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Advancing the Quorum in *Pseudomonas aeruginosa*: MvaT and the Regulation of *N*-Acylhomoserine Lactone Production and Virulence Gene Expression†

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Pseudomonas aeruginosa **regulates the production of many exoproteins and secondary metabolites via a hierarchical quorum-sensing cascade through LasR and RhlR and their cognate signal molecules *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and *N*-(butanoyl)-L-homoserine lactone (C4-HSL). In this study, we found that transcription of the quorum sensing-regulated genes *lecA* (coding for PA-IL lectin), *lasB* (coding for elastase), and *rpoS* appeared to be growth phase dependent and their expression could not be advanced to the logarithmic phase in cells growing in batch culture by the addition of exogenous C4-HSL and 3O-C12-HSL. To identify novel regulators responsible for this growth phase dependency, a *P. aeruginosa lecA*::*lux* reporter strain was subjected to random transposon mutagenesis. A number of mutants affected in *lecA* expression were found that exhibited altered production of multiple quorum sensing-dependent phenotypes. While some mutations were mapped to new loci such as *clpA* and *mvaT* and a putative efflux system, a number of mutations were also mapped to known regulators such as *lasR*, *rhlR*, and *rpoS*. MvaT was identified as a novel global regulator of virulence gene expression, as a mutation in *mvaT* resulted in enhanced *lecA* expression and pyocyanin production. This mutant also showed altered swarming ability and production of the LasB and LasA proteases, 3O-C12-HSL, and C4-HSL. Furthermore, addition of exogenous 3O-C12-HSL and C4-HSL to the *mvaT* mutant significantly advanced *lecA* expression, suggesting that MvaT is involved in the growth phase-dependent regulation of the *lecA* gene.**

Many bacterial species employ complex communication mechanisms linking cell density with gene expression. Diffusible signal molecules termed autoinducers accumulate in the extracellular environment during the growth of a bacterial population, thus reflecting its cell density. Once a critical threshold concentration has been reached, a response is triggered that leads to changes in gene expression and consequently the phenotype of the cells. This type of communication, which regulates many diverse physiological processes, has been termed quorum sensing (13). In gram-negative bacteria, the most intensely studied quorum-sensing systems rely upon the interaction of *N*-acylhomoserine lactone (AHL) signal molecules, synthesized by LuxI-type AHL synthases, with LuxR-type transcriptional regulator proteins. Together, the LuxR-type protein and its cognate AHL then activate the expression of specific target genes (for reviews, see references 52 and 59).

Pseudomonas aeruginosa, an opportunistic human pathogen, is known to possess at least two AHL-dependent quorum-sensing systems, the *las* and *rhl* systems, which are composed of the LuxRI homologues LasRI (14, 32) and RhlRI (23, 30), respectively. LasI directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) (33), whereas RhlI directs the synthesis of *N*-(butanoyl)-L-homoserine lactone (C4-HSL) (34, 60). Each system modulates a regulon comprising an overlapping set of genes. However, the *las* and *rhl* systems are not independent of each other but form a regulatory hierarchy in which LasR/3O-C12-HSL activates the transcription of *rhlR* (24, 36). Recently, a third LuxR homologue, termed QscR, has been identified that has been shown to regulate the transcription of *lasI* (6). Furthermore, an additional signaling molecule has been described that was shown to control the expression of *lasB*. This molecule was chemically characterized as 2-heptyl-3-hydroxy-4-quinolone, a compound related to the 4-quinolone antibiotics. This was termed the *Pseudomonas* quinolone signal (PQS), the production of which was shown to be dependent on LasR/3O-C12-HSL (37). In addition to *lasB*, the PQS was also shown to regulate *rhlI* expression (25). PQS concentrations were found to be highest in late stationary phase, suggesting that this molecule is not involved in cell density sensing (25). Genes regulated by either LasR/3O-C12-HSL or RhlR/C4-HSL include those coding for elastase (LasB), LasA protease, alkaline protease, exotoxin A, cytotoxic lectins, hydrogen cyanide, and pyocyanin. As well as controlling the expression of many genes for exoproteins and secondary metabolites, the quorum-sensing machinery in *P. aeruginosa* is known to influence the *xcp* secretion pathway (4), biofilm maturation (7, 9), catalase gene expression, twitching motility (16), and expression of the stationary-phase sigma factor *rpoS* (24). Indeed, Whiteley et al. (55) have estimated that 1 to 4% of all *P. aeruginosa* genes may be controlled, to some extent, by quorum sensing.

Although a few regulatory systems have been described that

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† For a commentary on this article, see page 2569 in this issue.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Reference or source
Strains		
P. aeruginosa		
PAO1	Wild type	Holloway collection
PAO1 *lecA*::*lux*	*lecA*::*luxCDABE* genomic reporter fusion in PAO1	62
PAO-P4	Tn*5*-B21 *lasR* mutant derived from PAO1 *lecA*::*lux*	This study
PAO-P8	Tn*5*-B21 mutant derived from PAO1 *lecA*::*lux*	This study
PAO-P9	Tn*5*-B21 *rpoS* mutant derived from PAO1 *lecA*::*lux*	This study
PAO-P10	Tn*5*-B21 *mvaT* mutant derived from PAO1 *lecA*::*lux*	This study
PAO-P19	Tn*5*-B21 mutant derived from PAO1 *lecA*::*lux*	This study
PAO-P34	Tn*5*-B21 *rhlR* mutant derived from PAO1 *lecA*::*lux*	This study
PAO-P47	*mvaT* chromosomal deletion mutant derived from PAO1	This study
PAO-P52	Tn*5*-B21 *clpA* mutant derived from PAO1 *lecA*::*lux*	This study
PAO-P69	Tn*5*-B21 mutant derived from PAO1 *lecA*::*lux*	This study
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	63
S17-1 λ*pir*	*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn7 λ*pir*	46
Plasmids		
pUC18	Multicopy cloning vector derived from pBR322 carrying polylinker inserted into *lacZ*α (Amp^r)	54
pUCP18	Same as pUC18 but containing an additional 1.8-kb stabilizing fragment for maintenance in *Pseudomonas* spp. (Amp^r)	44
pDM4	Suicide vector carrying the *sacBR* genes for sucrose sensitivity (Cm^r)	26
pSP73	Cloning vector	Promega Corp.: Madison, Wis.
pSUP102 Tn*5*-B21	Transposable promoter probe; B21 in pSUP102 (Cm^r Tet^r)	47
pSB1075	AHL reporter plasmid; *P. aeruginosa lasRI* and *luxCDABE* from *Photorhabdus luminescens* (Amp^r)	61
pSB536	AHL biosensor; *ahyR*″::*luxCDABE* in pAHP13 (Amp^r)	51
pUC*mvaT*	1.6-kb *Bam*HI-*Pst*I PAOI chromosomal DNA fragment containing *mvaT* in pUC18	This study
pUCΔ*mvaT*	Same as pUC::*mvaT* except contains a 327-bp *mvaT* deletion in pUC18	This study
pDM4Δ*mvaT*	pDM4 containing *mvaT* flanking regions and deletion-containing *mvaT* gene	This study
pUCP*mvaT*	1.6-kb PAO1 *Bam*HI-*Pst*I DNA insert containing *mvaT* gene in pUCP18	This study
pMW47.1	2-kb *Pst*I PAO1 DNA insert (*rhlRl*) in pUCP18	23

[a] Amp^r, Cm^r, and Tet^r stand for resistance to ampicillin, chloramphenicol, and tetracycline, respectively.

influence *las*- or *rhl*-mediated quorum sensing, no systematic approach has been undertaken to elucidate how the quorum-sensing systems of *P. aeruginosa* are integrated into the global regulatory network of the cell. A mutant defective in the response regulator *gacA* was shown to exhibit reduced and delayed formation of C4-HSL and also reduced expression of *lasR* (40). Furthermore, a CRP homologue termed Vfr was shown to be required for basal-level *lasR* expression (1). In addition, a regulator termed RsaL has been described that is thought to repress transcription of *lasI* (8). Whiteley et al. (56) have shown that the stationary-phase sigma factor RpoS negatively regulates C4-HSL production and that, in an *rpoS* mutant, expression of *rhlI*, *hcnA*, and *phzA* is advanced. Also, a deletion of *qscR* in *P. aeruginosa* resulted in premature expression of the quorum sensing-related genes *lasI*, *rhlI*, *hcnA*, and *phzA* (6). Furthermore, a deletion in the posttranscriptional regulator RsmA led to advanced expression of *lasI*, *rhlI*, and *hcnA* (37a).

