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Quorum-Sensing Regulation of the Biofilm Matrix Genes (*pel*) of *Pseudomonas aeruginosa*▽

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Quorum sensing (QS) has been previously shown to play an important role in the development of *Pseudomonas aeruginosa* biofilms (D. G. Davies et al., Science 280:295–298, 1998). Although QS regulation of swarming and DNA release has been shown to play important roles in biofilm development, regulation of genes directly involved in biosynthesis of biofilm matrix has not been described. Here, transcription of the *pel* operon, essential for the production of a glucose-rich matrix exopolysaccharide, is shown to be greatly reduced in *lasI* and *rhlI* mutants. Chemical complementation of the *lasI* mutant with 3-oxo-dodecanoyl homoserine lactone restores *pel* transcription to the wild-type level and biofilm formation ability. These findings thus connect QS signaling and transcription of genes responsible for biofilm matrix biosynthesis.

Bacteria form biofilms, matrix-enclosed multicellular assemblages that appear to provide increased survival ability under various stress conditions (3, 4). Cell-cell communication or quorum sensing (QS) has been shown to be involved in the formation of biofilm in several bacterial species (reviewed in references 11 and 12). *Pseudomonas aeruginosa*, a gram-negative and opportunistic human pathogen, possesses two hierarchical QS systems known as *las* and *rhl* (10, 15, 19). Upon increase in the population density, the concentration of the signaling molecule 3-oxo-dodecanoyl (3-*O*-C_{12}) homoserine lactone increases and is able to form a complex with LasR, a transcriptional regulator. This complex activates the transcription of *lasI*, *rhlR*, and a number of other genes. In a seminal paper that first indicated a role for cell-cell signaling in biofilm formation, Davies et al. showed that mutants defective in the Las QS system yielded unstructured and frail biofilms (5). Recent studies have demonstrated that QS-dependent regulation of swarming and DNA release play important roles in biofilm development of *P. aeruginosa* (1, 16). Because mutants lacking the Las QS system form defective biofilms that are completely flat and detergent sensitive and because the Las QS system controls expression of a large set of genes, it is highly likely that the biofilm-defective phenotype of the mutants is multifactorial. This includes above-mentioned defects in swarming, DNA release, and possibly defects in the production of other structural components of biofilm matrix.

Recently, our laboratory identified a cluster of *P. aeruginosa* genes, termed *pel*, whose products are responsible for the production of glucose-rich biofilm matrix exopolysaccharide in strain PA14 (7). Later, an independent study by Vasseur et al. showed that this was also the case in strain PAK (18). The glucose-rich polysaccharide is essential for the formation of a wrinkled colony and a surface-associated biofilm; thus, it is a major component of biofilm in this strain (7). The *pel* operon contains seven genes, *pelA* to *pelG*, that display sequence similarity with genes that encode sugar-processing enzymes. These include oligogalacturonide lyase (*pelA*), glycosyltransferases (*pelC* and *pelF*), sucrose synthase (*pelE*), and transmembrane proteins (*pelD* and *pelG*). Transposon insertion into or deletion of these genes resulted in severe defects in biofilm formation (7, 18).

To test whether some genes directly involved in the biosynthesis of a biofilm matrix component might be regulated by QS, we analyzed whether *las* mutants were defective in the expression of the *pel* genes. Colony morphology was first observed as an indicator of matrix production (7). Overnight, shaken liquid cultures of *P. aeruginosa* PA14 and several isogenic mutants, all grown at 37°C, were diluted in tryptone broth without NaCl to a final optical density at 600 nm of 0.0025. Ten microliters of each strain was spotted on tryptone broth lacking NaCl but containing 0.5% agar. After 4 days of incubation at room temperature, the wild-type strain formed a characteristically wrinkled colony (Fig. 1A) that was elastic and resisted breakage when transferred into water and subjected to vigorous vortexing, which is indicative of robust production of the glucose-rich exopolysaccharide produced by the *pel* gene products (7). The *pelA* mutant formed a flat colony as previously described (7) (Fig. 1A). The *rhlI* mutant formed a colony that appeared hyperwrinkled (Fig. 1A); however, the colony easily broke apart into fragments during vigorous vortexing (data not shown), indicating that the matrix structure was altered in the *rhlI* mutant. In contrast, the *lasI* mutant formed completely flat colonies, similar to those formed by the *pelA* mutant (Fig. 1A). We observed these results reproducibly in multiple isolates of these mutants. Because these mutants had been extensively characterized previously, including by genetic complementation, and because of the chemical complementation results described below, we did not carry out genetic complementation analyses here.

