNQluxIII to create pCol9. For the PAO1 chromosomal reporter construct, the 5′ and 3′ regions of the *lecA* gene were PCR amplified using the primers Col1 (CCCGGGCACCATTGTGTTTCCTGGCGTTCAGCCGACT TC) and Col2 (CCCGGGATTGACCGGAATTCCTCAGCTGTTGCCAATCT TCATGACCAG) and primers Col3 (CGACGTGCGGGGTACCCCTGGCAA TAACTCCGGCTCGTT) and Col4 (GGTACAGGTTGGCGGGGTACCGCG TGCAATCGTACAGGC), respectively. The 5′ PCR fragment was cloned in the correct orientation into the *Eco*RI site upstream of *luxCDABE* in pSB421 (70), while the 3′ PCR fragment was cloned in the correct orientation into the *Kpn*I site downstream of *luxCDABE* in pSB421, resulting in plasmid pCol2. The *Not*I fragment containing *luxCDABE* Km^r flanked by the lectin sequence was cloned into the unique *Not*I site in pKNG101 (36), resulting in plasmid pCol4. Finally, chromosomal integration was achieved using the protocol described by Kaniga et al. (36) and confirmed by Southern blotting and PCR analysis (data not shown).

Time- and cell-density-dependent measurement of bioluminescence. Bioluminescence was determined as a function of cell density using a combined, automated luminometer-spectrometer (the Anthos Labtech Lucy1). Overnight cultures of *P. aeruginosa* were diluted 1:100 in fresh medium, and 0.2 ml was inoculated into microtiter plates. Luminescence and OD_{495} were automatically determined every 30 min. Luminescence is given in relative light units (RLU) divided by OD_{495}. For bar charts, the sum of the RLU/OD_{495} readings taken over the growth curve between 4.5 and 10 h was calculated and presented as a percentage of the control (no exogenous AHL added) and denoted as relative cumulative units (percentage of control).

Nucleotide sequence accession number. The sequence data reported here have been submitted to the GenBank database under accession no. AF229814.

RESULTS

PA-IL and PA-IIL production is positively regulated by the *las* and *rhl* quorum-sensing systems. Whole-cell protein extracts prepared from *P. aeruginosa* PAO1 cells harvested at 2-h intervals throughout growth were analyzed by immunoblot analysis using antibodies specific for PA-IL or PA-IIL. Neither lectin was detected in samples taken during the exponential stage of growth; however, at high cell densities, during the transition to stationary phase, production of both lectins was observed (Fig. 1). To determine whether quorum sensing is involved in the regulation of PA-IL and PA-IIL, their production was studied in *P. aeruginosa* PANO67. This is a pleiotropic PAO1 mutant, which lacks the ability to produce many different virulence factors and secondary metabolites, and although it produces 3O-C12-HSL (indicative of a functional *lasRI* locus), it is unable to synthesize C4-HSL (34, 37, 38, 69). PANO67 can be restored to wild type by the introduction of *rhlRI* but not *lasR* on a multicopy plasmid (37). Immunoblot analysis of PANO67 extracts prepared from cells taken at different time points throughout growth revealed that PANO67 does not produce either PA-IL or PA-IIL. However, transformation of PANO67 with pMW47.1 (containing the *rhlRI* locus) restored the growth-phase-dependent production of PA-IL and PA-IIL (Fig. 1A and B), suggesting that the *rhl* locus is required for lectin synthesis in *P. aeruginosa*. PANO67 transformed with the pUCP18 vector alone did not produce either lectin (data not shown). Further evidence that both lectins are regulated via quorum sensing was provided by studying lectin production in the *P. aeruginosa* mutant strains PDO100 (*rhlI* negative) and PDO111 (*rhlR* negative) (6). In both strains, PA-IL and PA-IIL production was abolished. Addition of C4-HSL to cultures of PDO100 overcame the *rhlI* mutation and restored lectin levels to that of the wild type (Fig. 1A and B). Furthermore, in the *lasR* mutant strain PAOR, production of both lectins was severely down regulated such that they were detected only during the late stationary phase (Fig. 1A and B).

Cloning and sequence analysis of the *lecA* gene. The sequence published for the ATCC 27853 *pa-IL* gene (here renamed *lecA*) region contained only 18 bp upstream of the structural gene (3, 4). To obtain the complete promoter region for further sequence analysis and reporter gene construction, the *lecA* gene region of PAO1 was cloned, resulting in plasmids pCF1, pCF2, and pCF3 with inserts of 4.4, 3.1, and 0.6 kb, respectively (see Materials and Methods for details). Expression of the recombinant *lecA* gene in *E. coli* occurred only when it was transformed with the plasmid pCF3. *E. coli* transformed with the plasmid pCF1 or pCF2 failed to express the recombinant PA-IL protein, as determined by immunoblotting (data not shown). Plasmid pCF3 contains only a 627-bp *Eco*RI fragment. Thus, it seemed probable that expression in *E. coli* (pCF3) was under the control of the pUCP18 *lac* promoter.