We have recently demonstrated that expression of the RhlR/C4-HSL-dependent PA-IL lectin gene *lecA* cannot not be advanced to the logarithmic growth phase by the addition of either 3O-C12-HSL or C4-HSL and that *lecA* is expressed in a growth phase-dependent manner (62). This is intriguing because, for instance, in *Vibrio fischeri* and *Erwinia carotovora* subsp. *carotovora*, the quorum sensing-controlled phenotypes of bioluminescence (10, 28) and carbapenem production (58), respectively, could be induced prematurely by the addition of their cognate AHL signal molecules.

In order to define in more detail the observed superregulation of quorum sensing-dependent genes in *P. aeruginosa*, we isolated transposon mutants that exhibited either increased, decreased, or delayed expression of a quorum sensing-dependent *lecA*::*lux* fusion. Further analysis of these mutants revealed that multiple quorum sensing-dependent phenotypes were affected. We describe here the identification of a new regulator involved in the modulation of cell density-dependent gene expression and propose that quorum-sensing regulation of virulence gene expression is linked with the growth phase and metabolic state of the cell.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used for cloning experiments, and *E. coli* S17-1 λpir was used for conjugation experiments. Bacteria were grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates or Pseudomonas Isolation Agar (Difco). Where indicated, C4-HSL or 3O-C12-HSL (or a combination of the two) was added to the growth medium prior to inoculation at a concentration of either 10 or 100 μM. Standard methods were used for the preparation of competent cells and for plasmid electroporation into *E. coli* and *P. aeruginosa* (42, 48). Conjugal transfer was performed as described by Kaniga et al. (18). Where required, tetracycline, chloramphenicol, and ampicillin were added at 40, 34, and 50 μg/ml, respectively, for *E. coli*. For *P. aeruginosa*, kanamycin, tetracycline, carbenicillin, and chloramphenicol were added at 50, 40, 300, and 400 μg/ml, respectively.

DNA manipulation. DNA was manipulated by standard methods (42). Restriction enzymes (Promega United Kingdom Ltd.) were used in accordance with the manufacturer's instructions. Agarose gel electrophoresis and Southern blot transfer were done essentially as described by Sambrook et al. (42). DNA probes

were labeled with digoxigenin and detected with the Alkaline Phosphatase Direct kit from Amersham. The IsoQuick kit (ORCA Research Inc.) was employed for isolation of chromosomal DNA from *P. aeruginosa*. For isolation of plasmid DNA from *E. coli*, the Qiagen Mini and Midi kits (Qiagen Ltd.) were used.

RNA analysis. Total RNA of *P. aeruginosa* was isolated as described previously (62). For Northern blot assays, RNA was separated in denaturing formaldehyde gels and transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech Ltd.) as described by Sambrook et al. (42). RNA dot blots were prepared with Hybond N+ nylon membranes in accordance with the manufacturer's instructions by using a dot blot apparatus from Bio-Rad. Probes were generated by PCR with chromosomal DNA from *P. aeruginosa* PAO1 and primers lecA1F (5′-ATATATCGGAGATCAATCATGGCTTGG-3′) and lecA1R (5′-CGTTCAGACCGAAGCGTGTTGAAGC-3′) (62) for *lecA*, lasB1F (5′-CGATCATGGGTGTTTCGCCG-3′) and lasB1R (5′-GCTCTTGCCGGGACCCTTGA-3′) for *lasB*, and rposF (5′-CACATCGACTACACGCGCGC-3′) and rposR (5′-GCTCGATGGTCTGGCGGATC-3′) for *rpoS*. For *rhlR*, a 0.5-kb *rhlR*-specific fragment was isolated from pMW47.1 (23) after restriction digestion with *Bam*HI and *Bgl*II. Probes were labeled with [α-^{33}P]ATP by using the Random Primers Labeling kit (Life Technologies Inc.). Hybridizations and washings were performed as described by Gerischer and Dürre (15). Transcript sizes were estimated by comparison with the 0.16- to 1.77-kb RNA ladder from Life Technologies Inc.

Transposon mutagenesis and cloning of Tn*5*-B21 insertion sites. Equal volumes of overnight cultures of *P. aeruginosa* PAO1 *lecA*::*lux* (62) grown at 42°C and *E. coli* S17-1 λpir pSUP102 Tn*5*-B21 (47) grown at 37°C were mixed and incubated for 6 h at 30°C. Cells were then harvested by centrifugation, resuspended in 1 ml of LB broth, and pelleted at 19,000 × *g* for 5 min before being resuspended in 50 μl of LB broth. A 25-μl volume was then plated on LB agar containing 40 μg of tetracycline per ml and 50 μg of kanamycin per ml and incubated overnight at 37°C. Approximately 6,000 *P. aeruginosa* Tc^r and Km^r colonies containing Tn*5*-B21 were restreaked onto selective antibiotic LB agar. Colonies were then screened for differences in light production throughout growth with a Luminograph LB 980 photon video camera (EG & G Berthold). A total of 38 mutants demonstrating increased, decreased, or no expression of the reporter fusion were selected and analyzed in greater detail with LUCYI (Anthos), a combined photometer-luminometer. Growth conditions and analysis of bioluminescence with LUCYI were as previously described (62). Tn *5*-B21 insertion sites were cloned into pSP73 after digestion of genomic DNAs obtained from the mutants with *Xho*I. Recombinant clones were analyzed on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates (50 μg/ml) for a color change due to the presence of the transposon-encoded *lacZ* gene. Alternatively, *Eco*RI-digested DNA was cloned into the same vector and screened for Tc^r, indicating the presence of the Tn*5*-B21-encoded gene for Tc^r. DNA flanking the transposon was sequenced with the primer Tn*5*.seq (5′-ACGGGAAAGGTTCGTTCAGGAC-3′). The DNA sequences obtained were analyzed with the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/).

Construction of a PAO1 *mvaT* deletion mutant. A PAO1 *mvaT* chromosomal deletion mutant lacking 327 internal nucleotides was constructed as follows. With PAO1 DNA as the template, the intact *mvaT* gene (375 bp) plus 549 bp of upstream flanking DNA and 665 bp of downstream flanking DNA was amplified by using primers mvaTUF (containing a *Bam*HI restriction site [underlined]; 5′-AATGGGGATCCTTGCTGGCCATCAGC-3′) and mvaTDR (containing a *Pst*I restriction site [underlined]; 5′-TGCCGCTGCAGCTGAAGCAGATCCAGG-3′). The resulting PCR product was digested with *Bam*HI and *Pst*I and cloned into similarly digested pUC18, resulting in plasmid pUC*mvaT* (Table 1). To introduce a deletion of the recombinant *mvaT* gene, primers mvaTUR (5′-ATATTCGTTGATCAGGTGCACGTCAGGTACC-3′) and mvaTDF (5′-GAGAGCTGGGCCACCGTGCACGGCTAAACCA-3′) were used in conjunction with inverse PCR with pUC *mvaT* DNA as the template. The resulting blunt-ended PCR product, containing a 327-bp deletion in *mvaT*, was self-ligated, resulting in the plasmid pUCΔ*mvaT*. The PCR product was excised from the vector with *Bam*HI and *Sph*I and cloned into the vector pDM4 (26), digested with *Bgl*II and *Sph*I, resulting in the plasmid pDM4Δ*mvaT*. Allelic exchange using pDM4Δ*mvaT* contained in *E. coli* S17-1 λpir with PAO1 resulted in a *P. aeruginosa* strain (PAO-P47) containing an in-frame deletion of the *mvaT* gene. This deletion was confirmed by both PCR and Southern blot analysis (data not shown).

