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PqsE Functions Independently of PqsR-*Pseudomonas* Quinolone Signal and Enhances the *rhl* Quorum-Sensing System[▽]

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Pseudomonas aeruginosa **is an opportunistic pathogen that causes both acute and chronic infections in immunocompromised individuals. This gram-negative bacterium produces a battery of virulence factors that allow it to infect and survive in many different hostile environments. The control of many of these virulence factors falls under the influence of one of three *P. aeruginosa* cell-to-cell signaling systems. The focus of this study, the quinolone signaling system, functions through the *Pseudomonas* quinolone signal (PQS), previously identified as 2-heptyl-3-hydroxy-4-quinolone. This signal binds to and activates the LysR-type transcriptional regulator PqsR (also known as MvfR), which in turn induces the expression of the *pqsABCDE* operon. The first four genes of this operon are required for PQS synthesis, but the fifth gene, *pqsE*, is not. The function of the *pqsE* gene is not known, but it is required for the production of multiple PQS-controlled virulence factors and for virulence in multiple models of infection. In this report, we show that PqsE can activate PQS-controlled genes in the absence of PqsR and PQS. Our data also suggest that the regulatory activity of PqsE requires RhlR and indicate that a *pqsE* mutant can be complemented for pyocyanin production by a large excess of exogenous *N*-butyryl homoserine lactone (C_4-HSL). Finally, we show that PqsE enhances the ability of *Escherichia coli* expressing RhlR to respond to C_4-HSL. Overall, our data lead us to conclude that PqsE functions as a regulator that is independent of PqsR and PQS but dependent on the *rhl* quorum-sensing system.**

Pseudomonas aeruginosa is a serious opportunistic pathogen that can cause infections in a diverse group of organisms from the animal, plant, and insect kingdoms (9, 47, 53). In humans, *P. aeruginosa* can cause multiple types of infections that are generally difficult to treat due to the high level of antibiotic resistance exhibited by this microbe (15). These infections are complex and generally involve numerous virulence determinants, with some factors playing a larger role than others in different types of infections (16). One aspect of *P. aeruginosa* virulence that seems to be important in most infections is the ability of the bacteria to communicate via intercellular signals (11). *P. aeruginosa* uses at least three cell-to-cell signaling systems to control the expression of assorted virulence factors. There are two classical acyl-homoserine lactone (HSL)-based quorum-sensing systems, the *las* and *rhl* systems (see reference 43 for a review of *P. aeruginosa* quorum sensing). The *las* and *rhl* quorum-sensing systems function via the signals *N*-(3-oxododecanoyl) HSL (3-oxo-C_{12}-HSL) and *N*-butyryl HSL (C_4-HSL), respectively (40, 41). When at a threshold concentration, 3-oxo-C_{12}-HSL and C_4-HSL serve as coinducers for the transcriptional activators LasR and RhlR, respectively (21, 35, 36, 39). The other cell-to-cell signaling system of *P. aeruginosa* functions through the quinolone compound 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal [PQS]) (44). This signal serves as a coinducer for the transcriptional activator PqsR (also known as MvfR), which positively regulates PQS production to create an autoregulatory loop (5, 32, 54, 58). These three cell-to-cell signaling systems are connected by multiple regulatory pathways. The *las* system appears to be at the top of a hierarchy as it positively regulates both the *rhl* quorum-sensing system and the quinolone signaling system (27, 44, 45). The *rhl* and quinolone systems are also connected in that C_4-HSL negatively affects PQS production, and PQS positively regulates *rhlR* (14, 26, 32, 33). With regard to genes that are controlled by cell-to-cell signaling, most are dominantly regulated by one system or the other (51, 55). Such is the case for *rhlA*, which is positively controlled by the *rhl* quorum-sensing system and is required for the production of rhamnolipids, surfactants that are important for virulence (35, 42, 49). However, there is also overlap between the systems with regard to some of the genes that are controlled. For example, *lasB*, which encodes the virulence factor LasB elastase, is directly regulated by both the *las* and *rhl* quorum-sensing systems and directly or indirectly regulated by quinolone signaling (14, 42, 44, 52). This complex interplay between the cell-to-cell signaling systems is one of the ways that *P. aeruginosa* finely tunes the expression of many genes in response to its environment.

With regard to PQS, the quinolone signaling system has been shown to be important for virulence, and PQS is produced in the lungs of cystic fibrosis patients infected by *P. aeruginosa* (7, 12, 19, 20, 28, 29, 48). PQS also can act as an iron chelator, and both the synthesis of PQS and the activity of PqsR-PQS are involved in iron homeostasis, another indication of the global importance of quinolone signaling (1, 37). In addition, PQS has a membrane-altering activity that is linked to the formation of membrane vesicles (30, 31). To date, quin-

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olone signaling has been shown to directly or indirectly control 92 or 143 genes as determined by two separate transcriptome analysis studies (1, 12). To activate genes, PqsR primarily responds to PQS, but it can also be activated by the PQS precursor, 2-heptyl-4-quinolone, which is 100-fold less potent than PQS (58). PqsR can also interact with and be negatively affected by fungus-produced sesquiterpenes, which share some structural similarities to PQS (8).