A 2,407-bp stretch of the putative *lecA* gene region of pCF1 was sequenced by primer walking, and the sequence was de-

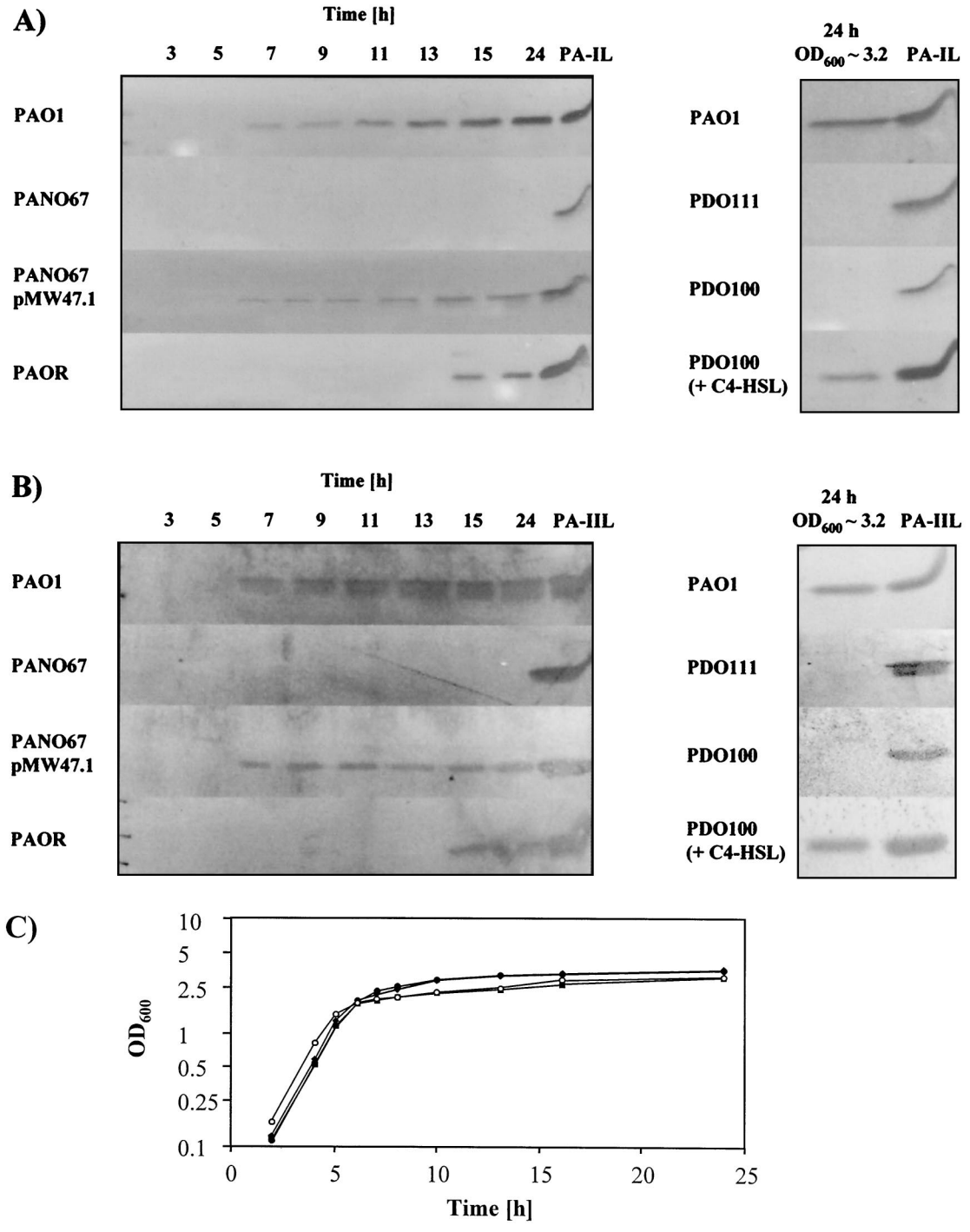

FIG. 1. Production of PA-IL (A) and PA-IIL (B) by *P. aeruginosa* PAO1, PANO67, PANO67(pMW47.1), PAOR, PDO100, and PDO111. For strains PAO1, PANO67, PANO67(pMW47.1), and PAOR, samples for immunoblot analysis were taken every 2 h and processed as described in Materials and Methods over the first 15 h and finally at 24 h (left panels). For PDO100 and PDO111, samples taken after 24 h were analyzed (right panels). As a control, the rightmost lane in each panel contained the purified PA-IL or PA-IIL lectin. For panel A, immunoblots were probed with a monospecific polyclonal antibody to PA-IL, and for panel B, immunoblots were probed with an antibody to PA-IIL. Growth curves (C) are shown for *P. aeruginosa* PAO1 (○), PANO67 (◆), PANO67(pMW47.1) (■), and PAOR (●). All strains were grown in LB medium at 37°C and entered stationary phase after approximately 6 h. Strains PDO100 and PDO111 showed very similar growth curves (data not shown).

posited as GenBank accession no. AF229814. DNA sequence analysis revealed an ORF of 369 bp, commencing with an ATG start codon and terminating with two consecutive stop codons, TGA and TAA. The putative PAO1 ORF is almost identical to the PA-IL lectin gene from *P. aeruginosa* ATCC 27853. One mismatch between the nucleotide sequences was identified, but the deduced amino acid sequences for the PA-IL proteins from the two strains were identical. Sequence comparison revealed a putative ribosome binding site (GGAGA) located 7 bp upstream of the ATG start codon, as has previously been described by Avichezer et al. (4) for strain ATCC 27853. A putative operator sequence, similar to the *lux* box upstream of