DNA sequencing and sequence analysis. Automated nonradioactive sequencing reactions were carried out with 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 package and the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/). For sequence comparisons, the programs Gap (complete protein sequences) and BestFit (for truncated protein sequences) were used.

Time- and cell density-dependent measurements of bioluminescence. Bioluminescence was determined as a function of cell density with a combined, automated luminometer-spectrometer (Anthos Labtech LUCYI). Overnight cultures of *P. aeruginosa* were diluted 1:1,000 in fresh LB medium, and 0.2 ml was inoculated into microtiter plates. The luminescence and turbidity (optical density at 495 nm [OD_{495}]) of the cultures were automatically determined every 30 min. Luminescence is given in relative light units (RLU) per unit of OD_{495}.

Assay for elastolytic activity. Elastolytic activity of bacterial supernatants was determined with the elastin Congo red (ECR; Sigma) assay (31). A 100-μl aliquot of bacterial supernatant was added to 900 μl of ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg of ECR and incubated with shaking at 37°C for 3 h. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control.

Assay for pyocyanin production. Pyocyanin was extracted from culture supernatants and measured by the method of Essar et al. (11). A 3-ml volume of chloroform was added to 5 ml of culture supernatant and mixed. The chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl. After centrifugation, the top layer (0.2 M HCl) was removed and its absorption at 520 nm was measured.

Staphylolytic activity assay. LasA protease activity was measured by determining the ability of *P. aeruginosa* culture supernatants to lyse boiled *Staphylococcus aureus* cells (20). A 30-ml volume of an overnight culture of *S. aureus* was boiled for 10 min and then centrifuged for 10 min at 10,000 × *g*. The resulting pellet was resuspended in 10 mM Na_2PO_4 (pH 7.5) to an OD_{600} of approximately 0.8. A 100-μl aliquot of bacterial supernatant was then added to 900 μl of *S. aureus* suspension, and the OD_{600} was determined after 2, 6, 10, 14, 18, 22, 26, 30, 45, and 60 min.

AHL detection and analysis. Aliquots of 900 μl of culture supernatant were taken at specific time intervals throughout growth. Bacterial cells were removed by centrifugation (19,000 × *g*, 5 min), and the resulting supernatant was filter sterilized through 0.2-μm-pore-size filters (Millipore). For accurate AHL quantification, culture supernatants were first acidified with 1 M HCl (100 μl) and then incubated with agitation at 20°C for 18 h. This was done to ensure that any AHLs hydrolyzed to the ring-open form during growth were recyclized. For detection of 3O-C12-HSL, 1 μl of acidified culture supernatant taken after 8 h of growth was spotted onto normal-phase thin-layer chromatography (TLC) plates (Silica gel 60F_{254}; Merck). For detection of C4-HSL, 5 μl of acidified culture supernatant taken after 8 h growth was spotted onto reverse-phase TLC plates (RP-18 F_{245}; Merck). TLC plates were overlaid with 50 ml of soft top agar containing either 1 ml of an *E. coli* strain harboring reporter plasmid pSB1075 (to detect 3O-C12-HSL) or pSB536 (to detect C4-HSL). Plates were incubated at 30°C for 18 h. Bioluminescence was detected and quantified with a Luminograph LB 980 photon video camera (EG & G Berthold). Each bacterial strain was tested in quadruplicate, and values were corrected for differences in cell density. Results are presented as percentages of those of the PAO1 *lecA*::*lux* parent strain.

SDS-PAGE and immunoblotting. *P. aeruginosa* cells were grown in LB broth, and 1-ml samples were taken throughout the growth. All strains demonstrated similar growth patterns. Cells were suspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and lysed by sonication. Samples were then boiled before being loaded onto SDS–15% polyacrylamide gels. Proteins were then electrophoretically transferred onto nitrocellulose membranes (Hybond C) and probed with an anti-PA-IL polyclonal antibody (1:200 dilution). Detection was achieved with a secondary anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (1:3,000 dilution) (Amersham Life Sciences) and developed with the ECL chemiluminescence system (Amersham Life Sciences).

Swarming motility assay. In order to analyze swarming motility, an assay based on that of Rashid and Kornberg (39) was used. Briefly, a 2-μl aliquot of an overnight culture of *P. aeruginosa* was inoculated onto the surface of a swarm plate and incubated overnight at 37°C. Swarm plates consisted of 2 g of Bacto Agar (Difco) and 3.2 g of Nutrient Broth No. 2 (Oxoid) in 400 ml of distilled water. After autoclaving, filter-sterilized 10% (wt/vol) D-glucose in distilled water was added to a final concentration of 0.5% (wt/vol). The ability to swarm was assessed by the distance of swarming from the central inoculation site.

Detection of siderophores. To detect siderophore production, 10 μl of an overnight culture was inoculated onto Chrome azurol S plates (45) and incubated

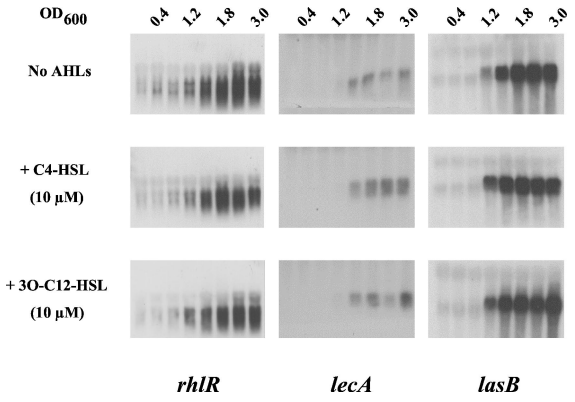

FIG. 1. Northern blot analysis of *rhlR*, *lecA*, and *lasB* in the presence and absence of exogenous AHLs. *P. aeruginosa* cells were grown in LB broth in the absence or presence of 10 μM C4-HSL or 3O-C12-HSL. RNA was isolated from cells harvested throughout growth and separated by horizontal electrophoresis as detailed in Materials and Methods. The *lecA* and *lasB* structural genes were amplified with primers lecA1F and lecA1R and primers lasB1F and lasB1R, respectively. The *rhlR* gene was derived from pMW47.1 (23). The resulting products were labeled with [α-^{33}P]ATP and used as probes.

for 18 h at 37°C. Siderophore production was detected by the appearance of orange halos around bacterial growth due to the release of free iron from an iron-dye complex within the plates.

Synthesis of *N*-acylhomoserine lactones. C4-HSL and 3O-C12-HSL were synthesized as previously described by Chhabra et al. (5). AHLs were dissolved in acetonitrile before addition to culture media at the indicated concentrations.

RESULTS

Transcription of *rhlR*, *rpoS*, *lecA*, and *lasB* cannot be advanced by exogenous AHLs. The RhlR/C4-HSL-dependent *lecA*::*lux* reporter fusion, which is expressed in a growth phase-dependent manner, could not be advanced by exogenous addition of either 3O-C12-HSL or C4-HSL (62). To test whether this is also the case for other quorum sensing-dependent genes or even components of the quorum-sensing cascade itself, the transcript levels of *rhlR*, *rpoS*, *lecA*, and *lasB* were analyzed in the presence of 10 μM C4-HSL or 3O-C12-HSL by Northern blotting (Fig. 1) and dot blot analysis (data not shown). Addition of these AHLs did not significantly advance the transcription of *rhlR*, *lecA*, *lasB* (Fig. 1), or *rpoS* (data not shown), indicating that a high autoinducer concentration, on its own, is insufficient to advance gene expression. The possibility remained that a high concentration of both the long- and short-chain AHLs is required for premature induction of these genes. This hypothesis was tested with the chromosomal *lecA*::*lux* fusion (62) and RNA dot blot analysis. Growth of *P. aeruginosa* in the presence of C4-HSL or a combination of C4-HSL and 3O-C12-HSL at 100 μM did not advance (or only very slightly advanced) expression of the *lecA*::*lux* fusion (Fig. 2) and *lasB* transcription (data not shown). Similar results were also obtained at lower concentrations (data not shown). However, in combination, both AHLs significantly elevated the expression of both genes. Thus, the quorum-sensing cascade and onset of quorum sensing-dependent gene expression appear to be tightly regulated.