It has been shown that PqsR-PQS directly activates at least the *pqsABCDE* and *phnAB* operons (5, 54). All of the members of these two operons, except for *pqsE*, appear to encode enzymes involved in the synthesis of 4-quinolone compounds, including PQS (10, 20). Curiously, mutation of the *pqsE* gene has no effect on the production of PQS or other 4-quinolone compounds, but the production of PQS-controlled virulence factors is negatively affected in *pqsE* mutants (13, 20). It has been reported that *pqsE* mutants do not produce pyocyanin or PA-IL lectin and that they produce much lower amounts of elastase and rhamnolipid (14, 20). In addition, like *pqsA* and *pqsR* mutants (which do not produce PQS), a *pqsE* mutant is avirulent in mice (12). Overall, the *pqsE* gene appears to play no role in the synthesis of PQS but seems to be absolutely required for the cells to respond to PQS. PqsE is predicted to be a 34.3-kDa protein that is most likely not secreted and is localized to the cytoplasm, according to in silico analysis with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/) and CELLO (version 2.5) subcellular localization predictor (http://cello.life.nctu.edu.tw/). A BLAST search of available genomes indicated that PqsE is related to the metallo-β-lactamase superfamily, members of which have a conserved motif able to bind up to two metal ions in their active sites (4). Unfortunately, this information provided no real indication of how PqsE allows the cell to respond to PQS. Therefore, we have begun a preliminary study of PqsE to try to understand how it can allow a cell to induce PQS-controlled virulence factors. We present data that suggest that PqsE serves a regulatory function and that this function is linked to the activation of RhlR.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference or source
E. coli DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U196 recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	57
P. aeruginosa strains		
PAO1	Wild type	24
PJF-QE1	*pqsE* deletion mutant derived from strain PAO1	This study
PJF-QA1	*pqsA* deletion mutant derived from strain PAO1	This study
PJF-QR1	*pqsR* deletion mutant derived from strain PAO1	This study
PAO-R1	*lasR*::Tet; derived from strain PAO1	21
PAO-JP1	*lasI*::Tet; derived from strain PAO1	42
PDO111	*rhlR*::Tn*501-11*; derived from strain PAO1	2
PDO100	*rhlI*Δ::Tn*501-2*; derived from strain PAO1	2
PAO-JP2	*lasI*::Tet *rhlI*Δ::Tn*501-2*; derived from strain PDO100	42
PAO-JP3	*lasR*::Tet *rhlR*::Tn*501-11*; derived from strain PDO111	42
PAO-JP2E	*lasI*::Tet *rhlI*Δ::Tn*501-2* Δ*pqsE*; derived from strain PAO-JP2	This study
Plasmids		
pMTP58	Cosmid vector containing *pqs* genes	25
pEX18Ap	Suicide vector for *P. aeruginosa*	23
pΔpqsE-suc	*pqsE* deletion suicide vector	This study
pΔpqsA-suc	*pqsA* deletion suicide vector	This study
pΔpqsR-suc	*pqsR* deletion suicide vector	This study
pACYC184	Cloning vector for *E. coli*	6
pEX1.8	*P. aeruginosa* expression vector	46
pZOE01	*tacp-pqsE* on pEX1.8	This study
pROSE04	*tacp-pqsE* on pACYC184	This study
pDSW7	*tacp-pqsA* on pEX1.8	This study
pDSW8	*tacp-pqsR* on pEX1.8	54
pECP60	*rhlR'-lacZ* translational fusion	56
pECP61.5	*rhlR'-lacZ* translational fusion; *tacp-rhlR*	42
pECP62.5	*lasB'-lacZ tacp-rhlR*	42
pECP64	*lasB'-lacZ tacp-lasR*	42
pPCS1002	*rhlR'-lacZ* transcriptional fusion	45

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are listed in Table 1. Strains of *P. aeruginosa* were maintained at −70°C in 10% skim milk (Becton Dickinson), and freshly plated bacteria were used to begin each experiment. Bacteria were cultured at 37°C in Luria-Bertani (LB) medium (50), peptone tryptic soy broth (PTSB) (38), or supplemented A medium (40) as noted below. When necessary to maintain plasmids, cultures were supplemented with 200 μg/ml carbenicillin for *P. aeruginosa* and 100 μg/ml ampicillin and/or 30 μg/ml chloramphenicol for *Escherichia coli*.

Plasmids used in this study are listed in Table 1. In order to generate an expression plasmid for PqsE, a 953-bp DNA fragment, which began at the *pqsE* start codon (ATG) and ended 47 bp downstream from the stop codon, was amplified by PCR using cosmid pMTP58 as a template. The oligonucleotide primers used for this amplification were engineered to contain a single HindIII site downstream from the stop codon (all oligonucleotide primer sequences are available upon request). Vector plasmid pEX1.8, which contains a *tac* promoter (*tacp*) to control gene expression, was digested with EcoRI and then treated with Klenow fragment to generate blunt-ended fragments. The plasmid was then digested with HindIII. The prepared plasmid DNA was ligated with the *pqsE*-containing PCR fragment, which had also been digested with HindIII, to produce the *tacp-pqsE* expression plasmid pZOE01. This construct was designed to contain optimal spacing between the ribosome binding site and the *pqsE* start codon. To create a PqsE expression plasmid for use in a two-plasmid reporter system, plasmid pZOE01 was digested with BamHI and ScaI, yielding an approximately 2-kb fragment carrying the *tacp-pqsE* fusion. This fragment was purified from an agarose gel and then ligated into vector plasmid pACYC184, which had been digested with BamHI and EcoRV. The resulting plasmid, pROSE04, harbors the *tacp-pqsE* fusion and a gene that confers resistance to chloramphenicol.