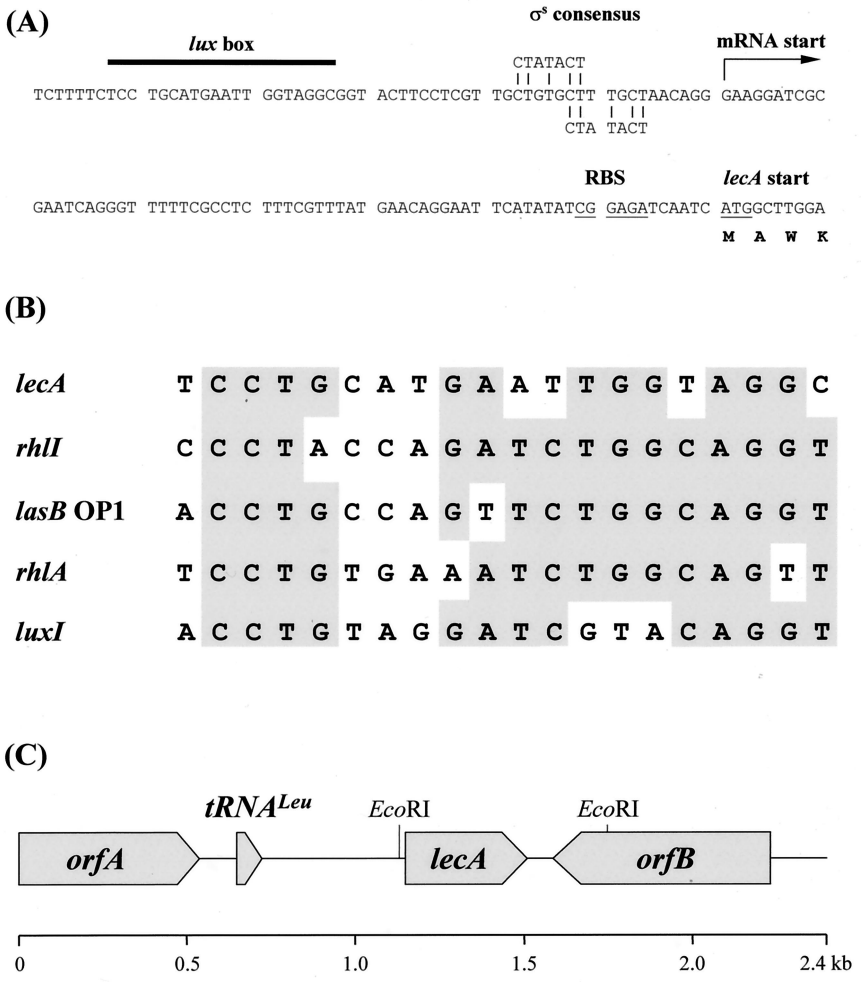

FIG. 2. The *lecA* upstream DNA sequence (A); a comparison of the *lux* box-like elements located upstream of the genes *rhlI*, *luxI*, *lecA*, *lasB*, and *rhlA* (B); and a schematic representation of the *lecA* locus (C). (A) The putative *lecA lux* box is indicated by a black bar, and the putative mRNA start point is indicated by an arrow. The proposed ribosome binding site (RBS) and the ATG start codon are underlined. Potential σ^S consensus sequences are aligned with the upstream region (short vertical lines). (B) Grey boxes indicate that at least four of five nucleotides are identical. *lux* box sequences were obtained from reference 9 (*luxI* and *rhlA*), reference 37 (*rhlI*), reference 55 (*lasB*-OP1), and this work (*lecA*). (C) The organization of *orfA*, the putative $tRNA^{Leu}$, *lecA*, and *orfB* is shown.

luxI of *Vibrio fischeri*, was identified centered 112 nucleotides upstream of the ATG start site. This 20-bp sequence, although not an inverted repeat, does show significant sequence similarity to the putative *luxI*, *rhlA*, and *rhlI* and the *lasB*-OP1 operators (Fig. 2A and B). A potential Rho-independent RNA polymerase terminator was present immediately downstream of *lecA*. Further analysis identified the presence of a putative $tRNA^{Leu}$ gene and a truncated ORF upstream of *lecA* (Fig. 2C). The deduced amino acid sequence of the truncated ORF (*orfA*) showed significant similarity to the AtoS sensor protein of *E. coli* and to other histidine protein kinases (data not shown). Downstream of *lecA*, a second ORF (*orfB*) was identified. Database searches using the deduced amino acid sequence of *orfB* did not reveal any significant similarity to any other proteins.

***lecA* transcript analysis.** For Northern blot analyses, RNA was isolated from *P. aeruginosa* cells harvested during mid-exponential, early stationary, and late stationary phase. A *lecA*-specific signal was obtained only with RNA isolated from stationary-phase cells with a transcript length of approximately 500 bp indicating a monocistronic organization (Fig. 3A). The transcriptional start site of *lecA* was determined by primer extension and S1 nuclease analysis, using oligonucleotide RNA2 (see Materials and Methods) and RNA isolated from stationary-phase cells. Identical signals, at position −70 relative to the ATG start codon, were obtained by both methods (Fig. 3B). Therefore, the putative *lecA lux* box is centered 42 nucleotides upstream of the transcriptional start site (Fig. 2A). Two potential σ^S −10 regions (CTGTGCT and CTTTGCT) were located in positions −18 to −12 and −13 to −7 upstream of the transcription start point (Fig. 2A), with five of the seven bases being identical to the consensus sequence (CTATACT) (12).