Isolation of mutants displaying altered *lecA*::*lux* expression. To identify new regulators that modulate quorum sensing-dependent gene expression, we subjected PAO1 *lecA*::*lux* to transposon mutagenesis. A collection of ~6,000 mutants was generated by random insertion of the transposon Tn*5*-B21 (47). The resulting mutants were screened for altered bioluminescence, due to changes in *lecA*::*lux* expression, with a photon imaging camera. A total of 38 mutants were isolated that demonstrated increased, decreased, or delayed expression of the reporter fusion. To quantify the expression of the reporter fusion throughout growth, each of these mutants was grown in LB broth and analyzed in a combined photometer-

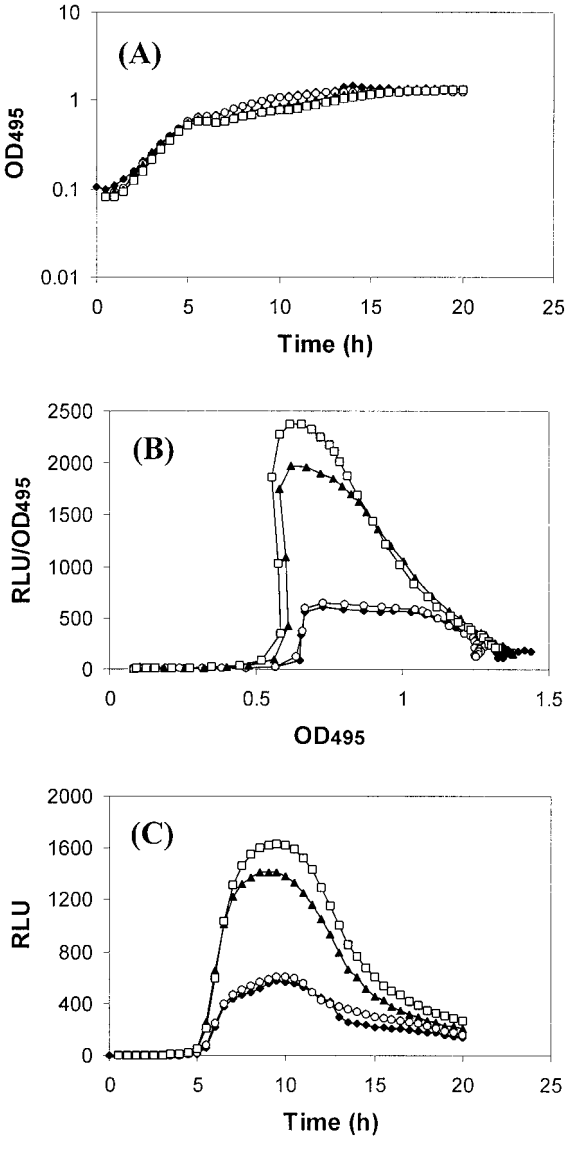

FIG. 2. Expression of *lecA*::*lux* in *P. aeruginosa.* PAO1 *lecA*::*lux* was grown in LB medium in the absence of exogenously added AHL (◆) or in the presence of C4-HSL (▲), 3O-C12-HSL (○), or a combination of the two (□) at 100 μM. RLU and OD_{495} were determined as described in Materials and Methods. The results represent a single experiment, although the experiment was repeated three times with similar results. Panels: A, growth of PAO1 *lecA*::*lux* in the presence or absence of AHLs; B, expression of *lecA* as a function of light output per cell; C, light output (*lecA* expression) of the culture with time.

TABLE 2. Identification of genes in mutants carrying a Tn*5*-B21 insertion[a]

Mutant	Cumulative index of *lecA*::*lux* expression (% of PAO1 *lecA*::*lux* expression)	Gene no. in *Pseudomonas* genome sequence	Tn*5*-B21 insertion site on PAO1 chromosome	Gene name or product	Reference
PAO-P4	4	PA1430	1558663	*lasR*	14
PAO-P8*	38	PA4341	4870418	Putative regulator	
PAO-P9	4	PA3622	4058513	*rpoS*	53
PAO-P10	223	PA4315	4844118	*mvaT*	41
PAO-P19	47	PA5265	5927833	Hypothetical protein	
PAO-P34	4	PA3477	3890556	*rhlR*	29
PAO-P52	191	PA2620	2963477	*clpA*	17
PAO-P69	10	PA4207	4707888	Probable RND-like efflux transporter	

[a] For each mutant, the assigned gene number in the *P. aeruginosa* complete genome sequence (49) is provided. Insertion sites were identified by comparing the DNA sequence flanking Tn*5*-B21 with the complete *P. aeruginosa* genome sequence. In PAO-P8*, Tn*5*-B21 was inserted just upstream of gene PA4341. The *lecA*::*lux* expression value for each mutant is shown as percentages of that of the parent PAOI *lecA*::*lux* strain. Each value was calculated by dividing each 30-min reading of RLU per unit of OD_{495} from the LUCYI experiment by that for the PAOI *lecA*::*lux* parent strain.

luminometer (Anthos Labtech LUCYI). A number of mutants showing significant alterations in the expression of the reporter fusion were selected for further analysis (Table 2). Southern blot analysis showed that all of the mutants contained only a single copy of the transposon and that it had not been inserted into the *lecA*::*lux* reporter fusion (data not shown). Of the mutants selected, four showed little (less than 15% compared to the parent strain) or no expression of the fusion. In contrast, two mutants demonstrated a significant up-regulation (greater than 190% compared to the parent strain) of bioluminescence and, hence, *lecA* expression (Table 2). The transposon-flanking regions were cloned and sequenced, and the genes identified were compared with the genome databases (Table 2). The genes found to be inactivated included the known quorum-sensing regulators *lasR* (mutant PAO-P4) and *rhlR* (PAO-P34), as well as the gene for the stationary-phase sigma factor RpoS (mutant PAO-P9), but also a set of new loci that had not been described in the context of cell-cell communication or virulence gene expression.

In mutant PAO-P10, Tn*5*-B21 had been inserted into a gene termed *mvaT* (49), as the putative gene product shows 82% similarity to the P16 subunit encoded by *mvaT* in the soil bacterium *Pseudomonas mevalonii* (41). In *P. mevalonii*, MvaT is a heteromeric transcriptional regulator of the *mvaAB* operon (41). Disruption of this gene in *P. aeruginosa* significantly increased (223%) expression of the *lecA*::*lux* reporter fusion (Table 2).

PAO-P8 showed a Tn*5*-B21 insertion site upstream of a gene (PA4341) with similarity to the IclR family of transcriptional regulators. Members of the IclR family have been shown to act as both repressors and activators of genes. In PAO-P8, expression of the *lecA*::*lux* reporter was greatly reduced (to 38% of that of PAO1 *lecA*::*lux*).

PAO-P52 showed an insertion within the putative *clpA* gene (PA2620). ClpA in *E. coli* can interact with ClpP to form a protease involved in the degradation of misfolded proteins (17), but it also acts as a chaperone.

In PAO-P69, the transposon had disrupted open reading frame (ORF) PA4207, coding for a putative efflux pump component. Expression of the *lecA*::*lux* fusion in this mutant was significantly down-regulated (10% of that in the parent strain).