To generate a PqsA expression plasmid for complementation experiments, a 1,553-bp fragment, which began at the *pqsA* start codon and ended at the *pqsA* stop codon, was amplified by PCR. The oligonucleotide primers used for this reaction were engineered to contain a PstI site upstream from the *pqsA* start codon and a HindIII site downstream from the stop codon. The PCR fragment was digested with PstI and HindIII and then ligated into pEX1.8, which had been digested with the same enzymes. This reaction produced pDSW7, which carries a *tacp-pqsA* fusion for expression of PqsA.

Generation of mutant strains. Mutant *P. aeruginosa* strains were derived using a modified version of our previous protocol (18). Mutant alleles for *pqsE*, *pqsA*, and *pqsR* were generated using PCR as described elsewhere (18). Alleles were constructed to contain in-frame deletions in the coding DNA sequence corresponding to amino acids 18 to 285 for *pqsE* (89% of protein sequence), 44 to 504 for *pqsA* (89% of protein sequence), and 7 to 267 for *pqsR* (78% of protein sequence). The mutated fragments were also designed to contain the following at each end: BamHI sites for *pqsE*, PstI sites for *pqsA*, and EcoRI sites for *pqsR*. Each fragment was digested with the appropriate restriction enzyme and then ligated into pEX18Ap, which had been previously digested with the same enzyme, to produce suicide plasmids pΔpqsE-suc, pΔpqsA-suc, and pΔpqsR-suc. To transfer the mutant alleles onto the *P. aeruginosa* strain PAO1 chromosome,

each suicide plasmid was transferred via conjugation from *E. coli* strain SM10 into strain PAO1, and integrants were selected on LB medium containing carbenicillin and 25 μg/ml triclosan. Mutants were then selected by plating integrants on medium containing 6% sucrose to remove the vector sequence from the chromosome. Potential mutants were screened by PCR using appropriate flanking primers, and mutants were further confirmed by determining the DNA sequence of the PCR products.

Assays for pyocyanin, rhamnolipid, and elastase. To check for pyocyanin production, bacteria from frozen skim milk stocks were plated onto LB medium containing carbenicillin as necessary to maintain plasmids. After approximately 24 and 48 h of incubation at 37°C, the plates were inspected visually for the distinct blue-green color indicating the presence of pyocyanin. Alternatively, freshly plated cells were used to inoculate 10-ml cultures of LB medium, which were then incubated at 37°C for approximately 6 h with vigorous shaking. Cells from each culture were then washed in fresh LB medium and used to inoculate 10-ml cultures of fresh LB medium to an optical density at 660 nm (OD_{660}) of 0.05. Subcultures were incubated at 37°C with vigorous shaking for 18 h, and then aliquots of each culture were transferred to glass test tubes and photographed. For experiments where cultures were supplemented with C_4-HSL, aliquots of C_4-HSL dissolved in acidified ethyl acetate (44) were dried in flasks under nitrogen before they were added to subcultures.

To quantitatively measure the amount of pyocyanin in culture supernatants, a modification of the method of Essar et al. (17) was used. Samples of cultures grown for 18 h were centrifuged to remove bacterial cells, and 500-μl aliquots of culture supernatants were extracted with 300 μl of chloroform. The organic phase was then extracted with 100 μl of 0.2 N HCl to give a pink solution containing pyocyanin. The absorbance of this solution at 520 nm was measured using a NanoDrop ND-1000 spectrophotometer, and the amount of pyocyanin present was calculated by comparison with results obtained using known quantities of pyocyanin (Cayman Chemical, Ann Arbor, MI). The range of detection for the assay was from 10 μM to 300 μM pyocyanin.

For rhamnolipid and elastase assays, bacteria were cultured in LB medium as described above for the detection of pyocyanin. After 18 h of incubation at 37°C, aliquots of each culture were centrifuged to remove bacterial cells. The cleared culture supernatant was filtered through a 0.45-μm-pore-size syringe filter, and samples were extracted immediately for the detection of rhamnolipids or stored at −80°C prior to being assayed for elastolytic activity.

The concentration of rhamnolipid in culture supernatants was determined using an orcinol assay as described elsewhere (35). Briefly, 300-μl samples of filtered culture supernatants were extracted twice with 600 μl of diethyl ether. The ether fractions were pooled in glass tubes and dried under a continuous stream of N_2. Then, 100 μl of water, 100 μl of 1.6% orcinol (Sigma), and 800 μl of 60% H_2SO_4 were added to each tube, and the tubes were incubated at 80°C for 30 min. After tubes were cooled at room temperature for 10 min, the absorbance at 421 nm (A_{421}) of each sample was measured. These data were compared with results obtained using known quantities of rhamnolipid (a mixture of mono- and dirhamnolipid; Jeneil Biosurfactant Co., Saukville, WI) to calculate the amount of rhamnolipid in each sample. The range of detection for the assay was from 2.5 μg to 25 μg of rhamnolipid.