RhlR and its cognate autoinducer C4-HSL are the transcriptional activators of *lecA* expression. To characterize the transcriptional regulation of *lecA*, *lecA*::*luxCDABE* gene fusions were constructed in both *P. aeruginosa* and *E. coli* (for details, see Materials and Methods). The *P. aeruginosa*-based reporter contains a single, chromosomally located *lecA*::*luxCDABE* Km^r fusion (*lecA*::*lux*). The *E. coli* reporter contains the plasmid pCol9, which is based on the *lecA*::*luxCDABE* gene fusion cloned into the medium-copy-number vector pNQ705.

In *P. aeruginosa*, the PA-IL lectin reporter fusion was induced at the beginning of stationary phase (Fig. 4A), confirming the data obtained by immunoblot analysis. Addition of exogenous C4-HSL or C6-HSL (from 0 to 100 μM) at the time

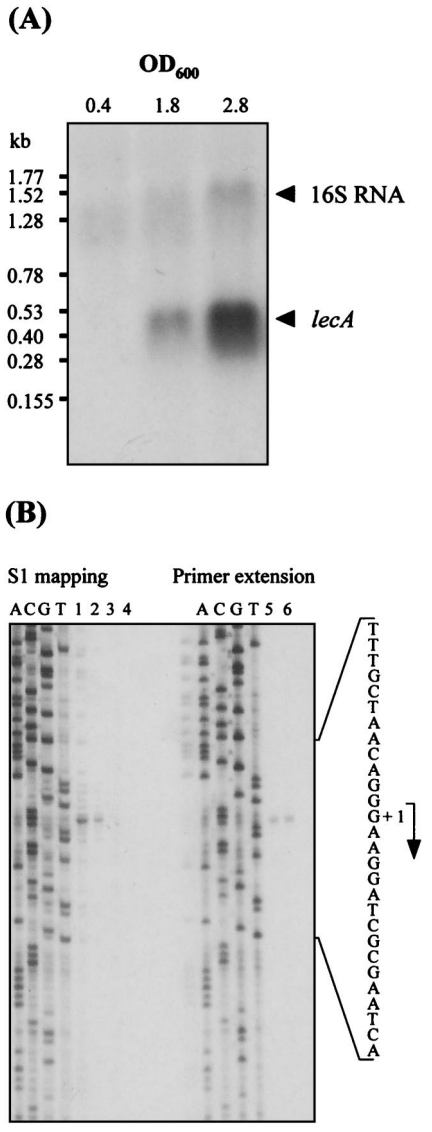

FIG. 3. Northern blot analysis of *lecA* transcripts (A) and mapping of the 5′ end of *lecA* transcripts (B). (A) RNA was isolated from cells harvested during exponential phase (OD_{600} = 0.4), early stationary phase (OD_{600} = 1.8), and late stationary phase (OD_{600} = 2.8). The *lecA* structural gene was amplified using the primers lecA.1F and lecA.1R and the template pCF1. The resulting PCR product was labeled with [α-^{33}P]ATP and used as a probe. (B) For S1 nuclease analysis, ^{35}S-radiolabeled DNA was synthesized using oligonucleotide RNA2 and hybridized to 10 μg of total RNA isolated from cells harvested in early stationary phase (OD_{600} = 1.8). After S1 nuclease digestion, 50% (lane 1), 20% (lane 2), and 5% (lane 3) of the radiolabeled DNA were subjected to electrophoresis. A control reaction mixture containing radiolabeled DNA but no RNA is shown in lane 4. The sequencing ladders were obtained by using the same oligonucleotide in DNA sequencing reactions. For primer extension analysis, the ^{33}P-radiolabeled oligonucleotide RNA2 complementary to the mRNA of the *lecA* gene was hybridized to 10 μg of total RNA isolated from early-stationary-phase cells (OD_{600} = 1.8) of *P. aeruginosa*. Of the resulting cDNA, 10 and 20% were subjected to electrophoresis on the gel presented (lanes 5 and 6, respectively).

of inoculation increased reporter expression in a dose-dependent manner (Fig. 4B) but did not advance it (Fig. 4A). C4-HSL was, however, more active than C6-HSL, increasing the expression more than twofold. In contrast, the addition of exogenous 3O-C12-HSL up to 100 μM had no effect on *lecA* expression (Fig. 4). Cultures grown in the presence or absence of AHLs showed very similar growth curves (data not shown). The rapid decay of light output (Fig. 4A) in stationary phase probably reflects the exhaustion of the substrates (reduced flavin mononucleotide and long-chain fatty aldehyde) required for the bioluminescence reaction (43, 70) since both Western blot (Fig. 1) and Northern blot (Fig. 3) data indicate that *lecA* is expressed in stationary phase and a comparable decay of light output was also observed with a *P. aeruginosa lecA*::*lux* mutant strain constitutively expressing *lecA*::*lux* due to a Tn*5* insertion upstream of the reporter (K. Winzer and P. Williams, unpublished data).