The Tn*5*-B21 insertion site in the mutant PAO-P19 was shown to be in an ORF termed PA5265 (49). This ORF had no similarity to any known genes, but a mutation did affect *lecA*::*lux* expression, showing 47% of the expression in PAO1 *lecA*::*lux*.

Multiple quorum sensing-regulated phenotypes are altered in Tn*5*-B21 mutants. To determine whether any of the genes listed in Table 2 control lectin expression directly via quorum sensing rather than as a consequence of impaired growth, we first examined the growth of each Tn*5*-B21 mutant in LB. The mutants examined possessed similar rates of growth in LB (Fig. 3). This suggests that any phenotypic differences observed between each mutant and the parent strain could not be attributed to differences in growth. The production of pyocyanin, elastase, LasA protease, and siderophore, as well as swarming motility, was analyzed in a selection of the Tn*5*-B21 mutants. Production of the phenazine pigment pyocyanin 8 h postinoculation was shown to be altered in all of the mutants tested compared with that in PAO1 and PAO1 *lecA*::*lux* (Fig. 4A). As expected, very little pyocyanin was detected in PAO-P34 (*rhlR* mutant), a finding in keeping with that of previous workers (3). Both PAO-P9 (*rpoS* mutant) and PAO-P10 (*mvaT* mutant) showed very high levels of pyocyanin production (1,226 and 912% of the parent strain level, respectively). Interestingly, levels of pyocyanin in the *lasR* mutant (PAO-P4) increased to levels greater than those in PAO1 *lecA*::*lux*, but only after 14 h of growth. For all of the other mutants, the comparative pyocyanin levels, calculated as a percentage of the PAO1 *lecA*::*lux*

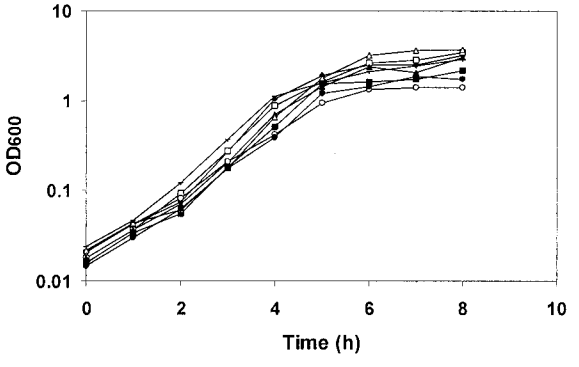

FIG. 3. Growth curves of *P. aeruginosa lecA*::*lux* Tn*5*-B21 mutants. Strains were grown at 37°C for 8 h in 100 ml of LB medium with shaking at 200 rpm, and the OD_{600} was determined. Growth of PAO1 *lecA*::*lux* (◆), PAO-P4 (□), PAO-P8 (▲), PAO-P9 (○), PAO-P10 (■), PAO-P34 (△), PAO-P52 (●), and PAO-P69 (+) is shown.

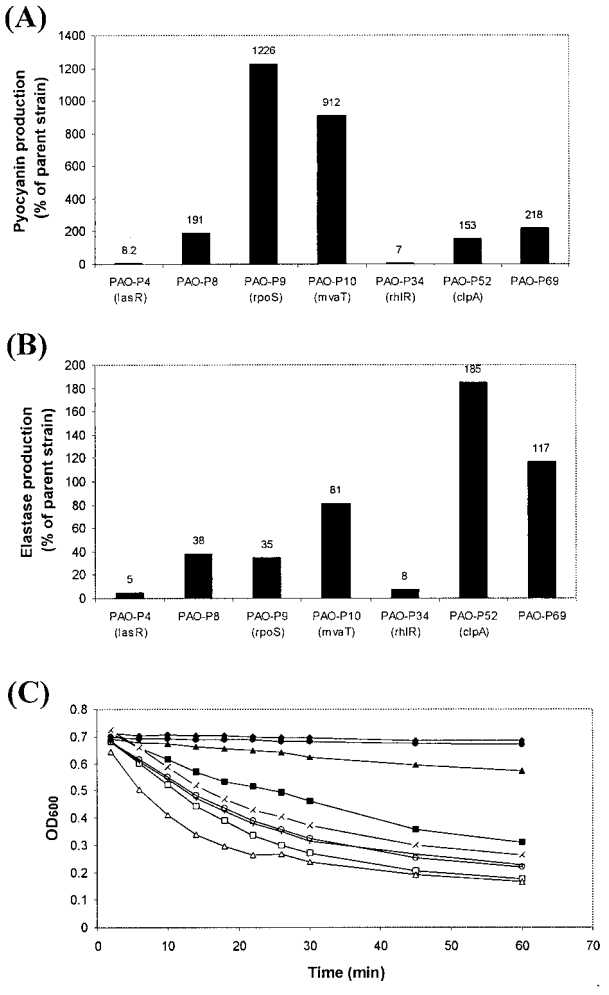

FIG. 4. Influence of Tn*5*-B21 mutations on pyocyanin production (A), elastolytic activity (B), and LasA protease activity (C). Samples for pyocyanin analysis were taken after 8 h of growth, after which all strains were in stationary phase. Pyocyanin levels are expressed as percentages of that of the parent strain, PAO1 *lecA*::*lux*. For elastolytic and LasA protease activities, culture supernatants were analyzed after 13 h of growth. Elastase activity is shown as a percentage of that of the parent strain. LasA protease activity was determined by measuring the changes in OD_{600} caused by the lysis of *S. aureus* cells after 2, 6, 10, 14, 18, 22, 26, 30, 45, and 60 min. LB culture medium was used as a control. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. The results shown are for the control (◆), PAO1 *lecA*::*lux* (□), PAO-P4 (▲), PAO-P8 (○), PAO-P9 (■), PAO-P10 (X), PAO-P34 (●), PAO-P52 (△), and PAO-P69 (+).

reporter, after 14 h of growth were the same as those found after 8 h.

Elastase, a zinc metalloprotease encoded by *lasB* (2), is capable of degrading and inactivating a wide range of biological and immunological tissues. As with pyocyanin, elastase production was affected in all of the mutants tested. Figure 4B shows the results of ECR assays (31) of culture supernatants sampled after 13 h of growth. As expected, both the *lasR* (PAO-P4) and *rhlR* (PAO-P34) mutants produced very little elastase. PAO-P52 (*clpA* mutant) showed high levels of elastase production compared to the parent strain (185%), and in contrast, both PAO-P9 (*rpoS* mutant) and PAO-P10 (*mvaT* mutant) showed reduced elastase production compared with the parent strain (35 and 81%, respectively).

Analysis of LasA protease activity with a staphylolytic assay (20) showed LasA to be affected in all of the transposon mutants tested (Fig. 4C), with PAO-P4 (*lasR* mutant) and PAO-P34 (*rhlR* mutant) showing little or no activity. PAO-P9 (*rpoS* mutant) and PAO-P10 (*mvaT* mutant) both demonstrated reduced levels compared with those of PAO1 *lecA*::*lux*. Interestingly, PAO-P52 (*clpA* mutant) showed greater LasA activity than the PAO1 *lecA*::*lux* parent strain.

A selection of mutants was also analyzed for siderophore production with the Chrome azurol S assay. Both PAO-P4 (*lasR* mutant) and PAO-P10 (*mvaT* mutant) showed levels of siderophore activity similar to those of PAO1 *lecA*::*lux*, but activity was reduced in both PAO-P8 and PAO-P9 (*rpoS* mutant) as determined by halo size (data not shown). PAO-P34 (*rhlR* mutant) exhibited virtually no siderophore activity (data not shown).

Finally, swarming motility was tested in a selection of mutants (Fig. 5). Compared with PAO1 *lecA*::*lux*, strains PAO-P4 (*lasR* mutant), PAO-P10 (*mvaT* mutant), PAO-P52 (*clpA* mutant), and PAO-P69 exhibited reduced swarming behavior while PAO-P8 and PAO-P9 (*rpoS* mutant) showed very little swarming. The *rhlR* mutant (PAO-P34) did not swarm, a finding in keeping with the observations of Kohler et al. (21).