The elastolytic activity of filtered culture supernatants was measured using a modification of the elastin-Congo red (ECR) assays described elsewhere (42). Duplicate 25-μl aliquots of culture supernatants were added to tubes containing 20 mg of ECR (Sigma) and 1 ml of ECR buffer (0.1 M Tris [ph 7.2], 1 mM $CaCl_2$). Tubes were incubated at 37°C with rotation for 16 h, and then 100 μl of 0.12 M EDTA was added to each, and the tubes were placed on ice. Insoluble ECR was removed from samples by centrifugation, and the cleared samples were diluted 1:1 in ECR buffer. The A_{495} of each diluted sample was measured, and the values were corrected for the absorption due to *P. aeruginosa* pigments by subtracting the A_{495} of each sample that had been incubated in the absence of ECR.

β-Gal assays in *P. aeruginosa*. For experiments involving the addition of both PQS and C_4-HSL (see Fig. 3), cells from overnight cultures of *P. aeruginosa* grown in PTSB medium were washed and resuspended in fresh medium at an OD_{660} of 0.05 and then incubated at 37°C with vigorous shaking until cells reached late-logarithmic phase. Then, cells from subcultures were washed and resuspended in fresh PTSB medium, and 1-ml aliquots of cell suspensions were transferred to tubes containing either PQS, C_4-HSL, both, or neither. After 18 h of incubation with vigorous shaking at 37°C, cells were harvested from 1-ml cultures by centrifugation and suspended in 1 ml of supplemented A medium. The β-galactosidase (β-Gal) activity of each sample was assayed in duplicate. Data are reported in Miller units (34) as the mean ± standard deviation (SD) of at least three separate experiments.

For experiments involving the addition of C_4-HSL only (see Fig. 4), cells from cultures of *P. aeruginosa* grown in PTSB medium for 20 to 24 h were washed and resuspended in fresh medium at an OD_{660} of 0.05, and then 1-ml aliquots were transferred to tubes containing C_4-HSL. After 18 h of incubation with vigorous shaking at 37°C, cells were diluted in supplemented A medium and assayed for β-Gal activity. Data are reported in Miller units as the mean ± SD of three separate experiments.

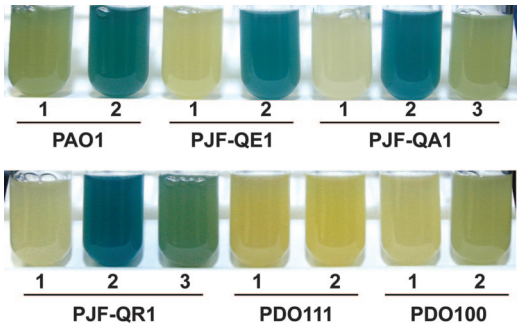

FIG. 1. The overexpression of *pqsE* in *P. aeruginosa* causes overproduction of pyocyanin. *P. aeruginosa* strains carrying either a vector plasmid (tube 1), a plasmid containing *tacp-pqsE* (tube 2), or a plasmid carrying a complementing genetic allele (*pqsA* for strain PJF-QA1 or *pqsR* for strain PJF-QR1) (tube 3) were grown in LB medium for 18 h, and aliquots of cultures were photographed to document the production of the blue-green pigment pyocyanin. Strain designations: PAO1, wild type; PJF-QE1, *pqsE* mutant; PJF-QA1, *pqsA* mutant; PJF-QR1, *pqsR* mutant; PDO111, *rhlR* mutant; and PDO100, *rhlI* mutant.

Analysis of C_4-HSL production. To measure C_4-HSL production, culture supernatants from *P. aeruginosa* strains grown in LB medium for 18 h at 37°C with vigorous shaking were extracted with ethyl acetate as described elsewhere (44). The amount of C_4-HSL in ethyl acetate extracts was determined using the C_4-HSL bioassay (42).

β-Gal assays in *E. coli*. To test the ability of *pqsE* to affect the function of RhlR-C_4-HSL or LasR-3-oxo-C_{12}-HSL in *E. coli*, a two-plasmid system was utilized in which one plasmid carried either *tacp-rhlR* or *tacp-lasR* and a reporter gene fusion, and the second plasmid was either the vector plasmid or a plasmid carrying *tacp-pqsE*. Cells from overnight cultures of *E. coli* carrying the appropriate plasmids and grown in supplemented A medium were diluted to an OD_{600} of 0.08 in fresh medium. Subcultures were incubated at 37°C with vigorous shaking for 3 h, and then 1-ml aliquots were transferred to tubes that contained dried signal compounds. After 90 min of incubation at 37°C with vigorous shaking, the β-Gal activity produced by each culture was measured. Data are reported in Miller units as the mean ± SD of at least three independent experiments.