For *E. coli* S17-1(pCol9), only background levels of *lecA*

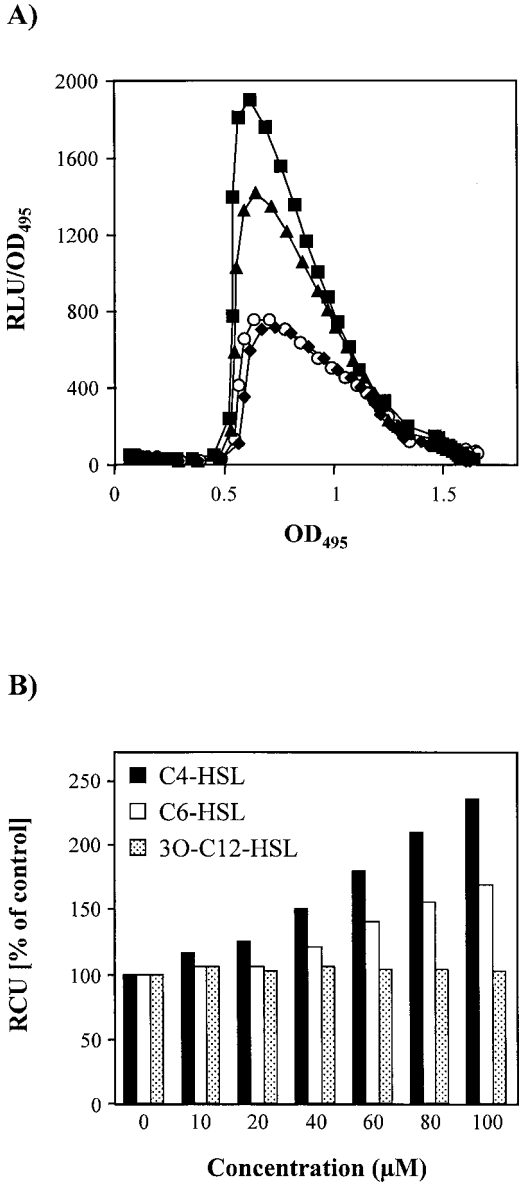

FIG. 4. Expression of *lecA*::*lux* in *P. aeruginosa*. (A) *P. aeruginosa lecA*::*lux* was grown in LB medium in the absence of exogenously added AHL (○) or in the presence of 100 μM C4-HSL (■), C6-HSL (▲), or 3O-C12-HSL (◆). RLU and OD_{495} were determined as described in Materials and Methods. The results represent a single experiment, although the experiment was repeated three times with similar results. (B) Response of the *P. aeruginosa lecA*::*lux* fusion to C4-HSL, C6-HSL, and 3O-C12-HSL. Relative cumulative light units (RCU) were calculated as a percentage of the control (no exogenous AHL added) as described in Materials and Methods. For each AHL concentration, the experiment was repeated three times.

expression were detected, consistent with the immunoblot data described earlier. This indicates that the *lecA* gene, when under the control of its native promoter, is not expressed in the heterologous genetic background of *E. coli*. When *E. coli* S17-1 was transformed with p*lasR*, pMW471.2 (*rhlR*), and pMW47.1 (*rhlRI*), only pMW47.1 induced an approximately threefold increase in the level of *lecA* transcription (Fig. 5A). In further experiments, C4-HSL or 3O-C12-HSL was added to either *E. coli* S17-1(pCol9)(pMW471.2) or *E. coli* S17-1(pCol9)(p*lasR*), at concentrations of 5, 10, and 15 μM. While the addition of exogenous C4-HSL to S17-1(pCol9)(pMW471.2) induced a concentration-dependent increase in the level of *lecA* expression (Fig. 5A), the addition of 3O-C12-HSL to S17-1(pCol9)(p*lasR*) failed to increase expression (data not shown). Addition of C4-HSL to S17-1(pCol9) and S17-1(pCol9)(pUCP18) also failed to induce *lecA* transcription (data not shown). These results suggest that *lecA* expression is directly regulated by RhlR/C4-HSL but not by LasR/3O-C12-HSL.

When *E. coli* S17-1(pCol9)(pMW471.2) was grown in the presence of C4-HSL (20 μM) together with 3O-C12-HSL (10 and 20 μM, respectively), a concentration-dependent decrease in expression was observed, relative to the levels detected following the addition of exogenous C4-HSL alone (Fig. 5B). 3O-C12-HSL, therefore, interferes with RhlR/C4-HSL-dependent activation of *lecA*::*lux* in an *E. coli* background. In contrast, addition of 10 or 20 μM C6-HSL to *E. coli* S17-1(pCol9)(pMW471.2) in the presence of 20 μM C4-HSL enhanced RhlR-dependent *lecA* expression (Fig. 5B).

Production of PA-IL and PA-IIL is RpoS dependent. Given the presence of an RpoS (σ^S) consensus sequence upstream of the *lecA* transcription start site, we used immunoblot analysis to monitor the production of both PA-IL and PA-IIL throughout growth. Figure 6 shows that synthesis of both lectins is abolished in a *P. aeruginosa rpoS* mutant.

DISCUSSION

The *lasRI* and *rhlRI* quorum-sensing circuits of *P. aeruginosa* have previously been shown to regulate numerous virulence factors (including elastase, alkaline protease, LasA protease, exotoxin A, pyocyanin, pyoverdine, and hemolysin), components of the Xcp secretion apparatus, the stationary-phase sigma factor RpoS, cyanide, rhamnolipids, twitching motility, superoxide dismutases, catalase, and biofilm formation in a cell-density-dependent manner (18, 31, 37, 38, 47, 50, 65, 69). Quorum sensing thus appears to constitute a global regulatory system in *P. aeruginosa*. Indeed, Whiteley et al. (67) have estimated that up to 4% of *P. aeruginosa* genes are regulated by quorum sensing. By screening a library of random *lacZ* transcriptional fusions in a *lasI-rhlI* double mutant on media supplemented or not with 3O-C12-HSL and C4-HSL, they identified mutations in some 39 *P. aeruginosa* genes, many of which have no homologues in the databases (67). Their approach did not, however, identify the genes coding for either PA-IL or PA-IIL, which, from the present work, we can now add to the repertoire of gene products now known to be controlled via quorum sensing in *P. aeruginosa*. The genes examined by Whiteley et al. did not represent the entire set of quorum-sensing-dependent genes, as no saturation mutagenesis was performed, and consequently, some genes that have been described as quorum sensing regulated have not been identified in their experiments (67).