A pellicle is a biofilm that assembles at the air-liquid inter-

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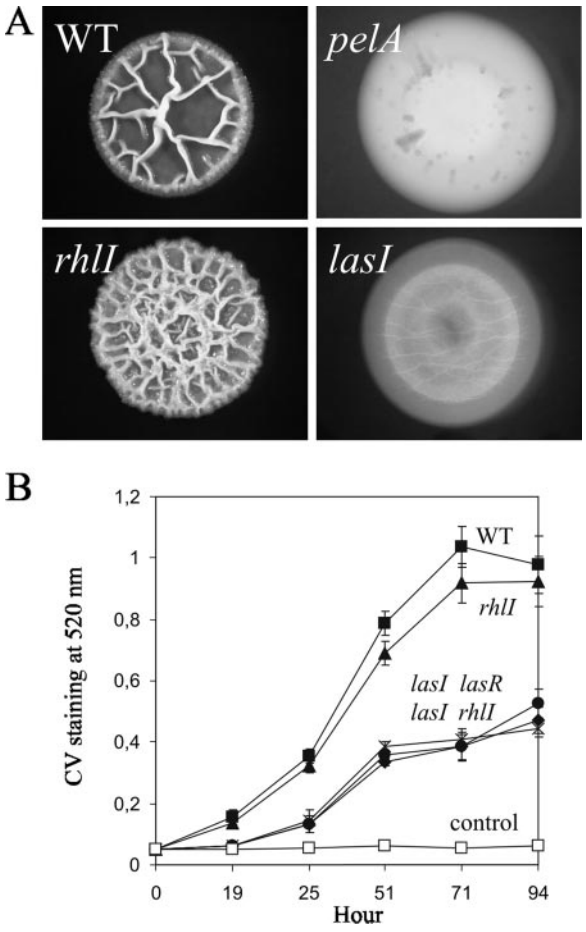

FIG. 1. Biofilm formation of the wild type (WT), *pelA* mutant, and QS mutants (*lasI* and *rhlI* mutants) of *P. aeruginosa* PA14. (A) Colony morphologies. (B) CV staining of the solid-surface-associated biofilm. The control was medium alone.

face of a standing liquid culture and was examined as an independent readout of matrix production. The wild-type strain and the *rhlI* mutant grown in tryptone broth without NaCl formed thick and visible pellicles by 48 h at room temperature, while the *pelA* mutant did not form a pellicle, which is consistent with previous results (7) (data not shown). The *lasI* mutant formed only a thin layer of film that was frail and could easily be broken apart upon gentle shaking (data not shown). These results are in good agreement with colony morphologies and suggested that the *lasI* mutant is defective in matrix production.

Development of the solid-surface-associated biofilm was examined by using crystal violet (CV) staining (7). Standing cultures in tryptone broth without NaCl in polystyrene microtiter plates were subjected to rigorous washes in water prior to CV staining as described by Friedman and Kolter (7). The wild type showed a dramatic increase of the solid-surface-associated biofilm between 19 h and 71 h (Fig. 1B). The *rhlI* mutant formed only slightly less solid-surface-associated biofilm than the wild type did (Fig. 1B). On the other hand, the *lasI*, *lasR*, and *lasI rhlI* mutants formed significantly decreased solid-surface-associated biofilm with at least 60% reduction in the CV staining compared to the wild type (Fig. 1B). This level of reduction is very similar to the level of reduction previously reported for the *pel* mutant (ca. 75%) (7). Taken together, these results indicated that the *las* QS mutants are defective in biofilm formation characterized by colony morphology, pellicle formation, and the solid-surface-associated biofilm and suggested that the Las QS system could be involved in the regulation of a biofilm matrix component.