RESULTS

PqsE acts as a PQS-independent regulator. Since the discovery of the PQS synthetic gene cluster (10, 20), the function of PqsE has been a mystery. *pqsE* is the final gene of a five-gene operon, of which the first four genes are required for PQS synthesis (10, 20, 32). However, mutation of *pqsE* has no effect on PQS synthesis (20). Instead, *pqsE* appears to be necessary for *P. aeruginosa* to respond to PQS and to produce PQS-controlled virulence factors such as pyocyanin (20). In order to begin studying the role of PqsE, we first constructed isogenic, in-frame deletion mutants for *pqsE*, *pqsA*, and *pqsR*. As observed previously (20), the *pqsA* and *pqsR* mutants did not make PQS, and PQS production was not affected in the *pqsE* mutant (data not shown). Furthermore, none of the three mutant strains produced pyocyanin (Fig. 1 and Table 2), which was as expected from previous studies (20). Production of both pyocyanin and PQS was restored to the *pqsA* and *pqsR* mutant strains by genetic complementation with a plasmid carrying a

TABLE 2. Pyocyanin production

Strain	Pyocyanin production (μM) in strain carrying[a]:	
	pEX1.8	pZOE01
PAO1	42.6 ± 8.3	284.4 ± 19.3
PJF-QE1	ND	276.5 ± 18.2
PJF-QA1	ND	261.5 ± 29.7
PJF-QR1	ND	233.6 ± 44.3
PDO111	ND	ND
PDO100	ND	15.3 ± 6.5

[a] Data are the means ± SDs of results from at least three independent experiments. pEX1.8, control plasmid; pZOE01, PqsE overexpression plasmid. ND, not detectable. Strains PJF-QA1 and PJF-QR1 produced 34.3 ± 8.4 and 60.8 ± 5.6 μM pyocyanin, respectively, when complemented by plasmids containing a wild-type copy of the gene that had been mutated.

copy of each respective gene (Fig. 1 and Table 2; also data not shown). As a control, transfer of the vector plasmid into the strains being tested did not affect either pyocyanin or PQS production (data not shown). When we complemented the *pqsE* mutant with a plasmid carrying a *tacp-pqsE* fusion, pyocyanin production by the complemented strain greatly exceeded the level of the wild-type strain (Fig. 1 and Table 2). Interested by this result, we then transferred the *tacp-pqsE* plasmid into the wild type and the *pqsA* and *pqsR* mutant strains. Much to our surprise, we found that the overexpression of PqsE in these mutants stimulated overproduction of pyocyanin in all three strains (Fig. 1 and Table 2). This result was unexpected because all prior data showed that PqsR and PQS were required for pyocyanin production (5, 13, 14, 20). Our data indicated that PqsE was able to regulate pyocyanin production in the absence of both PQS and PqsR, implying that PqsE is an independent regulator.

Because pyocyanin production is also controlled by the other *P. aeruginosa* intercellular signaling systems (51), the *las* and *rhl* systems, we were interested in testing whether PqsE could act independently of these regulators as well. Therefore, we transferred the PqsE overexpression plasmid into various quorum-sensing mutant strains and observed the effects on pyocyanin production. In agreement with other reported observations (14, 22), the *lasR* and *lasI* mutants displayed delayed, but increased, production of pyocyanin (data not shown). The overexpression of PqsE in these strains did not alter this phenotype, nor was it able to restore pyocyanin to the *lasR rhlR* or *lasI rhlI* double mutants (data not shown), indicating that PqsE is unable to induce pyocyanin production in the absence of both the *las* and *rhl* quorum-sensing systems. Interestingly, the large positive effect of PqsE on pyocyanin production was abolished in an *rhlR* mutant containing the *pqsE* expression plasmid (Fig. 1 and Table 2). However, a slight positive effect on pyocyanin production was seen in an *rhlI* mutant carrying this same plasmid (Fig. 1 and Table 2). These data indicated that RhlR is necessary for PqsE to regulate pyocyanin production. This finding led us to speculate that PqsE might be functioning directly or indirectly through RhlR, which will be examined further below.

In addition to pyocyanin, numerous other virulence factors are known to be controlled by both PQS signaling and the *rhl* quorum-sensing system (1, 12, 14). To further assess the regulatory effects of PqsE, we examined the production of two other PQS- and *rhl*-controlled virulence factors, elastase and rhamnolipid. The results obtained were similar to those for pyocyanin production. Rhamnolipid production was decreased approximately 50% in the *pqsE*, *pqsA*, and *pqsR* mutants, and all three of these strains, along with the wild type, produced a much greater amount of rhamnolipid when they carried the *pqsE* expression plasmid (Fig. 2A). This effect did not occur in an *rhlR* mutant, which produced no rhamnolipid with or without the *pqsE* expression plasmid (Fig. 2A). Interestingly, rhamnolipid production was partly restored in an *rhlI* mutant that expressed PqsE (Fig. 2A), which is similar to what occurred for pyocyanin production (Fig. 1). A similar result was seen with elastase production assays. The *pqsE*, *pqsA*, and *pqsR* mutants all doubled their elastase production when they harbored the *pqsE* expression plasmid (Fig. 2B). It should be noted that under our growth conditions, elastase production in these three mutants was similar to that of the wild-type strain, which demonstrates that the *las* and *rhl* quorum-sensing systems are the dominant regulators of elastase production. In addition, as seen with pyocyanin and rhamnolipid production,