AHL analysis. To determine whether the observed pleiotropic effects in the Tn*5*-B21 mutants were due to alteration of the quorum-sensing cascade, we analyzed the production of both C4-HSL and 3O-C12-HSL in these mutants. Samples of *P. aeruginosa* culture (900 μl) were taken 8 h postinoculation and acidified with 100 μl of 1 M HCl. The detection and quantification of AHLs are described in Materials and Methods. Figure 6 shows that PAO-P9 (*rpoS* mutant), PAO-P10 (*mvaT* mutant), PAO-P52 (*clpA* mutant), and PAO-P69 all produced high levels of both C4-HSL (Fig. 6A) and 3O-C12-HSL (Fig. 6B) compared with PAO1 *lecA*::*lux*, suggesting that these genes influence the quorum-sensing circuitry to some extent.

Addition of C4-HSL and 3O-C12-HSL to *mvaT* mutant PAO-P10 advances *lecA*::*lux* expression. Since *mvaT* appeared to encode an important novel regulator of the expression of a number of quorum sensing-regulated phenotypes, we sought to determine whether this gene plays a role in controlling the growth phase-dependent expression of *lecA*. Firstly, experiments were performed to confirm that the observed effects on *lecA* expression in PAO-P10 were indeed due to inactivation of *mvaT* and not a secondary mutation on the chromosome. It was possible to reduce expression of the *lecA*::*lux* fusion in PAO-P10 to levels lower than those in the parent strain by expressing intact *mvaT* on pUCP18 (Fig. 7A). In addition, pyocyanin production was also significantly decreased in this *trans*-complemented mutant (data not shown). Furthermore, a defined *mvaT* deletion mutant (PAO-P47) form of PAO1 was constructed (see Materials and Methods) that showed significantly enhanced PA-IL lectin (Fig. 7B) and pyocyanin (data not shown) levels compared with the PAO1 wild type. In addition, elastase and LasA protease levels were decreased in this mutant compared with those in strain PAO1 (data not shown).

To investigate whether expression of *lecA*::*lux* could be advanced in the absence of a functional *mvaT* gene, PAO-P10 was grown in the presence of either C4-HSL, 3O-C12-HSL, or

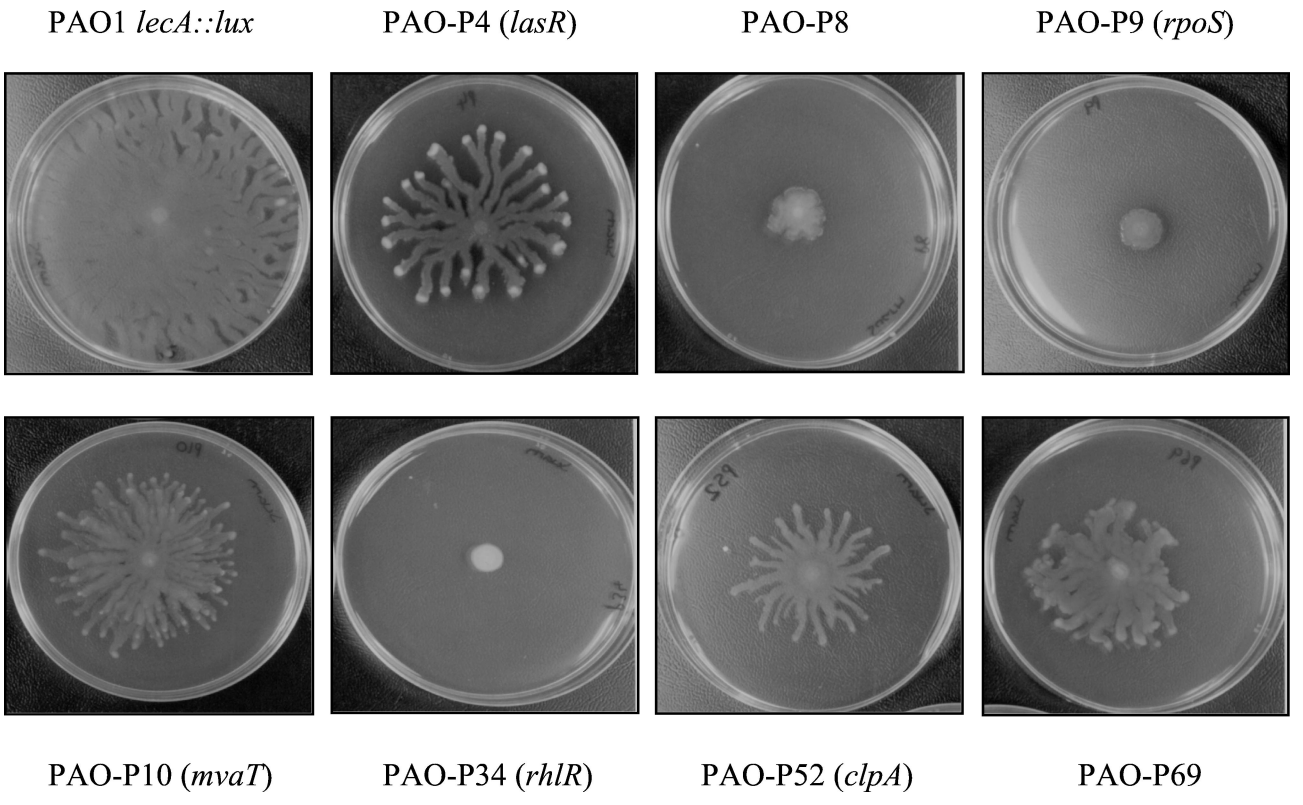

FIG. 5. Swarming motility assessed with swarm plates. The ability to swarm was determined by measuring growth from the central inoculation site as previously described (39).

a combination of the two at a 10 μM concentration. In contrast to that in the PAO1 *lecA*::*lux* parental strain (Fig. 2), expression of *lecA*::*lux* was advanced in PAO-P10 with respect to time (Fig. 8A) and cell density (Fig. 8B) when both AHLs were added in combination to the medium.

DISCUSSION

In *P. aeruginosa*, many virulence genes have been reported to be regulated by quorum sensing. By definition, quorum sensing is a mechanism by which bacterial cells regulate specific target genes in response to a critical concentration of signal molecules, which is, in turn, a measurement of the cell density of the population (13). Previous work has shown that deletion of *qscR* (6), *rpoS* (56), and *rsmA* (37a) in *P. aeruginosa* results in advanced expression of quorum sensing-regulated genes. In addition, work by Whiteley et al. (55) with a *lasI rhlI* double mutant has shown that expression of a number of genes can be induced prematurely in the presence of AHLs. This is, in fact, the case for bioluminescence and carbapenem production in wild-type strains of *V. fischeri* (10, 28) and *E. carotovora* subsp. *carotovora* (58), respectively. In contrast, the present report and also a previous publication (62) demonstrate that at least some quorum sensing-regulated virulence genes in *P. aeruginosa* are "superregulated" in a growth phase-dependent manner. For instance, under the conditions employed in this study, induction of *lecA* and *lasB* occurred during the transition to stationary phase and transcription of these genes could not be advanced by addition of exogenous C4-HSL and 3O-C12-HSL, indicating that a critical autoinducer concentration is required but is not sufficient for their expression. Similarly, expression of *rhlR*, the gene encoding one of the LuxR homologues that mediate quorum sensing in *P. aeruginosa*, could not be advanced under these conditions. Thus, it appears that growth phase-dependent superregulation of quorum sensing is conducted on at least two levels, (i) control of the quorum-sensing cascade itself and (ii) target gene expression. To identify new genes involved in quorum sensing or growth phase-dependent regulation of the *lecA* gene, we used the bioluminescent *lecA*::*lux* reporter strain (62) as a target for transposon mutagenesis. This approach resulted in the isolation of mutants that were all affected in multiple quorum-sensing phenotypes. Furthermore, the isolation of *lasR* and *rhlR* mutants demonstrated that this approach could also be used for the identification of regulators involved in control of the quorum-sensing hierarchy itself. Among the mutants isolated, only PAO-P10 showed a greater-than-twofold increase in *lecA*::*lux* expression. Furthermore, in contrast to the parental strain, addition of exogenous C4-HSL and 3O-C12-HSL in combination advanced *lecA*::*lux* expression in PAO-P10 significantly. This suggested that the growth phase-dependent control mechanisms observed with the parent strain were no longer fully functional in this mutant. The locus inactivated in PAO-P10 was highly similar to a gene encoding the P16 subunit of MvaT, a novel heteromeric transcriptional regulator in *P. mevalonii* (41). Hence, the putative *mvaT* homologue of *P. aeruginosa* merited a more detailed analysis. In *P. mevalonii*, MvaT positively regulates the *mvaAB* operon encoding 3-hy-