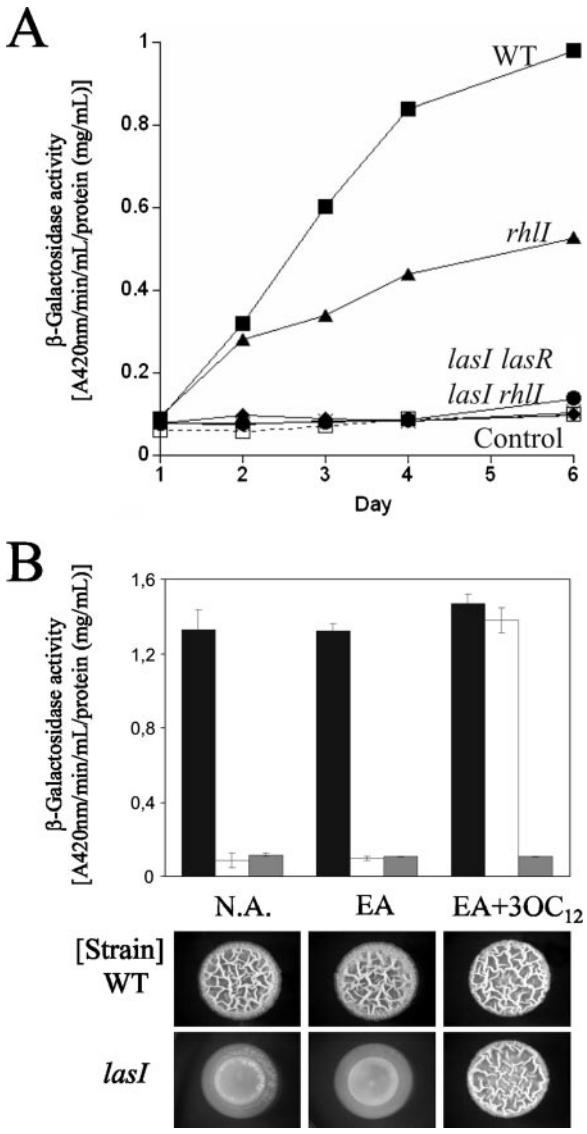

FIG. 2. Requirement of QS in *pelA* transcription and wrinkled-colony formation in *P. aeruginosa* PA14. (A) β-Galactosidase activities of the *pel* promoter-*lacZ* transcriptional fusion in the wild type (WT) and QS mutants (*lasI*, *lasR*, *rhlI*, and *lasI rhlI* mutants). (B) β-Galactosidase activities of the *pel* promoter-*lacZ* transcriptional fusion (top panel) and colony morphologies (bottom panel) of the wild type and the *lasI* mutant in the presence of 10 μM 3-*O*-C_{12} homoserine lactone ($3OC_{12}$). The promoter-less *lacZ* construct was used as a control. N.A., no autoinducer added; EA, ethyl acetate-glacial acetic acid (1,000:1, vol/vol).

Because the *las* mutants showed the flat colony morphology, frail thin pellicle, and a complete lack of surface-adhered biofilm similar to those observed for the *pelA* mutant, we asked

whether *pelA* expression is somehow altered in the *las* mutants. To test this, a transcriptional fusion composed of the 785-bp upstream sequence of *pelA* and a promoterless *lacZ* gene was constructed in the vector mini-CTX-lacZ (2). This construct was introduced as a single copy into the chromosome at the unique *attB* site (2). Spot colonies were made as described above and harvested at various time points by resuspension in 50 mM sodium phosphate, pH 7.4, and cells were lysed by ultrasonication. β-Galactosidase activities were determined by using cleared supernatants after centrifugation, and the results were normalized to the total protein concentration. The β-galactosidase activity of the wild type increased linearly for the first 4 days and subsequently reached a plateau (Fig. 2A). The *rhlI* mutant showed a somewhat slower rate of increase of β-galactosidase activity, reaching levels about 50% of those of the wild type (Fig. 2A). In contrast, the *lasI* and *lasR* single mutants showed no activity above the background level (Fig. 2A). Likewise, the *lasI rhlI* double mutant showed no activity (Fig. 2A). These results indicate that transcription of the *pel* genes is activated by the Las QS signaling system. In addition, it appears that part of the activation is mediated through Rhl QS.