For both PA-IL and PA-IIL, mutation of *lasR* delayed but did not abolish lectin production. The loss of both lectins in PANO67 and their restoration following the introduction of

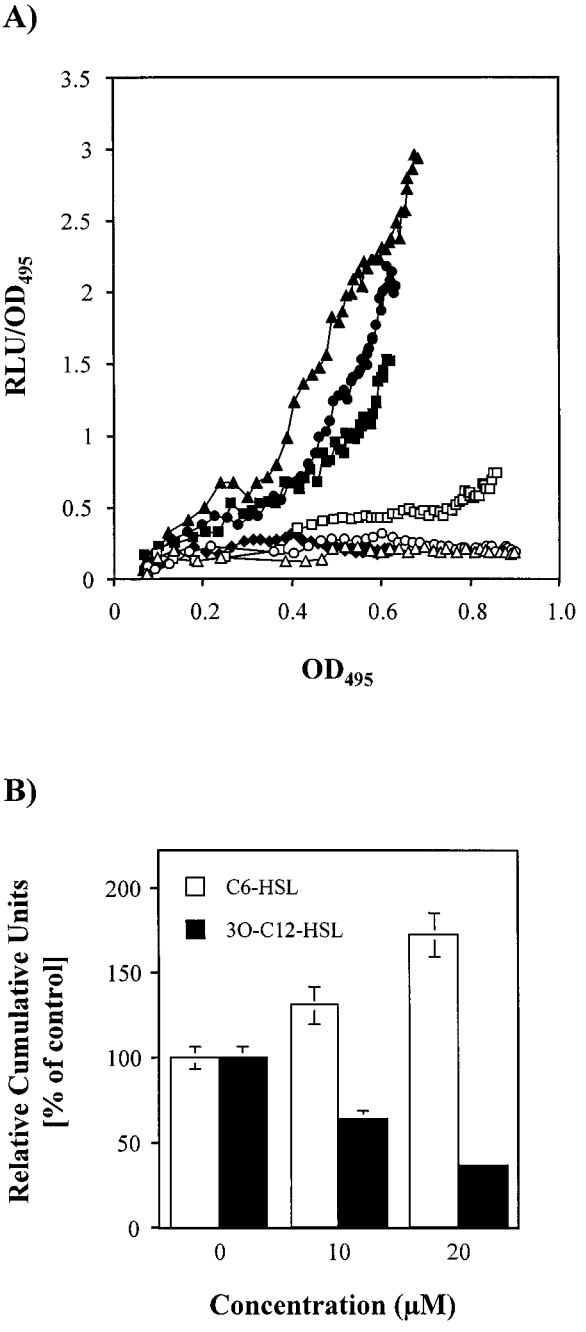

FIG. 5. Effect of *rhlR*, *rhlRI*, and *lasR* on the expression of *lecA*::*lux* in *E. coli* S17-1(pCol9) (A) and influence of C6-HSL and 3O-C12-HSL on RhlR/C4-HSL-driven expression of *lecA*::*lux* in *E. coli* S17-1 [pCol9] pMW47.1 (B). (A) Expression of *lecA*::*lux* in *E. coli* S17-1(pCol9) in the presence of pMW47.1 (*rhlRI*) (□), p*lasR* (△), pUCP18 (○), and pMW471.2 (*rhlR*) with no C4-HSL (◆), 5 μM C4-HSL (■), 10 μM C4-HSL (●), or 15 μM C4-HSL (▲) added. Strains were grown in LB medium in the presence of the indicated additives. RLU and OD_{495} were measured every 30 min as described in Materials and Methods. The results represent a single experiment, although the experiment was repeated three times with similar results. (B) Response of *E. coli* S17-1(pCol9)(pMW471.2) grown in the presence of 20 μM C4-HSL to C6-HSL and 3O-C12-HSL. RLU and OD_{495} were determined every 30 min. Relative cumulative light units were calculated as a percentage of the control (no exogenous AHL added) as described in Materials and Methods. For each AHL concentration, the experiment was repeated three times.