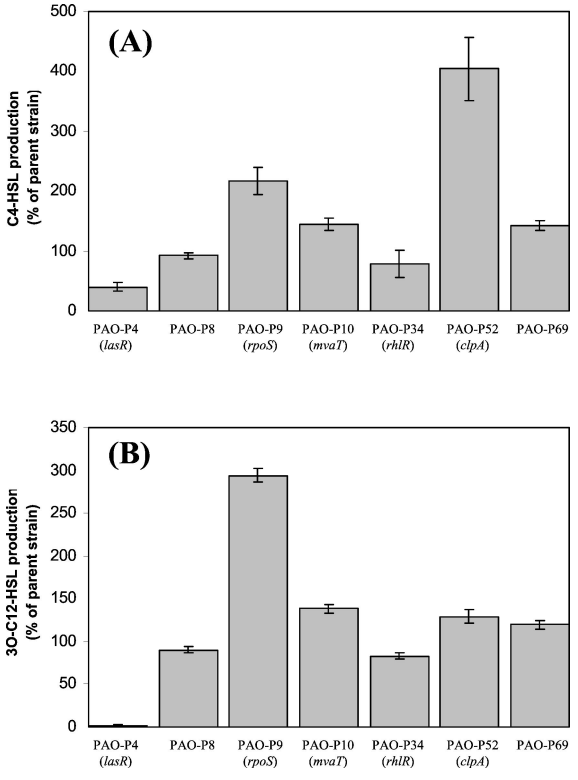

FIG. 6. Influence of Tn*5*-B21 mutations on C4-HSL (A) and 3O-C12-HSL (B) production. Bacterial culture supernatants were taken after 8 h of growth and acidified with 1 M HCl. Culture supernatants were spotted onto either reverse-phase TLC plates and overlaid with soft top agar containing an *E. coli* bioluminescent reporter strain to detect C4-HSL or onto normal-phase TLC plates and overlaid with an *E. coli* bioluminescent reporter strain to detect 3O-C12-HSL. C4-HSL and 3O-C12-HSL levels were determined by quantifying the bioluminescence observed with the reporter strains. Values were adjusted for differences in OD_{600}, and the level observed for the parent strain (PAO1 *lecA*::*lux*) was defined as 100%.

droxy-3-methylglutaryl coenzyme A reductase and 3-hydroxy-3-methylglutaryl coenzyme A lyase, enzymes that catalyze the initial reactions of mevalonate catabolism in this organism. In order to determine whether MvaT regulates a similar operon in *P. aeruginosa*, we searched for homologues of *mvaA* and *mvaB* with the complete *Pseudomonas* genome sequence. A putative homologue of *mvaB* was identified that showed 74% similarity to *mvaB* from *P. mevalonii.* However, no homologue to *mvaA* was found. Analysis of the *mvaT* gene region in *P. aeruginosa* with the genome sequence revealed only two genes with an assigned function. Downstream of *mvaT* and convergently transcribed is the *sbcB* gene coding for the protein exo-DNase I (also known as exonuclease I). This protein showed 66% similarity to exonuclease I in *E. coli*, where it degrades single-stranded DNA from the 3′ end and is involved in DNA replication, recombination, modification, and repair (38). Upstream of *mvaT* and divergently transcribed is the *purU1* gene, which encodes a putative formyltetrahydrofolate deformylase (27). This protein showed 67% similarity to the formyltetrahydrofolate deformylase of *Aquifex aeolicus.* It seems unlikely that polar effects of the transposon insertion were responsible for the observed phenotypic changes, as *mvaT* does not form an operon with these genes. Proteins similar to MvaT may represent a new family of transcriptional regulators that are unique to pseudomonads, as database analysis revealed putative homologues in *P. putida*, *P. syringae*, and *P. fluorescens* and also a second homologue (PA2667) in *P. aeruginosa* but not in other bacterial species. Interestingly, analysis of the gene region encoding one MvaT homologue in *P. putida* KT2440 revealed that this gene was also flanked by the *sbcB* and *purU1* genes in a manner identical to that seen in *P. aeruginosa*.

In *P. aeruginosa* PAO-P10, inactivation of *mvaT* not only resulted in increased expression of the *lecA*::*lux* reporter fusion but also caused ninefold overproduction of pyocyanin. As expected, these effects could be reversed by providing an intact *mvaT* gene in *trans*. Furthermore, increased production of the PA-IL lectin and pyocyanin was confirmed with an in-frame deletion of *mvaT* in *P. aeruginosa* PAO1. AHL analysis of PAO-P10 demonstrated that this mutant produced increased levels of both C4-HSL and 3O-C12-HSL, suggesting that MvaT may act as a repressor of quorum sensing in *P. aeruginosa.* The increase in AHLs could provide an explanation of why *lecA* expression and pyocyanin production are dramatically increased in PAO-P10. However, it is important to note that although AHL levels were increased, other quorum sensing-dependent phenotypes were down-regulated in this mutant. For instance, PAO-P10 showed reduced production of elastase and LasA protease and decreased swarming motility.

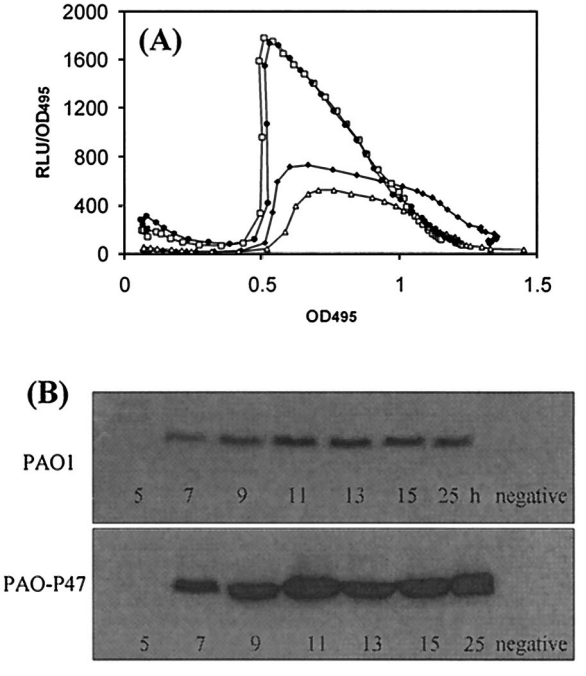

FIG. 7. (A) Complementation of PAO-P10 with plasmid-based *mvaT.* The results shown are for PAO1 *lecA*::*lux* (◆), PAO-P10 (□), PAO-P10 pUCP18 (●), and PAO-P10 pUCP *mvaT* (△). (B) PA-IL lectin production in PAO1 and an *mvaT* chromosomal deletion mutant (PAO-P47) after 5, 7, 9, 11, 13, 15, and 25 h of growth. Shown is a Western blot of a *P. aeruginosa* cellular lysate after SDS-PAGE. The blot was developed with PA-IL antisera as described in Materials and Methods. PAO-P34 (*rhlR* mutant) was used as a PA-IL-negative control.