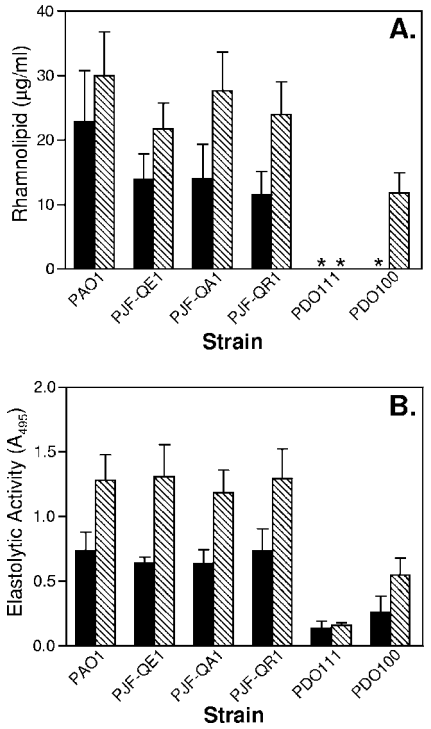

FIG. 2. The production of rhamnolipids and elastase by *P. aeruginosa* is increased in response to the overexpression of *pqsE*. *P. aeruginosa* strains carrying either a vector plasmid (black bars) or a plasmid containing *tacp-pqsE* (striped bars) were grown in LB medium for 18 h, and cells were removed from the medium by centrifugation and filtration. (A) The amount of rhamnolipid present in the cleared growth medium from each culture was determined using the orcinol assay as described in Materials and Methods. An asterisk indicates that the amount of rhamnolipid present was below the detectable limit for the assay. The data presented represent the mean ± SD of results from at least three independent experiments. (B) The elastolytic activity present in the growth medium from each culture was assayed using ECR as described in the Materials and Methods section. The data presented represent the mean ± SD of results from at least four individual experiments.

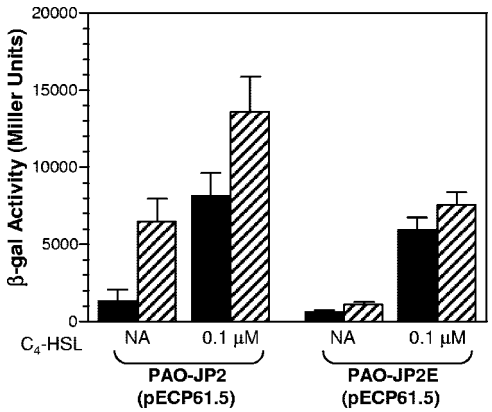

FIG. 3. The induction of *rhlA* by PQS and RhlR is dependent on *pqsE*. *P. aeruginosa* strains PAO-JP2 (*lasI rhlI*) and PAO-JP2E (*lasI rhlI pqsE*) carrying a plasmid harboring a *rhlA′-lacZ* fusion and *tacp-rhlR* were grown in PTSB medium supplemented with the indicated concentrations of C_4-HSL in the presence (hatched bars) or absence (black bars) of 20 μM PQS, which is within the range normally produced by wild-type cultures. After 18 h of incubation at 37°C, each culture was assayed for β-Gal activity. β-Gal activity is presented in Miller units as the mean ± SD of results from duplicate assays from at least three independent experiments.

the overexpression of PqsE in an *rhlR* mutant did not affect elastase production and caused only a slight increase in elastase activity from an *rhlI* mutant (Fig. 2B). Taken together, the data shown in Fig. 1 and 2 and in Table 2 indicated that PqsE can regulate multiple virulence factors independently of PqsR and PQS, but it cannot act independently of RhlR.

PqsE is required for PQS to activate the *rhl* quorum-sensing system. Since PqsE is involved in the complex intercellular signaling regulatory scheme of *P. aeruginosa*, we wanted to learn more about the effect of PqsE on PQS activity in the absence of both C_4-HSL and 3-oxo-C_{12}-HSL. To test this, we examined *rhlA′-lacZ* induction in a *P. aeruginosa lasI rhlI* double mutant that expressed *rhlR*, which is a system previously used as a bioassay for C_4-HSL activity (42). We also tested *rhlA′-lacZ* induction in strain PAO-JP2E, a derivative of strain PAO-JP2 (*lasI rhlI*) that also carries a mutation in *pqsE*. This system allowed us to assess the effects of PQS and C_4-HSL on the *rhl* system independently. (Note that we previously showed that strain PAO-JP2 does not produce PQS when grown under similar conditions due to the lack of a functional *las* system [3, 44].) These experiments showed that, as expected, *rhlA* was not expressed above a background level in the absence of C_4-HSL and 3-oxo-C_{12}-HSL in either strain (Fig. 3). Interestingly, the addition of exogenous PQS to strain PAO-JP2 caused a large increase in *rhlA* induction (Fig. 3), indicating that PQS was active in the absence of C_4-HSL and 3-oxo-C_{12}-HSL. Furthermore, adding PQS to strain PAO-JP2E that was expressing RhlR caused only a slight induction of *rhlA′-lacZ* (Fig. 3). While the induction of *rhlA* caused by PQS in strain PAO-JP2 was considerable, we also observed a similar induction in response to 0.1 μM C_4-HSL (Fig. 3), which is a concentration that is 200-fold lower than the tested concentration of PQS. When strain PAO-JP2 was supplemented with both PQS and C_4-HSL, there was an increase in *rhlA′-lacZ* activity beyond that caused by either signal alone (Fig. 3). However, in strain PAO-JP2E the activity produced by the combination of the signals was equivalent to that of C_4-HSL alone (Fig. 3). These data showed that PQS was able to induce *rhlA* expression in *P. aeruginosa* in the presence of RhlR and in the absence of acyl-HSL signals and that this effect required PqsE. Overall, our data suggested that a factor(s) that positively regulated *rhlA* was activated by both PQS and C_4-HSL and that this unknown regulator was also dependent on PqsE.