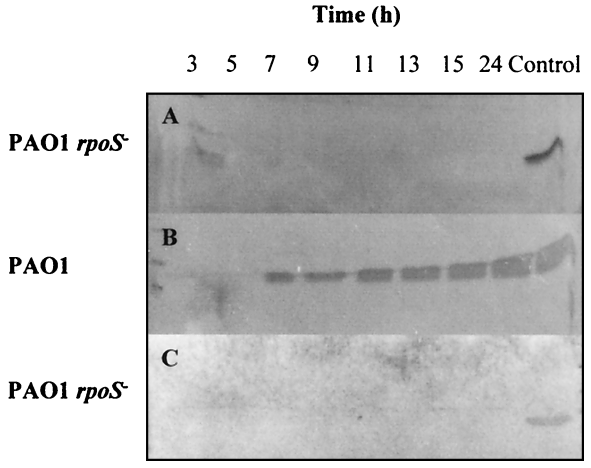

FIG. 6. Production of PA-IL (A and B) and PA-IIL (C) by *P. aeruginosa* strains PAO1 (B) and PAO1 *rpoS* negative (A and C). For each strain, samples for immunoblot analysis were taken every 2 h and processed as described in Materials and Methods over the first 15 h and finally at 24 h. All strains were grown in LB medium at 37°C and showed similar growth curves, each entering stationary phase after approximately 6 h (data not shown). For panels A and B, immunoblots were probed with a monospecific polyclonal antibody to PA-IL, and for panel C, immunoblots were probed with an antibody to PA-IIL. As a control, the rightmost lane in each panel contained the purified PA-IL (A and B) or PA-IIL (C) lectin. PAO1 wild-type samples probed with antibody to PA-IIL are shown in Fig. 1B.

the plasmid-borne *rhl* locus suggested that RhlR/C4-HSL rather than LasR/3O-C12-HSL was likely to be directly responsible for controlling their expression. In addition, these data suggest that, although the *las* system regulates the *rhl* system, in late stationary phase the *rhlRI* locus can be activated in a LasR/3O-C12-HSL-independent manner such that the loss, by mutation, of *lasR* delays, but does not abolish, lectin synthesis.

Immunoblot analysis of lectin production did not, however, reveal whether RhlR/C4-HSL or LasR/3O-C12-HSL could activate lectin production independently, as has been observed for the *lasB*, *rhlAB*, and *xcp* operons (6, 7, 47, 51, 52). Furthermore, the immunoblot data could not exclude the possibility that lectin synthesis was not directly activated by the quorum-sensing circuitry but by other, as yet unidentified regulators, which were themselves controlled via quorum sensing.

To obtain more detailed insights into the quorum-sensing-dependent control of PA-IL regulation required that we first clone the *P. aeruginosa* structural gene encoding PA-IL, *lecA*, and flanking DNA for sequence analysis and reporter gene construction. Since the immunoblot data indicated that regulation of the genes for PA-IL and PA-IIL was tightly coupled, it was possible that the two genes were contained within an operon. However, analysis of the *lecA* gene region of PAO1 failed to identify a gene which when translated matched the deduced N-terminal amino acid sequence of PA-IIL (data not shown). Moreover, the *lecA* transcript size of approximately 500 bp was in good agreement with the predicted size of 480 bp deduced from the proposed transcription start point and the Rho factor-independent terminator and clearly indicates that *lecA* is monocistronic. The genes coding for the two *P. aeruginosa* lectins are therefore unlinked despite the tight coupling of lectin production.

The failure of *E. coli* to express recombinant PA-IL from the original cosmid clones and plasmids pCF1 and pCF2 suggested that additional *P. aeruginosa* regulators were required. Expression from plasmid pCF3 was most probably driven by the pUCP18 *lac* promoter, as the ATG start codon of the *lecA* gene was only 19 bp from the 5′ end of the encoding DNA fragment, indicating that the *lecA* promoter sequence was lost during subcloning. To gain further insights into the regulation of *lecA*, the 5′ end of the *lecA* gene was mapped using both S1 mapping and primer extension. Both techniques indicated that the transcription start site was 70 bp upstream of the ATG codon. Analysis of the region upstream of the mRNA start point revealed two potential RpoS (σ^S) −10 elements in positions −18 to −12 and −13 to −7 relative to the transcription start point. This suggested a possible role for RpoS in lectin gene expression. RpoS was originally identified in gram-negative bacteria as an alternative sigma factor responsible for the activation of genes required for survival in the stationary phase, but it is now clear that RpoS is an important regulator of the general stress response (42). In *P. aeruginosa*, mutation of *rpoS* leads to enhanced susceptibility of stationary-phase cells to heat, low pH, high osmolarity, hydrogen peroxide, and ethanol, although the increased sensitivity was not as pronounced as that reported previously for *E. coli* (35, 63). Intriguingly, in a rat chronic lung infection model, the *rpoS* mutant was more virulent than the parent strain, suggesting that, in *P. aeruginosa*, RpoS influences virulence gene expression (63). This was suggested to be due to enhanced pyocyanin synthesis, since exotoxin A production was reduced by 50% and exoprotease production was relatively unaffected in the *rpoS* mutant (63). For *P. aeruginosa*, Latifi et al. (38) have demonstrated that *rpoS* expression is itself directly regulated by RhlR/C4-HSL. Thus, the data presented here indicate that, since *lecA* expression is RpoS dependent, RhlR/C4-HSL controls expression of the PA-IL lectin both directly (as demonstrated by the RhlR/C4-HSL-dependent regulation of the *lecA*::*lux* fusion in *E. coli*) and indirectly (via the RhlR/C4-HSL-dependent expression of *rpoS*).