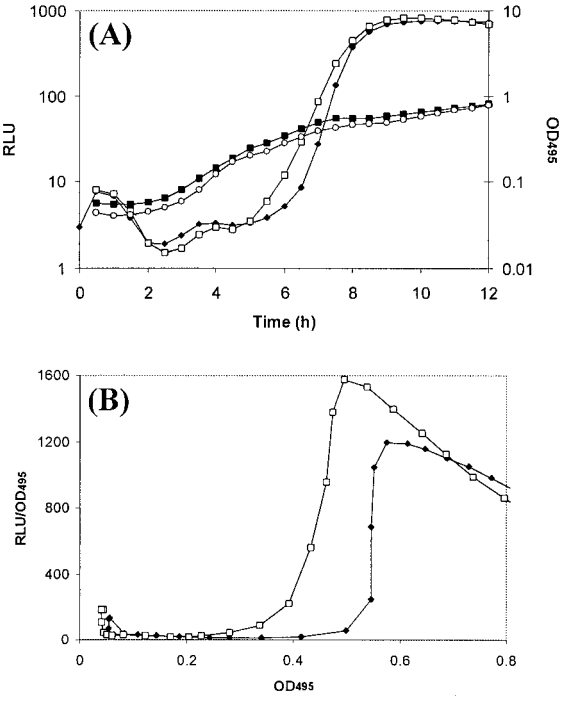

FIG. 8. Advancement of *lecA* expression in PAO-P10 (*mvaT* mutant). (A) Growth (OD_{495}) of PAO-P10 in the absence (■) or presence (○) of both C4-HSL and 3O-C12-HSL at 10 μM. Also shown is absolute *lecA* expression (RLU) with time in the absence (◆) or presence (□) of both C4-HSL and 3O-C12-HSL at 10 μM. (B) Representation of relative *lecA* expression with cell density in the absence (◆) or presence (□) of a combination of C4-HSL and 3O-C12-HSL at 10 μM. The results represent a single experiment, although the experiment was repeated three times with similar results.

Thus, in *P. aeruginosa*, MvaT appears to be a global regulator that modulates the expression of several virulence genes and is part of a growth phase-dependent control system.

Addition of both C4-HSL and 3O-C12-HSL is required, in the absence of MvaT, to bring *lecA*::*lux* expression forward (Fig. 8). Since the parent strain did not show advanced expression with regard to time and cell density in the presence of both AHLs (Fig. 2), it can be argued that a critical AHL concentration is required, but is not sufficient, for the expression of some quorum sensing-regulated genes. We hypothesize that early expression of these genes may be prevented by additional control elements that ensure that they are only switched on at a certain stage of growth. MvaT may represent one such control element. Interestingly, Whiteley et al. (55) also identified a number of genes (so-called class II and IV genes) whose expression was enhanced but not advanced by the addition of AHLs. Some of these genes may also be superregulated in a growth phase-dependent manner. Furthermore, the increase in *lecA* expression in the presence of both AHLs, taken together with data provided by Whiteley et al. (55), supports the idea that posttranslational regulation of the *rhl* system, due to the competition of C4-HSL and 3O-C12-HSL for RhlR binding (36), does not occur in *P. aeruginosa* but that both autoinducers can act in a synergistic manner.

It is not unreasonable to assume that growth phase-dependent control of quorum sensing-dependent genes is multifactorial. One of the control elements involved appears to be RpoS. Transcription of *rpoS* was shown to be induced upon entry into stationary phase (56), although transcripts are already detectable during mid-exponential phase (K. Winzer and P. Williams, unpublished). Mutations in either the *las* or *rhl* quorum-sensing system or addition of exogenous AHLs did not advance or inhibit induction of the *rpoS* gene (56). How *rpoS* is regulated in *P. aeruginosa* has not been studied in detail, but Kojic and Venturi (22) have shown that expression is largely dependent on PsrA, a regulator of the TetR family. Intriguingly, regulators belonging to this family are activated upon interaction with a diffusible molecule. Our results demonstrate that the stationary-phase sigma factor is required not only for *lecA* expression but also for the production of elastase and LasA protease, as well as swarming. This requirement of RpoS for swarming has not been described before. The requirement for RpoS could also explain the late expression of class II and IV genes described by Whiteley et al. (55). In a previous study by Suh et al. (50), only a small decrease in elastase and LasA activities was reported for an *rpoS* mutant whereas our mutant, PAO-P9, showed lower activity levels. Still, in both studies, pyocyanin production by *rpoS* mutants was shown to be significantly increased, a phenotype also reported by Whiteley et al. (56). It has been shown that *rhlI* expression is increased in an *rpoS* mutant (56). In this study, we showed that, accordingly, our *rpoS* mutant (PAO-P9) produced greater amounts of C4-HSL than did the parental strain. In addition, PAO-P9 also produced more 3O-C12-HSL, a finding not previously described. Whether the extremely high pyocyanin levels seen in this strain were due to high levels of both AHLs is unknown, although it is interesting that other mutants (PAO-P10, PAO-P52, and PAO-P69), up-regulated in pyocyanin and downregulated in other phenotypes, also produced higher levels of both AHLs compared with the parent strain.

Pearson et al. (35) have shown that 3O-C12-HSL, but not C4-HSL, accumulates in *P. aeruginosa* cells, with the intracellular concentration being three times higher than the external level. The *mexA-mexB-oprM*-encoded efflux pump is involved in the active efflux of 3O-C12-HSL, as the intracellular level in a *mexA-mexB-oprM* deletion mutant was eightfold higher than the external concentration (35). In our mutant, PAO-P69, a gene encoding a component of a putative active efflux system had been inactivated. This mutant was significantly different from the parent strain with regard to phenotypes regulated by quorum sensing. Intriguingly, this gene (PA4207) was identified by Whiteley et al. (55) as a class IV (late response) gene (J. Pearson, personal communication) that only responds to the addition of both C4-HSL and 3O-C12-HSL in a *lasI-rhlI* double mutant. This suggests that this gene is controlled by quorum sensing and is also involved in the regulation of a number of quorum sensing-dependent phenotypes. The expression of quorum sensing-regulated genes in the *mexA-mexB-oprM* mutant has not been described (35), but according to the present model (24, 36), earlier and stronger induction of LasR/3O-C12-HSL-dependent genes should be expected due to the high intracellular 3O-C12-HSL concentration. Earlier data were in agreement with this model, since Evans et al. (12) had demonstrated that hyperexpression of *mexA-mexB-oprM* resulted in a significant reduction of extracellular elastase and pyocyanin. However, mutant PAO-P69 showed, with the exception of pyocyanin production, the opposite of what was expected. Elastase

levels were shown to be similar to those of the parent strain, while LasA protease activity was reduced. The quorum-sensing system of *P. aeruginosa* is still not well understood and may involve complex cross-regulation between the *las* and the *rhl* systems, as well as the third LuxR homologue, QscR (6).

In *E. coli*, the ATPase ClpA is a chaperone and also provides substrate specificity to the ClpP protease (19, 57). How inactivation of *clpA* influences quorum sensing-regulated phenotypes in *P. aeruginosa* still needs to be established. Inactivation of *clpA* could reduce the concentration of properly folded regulatory proteins. Alternatively, ClpA, together with ClpP, could have a regulatory function like that of ClpX and ClpP in *E. coli*, which degrade RpoS during the exponential phase (43). It will be interesting to unravel the link between these proteases and virulence gene expression in *P. aeruginosa.*

In summary, we have shown that in *P. aeruginosa*, growth phase-dependent control of gene expression can override activation by quorum sensing. We have isolated and characterized a number of mutants, all of which are affected in multiple quorum-sensing phenotypes. A new global regulator, MvaT, was identified that is involved in the growth phase-dependent control of the *lecA* gene. However, more work is required to unravel the complicated regulatory network that links cell density-dependent gene expression with the growth phase and metabolic state of the cell.

ACKNOWLEDGMENTS

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