To further confirm the requirement of Las QS for transcription of the *pel* genes, we carried out a chemical complementation experiment. Synthetic 3-*O*-C_{12} homoserine lactone, the autoinducer of the Las QS signaling system, was dissolved in ethyl acetate-glacial acetic acid (1,000:1, vol/vol) and added to a final concentration of 10 μM in tryptone broth agar without NaCl and buffered with 5 mM 3-(*N*-morpholino)-propanesulfonic acid–NaOH, pH 7.0. Cell suspensions were then spotted on these plates and allowed to grow for 4 days at room temperature. The *lasI* mutant formed wrinkly colonies only when 3-*O*-C_{12}-HSL was present in the medium (Fig. 2B). At the same time, β-galactosidase activity was restored to wild-type levels (Fig. 2B). Taken together, these results indicate that the Las QS signaling system activates transcription of the *pel* genes and in so doing influences biofilm formation by *Pseudomonas aeruginosa* PA14.

Interestingly, *pel* transcription was found to be dependent on temperature. β-Galactosidase activity was detected at room temperature (ca. 25°C) (Fig. 3A), whereas little activity was detectable at 37°C in the wild type and in the *rhlR* mutant (Fig. 3B). No activity was detectable in the *lasR* mutant at both temperatures (Fig. 3A and B). The formation of the solid-surface-associated biofilm decreased dramatically at 37°C in the wild type and the *rhlR* mutant (Fig. 3C). These results indicate that *pel* transcription is subjected to thermocontrol which overrides the transcriptional activation by the Las QS system.

Here we demonstrate that Las QS is involved in transcription of the *pel* genes and biofilm formation in *Pseudomonas aeruginosa* PA14, thus providing an additional physical explanation for the involvement of cell-cell signaling in biofilm formation in this bacterium. Aside from swarming and DNA release being dependent on QS (1, 16), our results demonstrate that the expression of the genes whose products are involved in synthesis of a main component of the extracellular matrix, the Pel exopolysaccharide, is regulated by QS.

Our results indicate that Las QS influences *pel* transcription and that part of the effect, though by no means all, may be through its role in activating the Rhl QS signaling system. Phenotypic outcomes of the *rhl* mutants, however, were complex. The mutants showed only a slight reduction of the solid-surface-associated biofilms (Fig. 1B) while they formed hyperwrinkled colonies, which were nevertheless frailer than the