For LuxR-type regulators such as LasR and RhlR, *lux* box-like elements constitute the DNA-binding site. Recently, it has been shown that the *lux* box-like operator sequences OP1 and OP2, upstream of the *lasB* structural gene in *P. aeruginosa*, are important for the initiation of transcription (14, 55, 73). Furthermore, Zhu and Winans (74) established that TraR, a LuxR-type regulator in *Agrobacterium tumefaciens*, in vitro binds precisely to a *lux* box-like element (the *tra* box). The presence of a putative *lux* box upstream of *lecA* therefore suggests that it is directly regulated by LasR and/or RhlR. This *lux* box-like element is centered 42 bp upstream of the transcriptional start site. Very similar distances have been reported for the *lux* box elements upstream of *rhlAB* (51), *lasB* (OP1) (55), and *rhlI* (K. Winzer and P. Williams, unpublished data), as well as the elements upstream of *luxI* in *V. fischeri* (11) and TraR-dependent promoters (16). As a consequence, these operators are extremely close to, or even overlap with, the respective promoter regions. However, although the putative *lecA* box is similar to the *lux* box described for *V. fischeri*, it is not an inverted repeat, which is also true for other *lux* box-like elements. While OP1 upstream of *lasB* is an inverted repeat and matches 13 of the 20 bp of the *V. fischeri lux* box sequence, OP2 is not an inverted repeat but does match 10 of the 20 *lux* box nucleotides (55). However, deletion of the OP2 sequence negatively affects LasR-mediated expression of *lasB*, indicating that it is still a target for LasR in the activation of *lasB* (55).

Heterologous expression of the *lecA*::*lux* plasmid reporter in *E. coli* together with either *lasR* or *rhlR* revealed that RhlR, together with its cognate autoinducer C4-HSL, is sufficient for the activation of the *lecA* gene in an *E. coli* background. In the absence of C4-HSL, or in the presence of LasR/3O-C12-HSL, only background levels of expression were observed. These data demonstrate that RhlR/C4-HSL, but not LasR/3O-C12-

HSL, directly activates the *lecA* gene, while the data obtained with the *P. aeruginosa* mutants support the existence of a regulatory hierarchy in which LasR/3O-C12-HSL serves as the master regulator. Furthermore, 3O-C12-HSL has been suggested to be involved in the posttranslational control of RhlR/C4-HSL-dependent genes (52). This is because, in *E. coli*, an excess of 3O-C12-HSL drastically reduced the binding of radiolabeled C4-HSL to cells expressing RhlR. Pesci et al. (52) also showed that the ability of RhlR/C4-HSL to activate an *rhlA′-lacZ* fusion in *E. coli* decreased in a dose-dependent manner with increasing concentrations of 3O-C12-HSL. These data suggest that 3O-C12-HSL is able to block the binding of C4-HSL to RhlR, thereby controlling RhlR activity at a posttranslational level. In the present study, we show that the RhlR/C4-HSL-dependent expression of a *lecA*::*lux* fusion in *E. coli* is inhibited by 3O-C12-HSL. This finding is consistent with the antagonism exerted by long-chain AHLs on quorum-sensing-dependent genes activated by short-chain AHLs. For example, the C6-HSL-mediated activation of the purple pigment violacein in *Chromobacterium violaceum* is inhibited by AHLs with *N*-acyl chains of eight carbons or more (40). Similarly, the C4-HSL-dependent production of exoproteases in *Aeromonas hydrophila* is inhibited by AHL analogues with acyl side chains of 10, 12, or 14 carbons (64). *P. aeruginosa* is, however, unusual in producing both short (C4-HSL) and long (3O-C12-HSL) AHLs. When exogenous AHLs (either C4-HSL, C6-HSL, or 3O-C12-HSL) were added to the *P. aeruginosa lecA*::*lux* reporter, only C4-HSL and C6-HSL enhanced *lecA*::*lux* expression. C6-HSL is generated via RhlI as a minor *P. aeruginosa* AHL together with C4-HSL (69). The results obtained with the *P. aeruginosa lecA*::*lux* reporter are consistent with the ability of C6-HSL to activate the *rhlI* promoter via RhlR in an *E. coli* genetic background (69). However, when exogenous 3O-C12-HSL was added to the *P. aeruginosa lecA*::*lux* reporter, in contrast to the situation with *E. coli*, no inhibition of *lecA* expression was observed. This finding suggests that, at least for *lecA*, 3O-C12-HSL does not act as a posttranslational regulator of PA-IL production. Whether this will also prove to be the case for other RhlR/C4-HSL-dependent genes remains to be established.

The addition of exogenous 3O-C6-HSL at the time of inoculation overcomes the cell-density-dependent induction of bioluminescence in *V. fischeri* and carbapenem antibiotic production in *Erwinia carotovora* (43, 68). However, expression of the *P. aeruginosa lecA*::*lux* fusion could not be induced immediately or advanced by the addition of either C4-HSL or 3O-C12-HSL or both even at high, nonphysiological concentrations under the growth conditions employed in this study. This finding suggests that, in the homologous genetic background, other regulatory factors in addition to RhlR/C4-HSL are required but are presumably unavailable early in the growth cycle. Such factors presumably include RpoS, which is not expressed until the onset of stationary phase (38). Interestingly, Whiteley et al. (67) have divided a number of quorum-sensing-dependent genes in *P. aeruginosa* into four classes depending on whether, in a *lasI rhlI* double mutant, they responded immediately (class I) or after a delay (class II) to 3O-C12-HSL alone or immediately (class III) or after a delay (class IV) to both 3O-C12-HSL and C4-HSL. The elastase gene *lasB*, for example, was considered to exhibit the characteristics of a class IV quorum-sensing gene (67). On this basis, either *lecA* may be considered as a class IV gene, or it may belong to a new class since it can still be well expressed in the absence of LasR and 3O-C12-HSL.

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