Exogenous C_4-HSL can overcome a mutation in *pqsE*. While our results showed that PqsE required the presence of RhlR to regulate multiple virulence factors, it has been clearly shown that RhlR and C_4-HSL are able to initiate gene expression in the absence of PqsE (42). Interestingly, we found that multiple *rhl*-controlled factors, such as rhamnolipid and pyocyanin, are downregulated in a *pqsE* mutant despite the fact that the mutant produced an amount of C_4-HSL equivalent to that produced by the wild-type strain (data not shown), a finding also reported by others (14). This led us to investigate whether a *P. aeruginosa pqsE* mutant was still capable of responding to C_4-HSL. In our first experiment, we cultured the *pqsE* mutant in the presence of excess C_4-HSL and observed pyocyanin production. We found that exogenous C_4-HSL restored pyocyanin production to a *pqsE* mutant but that relatively large amounts of C_4-HSL were required to restore pyocyanin production to a level that was approximately 60% of that seen from the wild-type strain (Fig. 4A). We continued this line of experiments and examined the expression of an *rhlA′-lacZ* fusion in the *pqsE* mutant. As expected from our earlier data (Fig. 2), *rhlA* expression was decreased approximately 60% in the *pqsE* mutant (Fig. 4B). The addition of exogenous C_4-HSL caused *rhlA′-lacZ* induction to increase in a dose-dependent manner, but restoration to a wild-type level of expression required greater than 100 μM C_4-HSL (Fig. 4B) (Note that this strain already produces a wild-type level of C_4-HSL, which is approximately 10 to 20 μM [41; also data not shown].) To ensure that *rhlR* was still expressed, we analyzed *rhlR* transcription in strain PJF-QE1(pPCS1002) and found that only a minor decrease of 23% (from 158,118 ± 7,527 to 121,763 ± 10,450 Miller units of β-Gal activity) occurred in *rhlR′-lacZ* expression relative to that in the wild-type strain PAO1(pPCS1002). Taken together, these results demonstrated that a *pqsE* mutant is less sensitive to C_4-HSL but that it can respond to C_4-HSL, suggesting that RhlR is active in a *pqsE* mutant. The data also imply that PqsE may play a role in the ability of RhlR to respond to C_4-HSL.

PqsE affects the ability of RhlR to activate gene expression in a heterologous host. So far, our data have shown that factors controlled by the *rhl* system are overexpressed when *pqsE* is overexpressed (Fig. 1 and 2) and underexpressed when *pqsE* is absent (Fig. 4). This suggested that PqsE may have a role in the function of the *rhl* quorum-sensing system. Since the quorum-sensing circuitry of *P. aeruginosa* is complex and is modified by a wide array of factors, we wanted to try to establish a more direct link between PqsE and the *rhl* quorum-sensing system. To do this, we determined whether the effect of PqsE could be seen in *E. coli* cells by introducing two plasmids into *E. coli*. One contained *tacp-pqsE*, and the other contained *tacp-rhlR* and either *rhlA′-lacZ* or *lasB′-lacZ*. When these *E. coli* strains were grown in the presence of isopropyl-β-D-thiogalactopyranoside but with no exogenous signals, the strain containing the *pqsE* vector showed no increase in *rhlA* expression and was

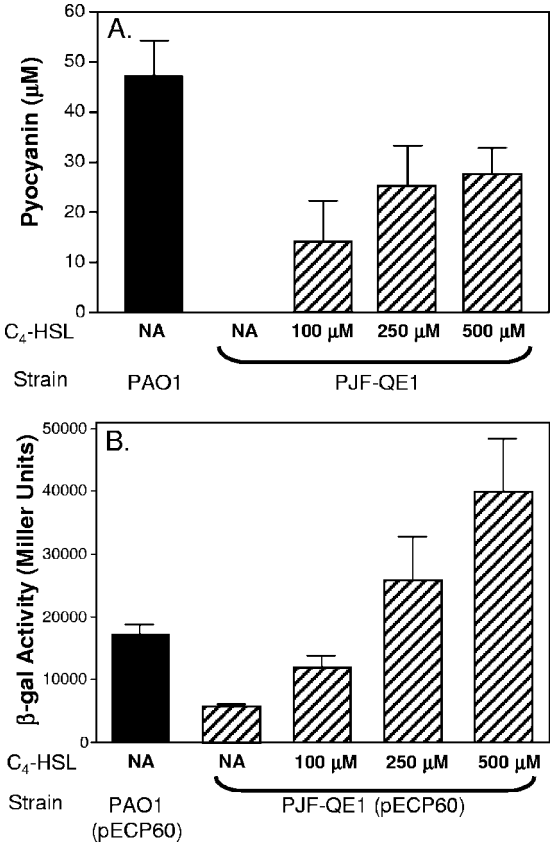

FIG. 4. Pyocyanin production and *rhlA* expression are restored to a *pqsE* mutant by the addition of exogenous C_4-HSL. (A) The wild-type (PAO1) and *pqsE* mutant (PJF-QE1) strains of *P. aeruginosa* were grown for 18 h in LB medium supplemented with the indicated concentrations of C_4-HSL. Pyocyanin was quantified as described in Materials and Methods, and data represent the average ± SD of at least three separate experiments. (B) The wild-type strain PAO1 (black bar) and *pqsE* mutant strain PJF-QE1 (striped bars) carrying a *rhlA′-lacZ* fusion on pECP60 were grown for 18 h in LB medium supplemented with the indicated concentrations of C_4-HSL. β-Gal activity produced in each culture was then assayed and is presented in Miller units as the mean ± SD of results from duplicate assays from at least three separate experiments. In both panels, the numbers below the bars represent the concentration of C_4-HSL. NA, no addition.