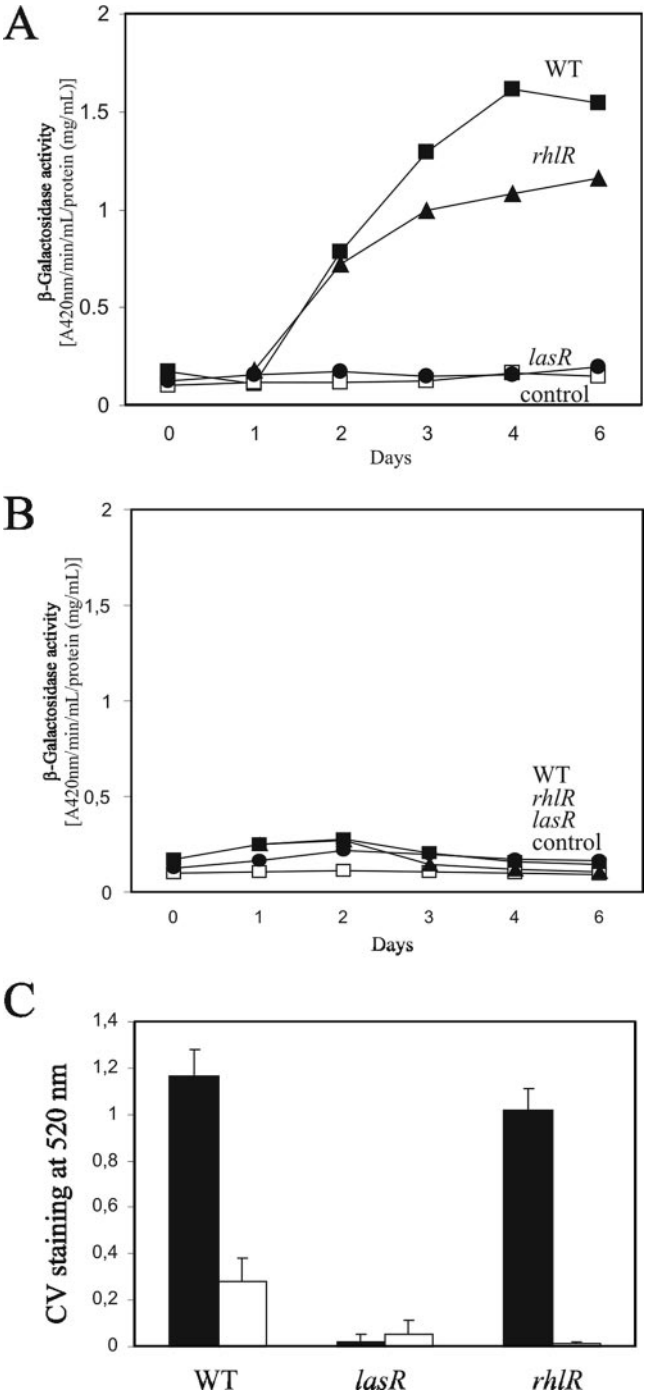

FIG. 3. Thermoregulation of *pel* transcription and biofilm formation in *P. aeruginosa* PA14. (A and B) β-Galactosidase activities of the *pel* promoter-*lacZ* transcriptional fusion in the wild type (WT) and QS mutants (*lasR* and *rhlR* mutants) at room temperature (A) and at 37°C (B). (C) CV staining of the solid-surface-associated biofilm at room temperature (solid bars) and at 37°C (open bars).

wild-type colonies (Fig. 1A). It is plausible that, in addition to *pel*, the Rhl QS system controls as-yet-unknown additional factors that influence biofilm matrix architecture.

We asked whether these QS systems activate *pel* transcription directly or not. Three pieces of evidence have led us to think it is indirect. First, LasR and RhlR recognition sequences have not been clearly defined, making sequence-based predictions difficult (14). Deletion of a 20-bp sequence that showed significant similarity to the predicted LasR/RhlR binding motif (20) and was located upstream of *pelA* did not alter its quorum-dependent transcription activity (results not shown). This suggests that the transcription of *pel* genes may be indirectly regulated by the QS systems, possibly by transcriptional factors whose expression itself is regulated by LasR and RhlR. Second, we observed that *pel* transcription is under thermoregulation, which overrides the transcriptional activation by the Las QS system (Fig. 3). These results suggest there exists a transcriptional regulator that controls *pel* expression. Third, results obtained in a recent study suggest a complex regulatory circuit of *pel* expression, which involves the two-component systems RetS and GacS/GacA, the small RNA molecule RsmZ, and the RNA-binding protein RsmA (8). RetS intercepts the GacS/GacA two-component regulatory circuit (8) that activates transcription of RsmZ (9). RsmZ antagonizes RsmA (9), which is assumed to bind leader sequences of target transcripts and posttranscriptionally represses their expression (6, 9, 17). Interestingly, it has been shown that RsmA negatively regulates *lasI* and *rhlI* translation (13). Therefore, it is plausible that *pel* expression is regulated at two levels, transcriptionally by the as-yet unknown transcriptional regulator that is regulated by the QS systems and posttranscriptionally by RsmA whose activity negatively regulates the QS systems.

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