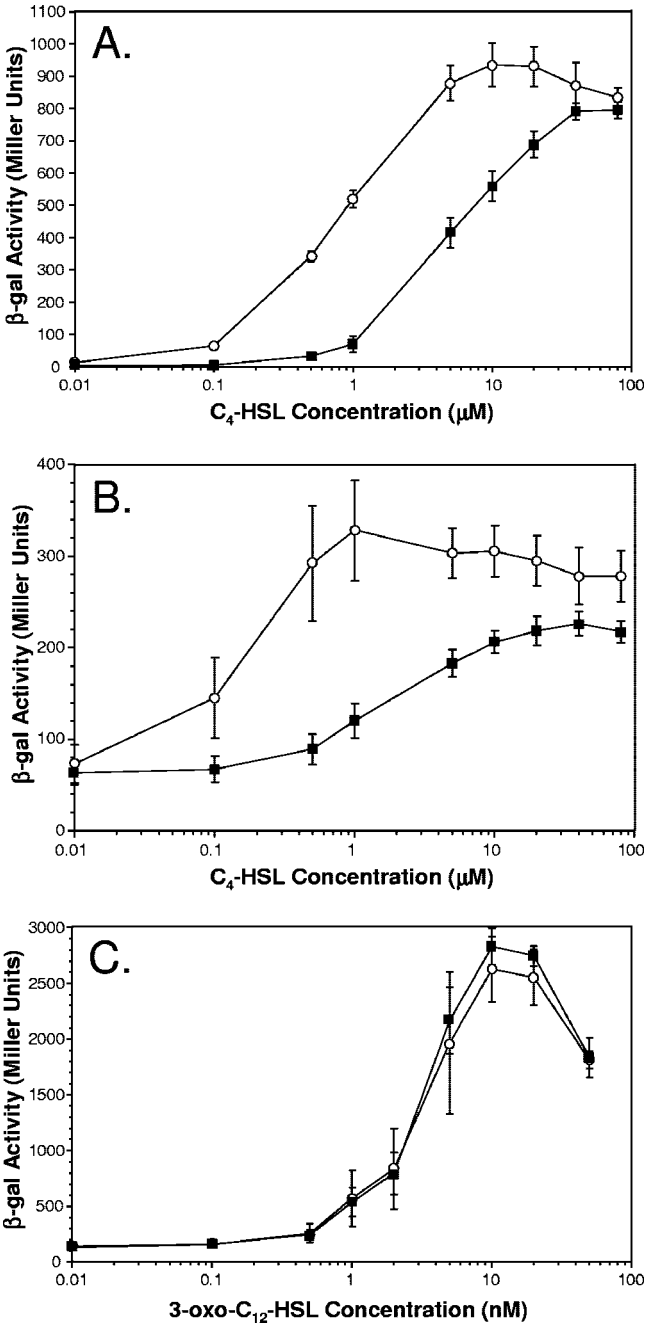

FIG. 5. PqsE enhances C_4-HSL activity in *E. coli*. *E. coli* strain DH5α carried either a vector plasmid (filled squares) or a plasmid harboring *tacp-pqsE* (open circles) and either a plasmid harboring *tacp-rhlR* and *rhlA′-lacZ* (A), a plasmid harboring *tacp-rhlR* and *lasB′-lacZ* (B), or a plasmid harboring *tacp-lasR* and *lasB′-lacZ* (C). Strains were cultured in the presence of exogenous signals as described in the Materials and Methods section, and β-Gal activity was measured. β-Gal activity is presented in Miller units as the mean ± SD of results from duplicate assays from at least three independent experiments.

identical to that seen from a strain containing a control vector (Fig. 5A). Most interestingly, as increasing amounts of C_4-HSL were added to the cultures, the strain that contained *pqsE* exhibited a much more sensitive response to C_4-HSL (Fig. 5A). This effect also occurred with another PQS- and C_4-HSL-controlled gene when *lasB′-lacZ* was substituted for *rhlA′-lacZ*. We found that RhlR was able to induce *lasB′-lacZ* at a much lower concentration of C_4-HSL when PqsE was present (Fig. 5B). In both strains that contained *pqsE*, the expression of the reporter gene (*rhlA* or *lasB*) in response to exogenous C_4-HSL peaked and then leveled off at an induction level similar to that seen from the strain containing the control vector (Fig. 5A and B). This enhanced responsiveness to exogenous signal was not seen when we tested the ability of PqsE to affect *lasB′-lacZ* induction by LasR and 3-oxo-C_{12}-HSL (Fig. 5C). In this case, both the control vector and *pqsE* vector strains expressed *lasB* at the same levels as the concentration of 3-oxo-C_{12}-HSL increased. Overall, these data showed that PqsE can enhance the ability of RhlR and C_4-HSL to activate gene expression in the absence of other *P. aeruginosa*-encoded factors, and this effect appeared to be specific for the *rhl* quorum-sensing system.

DISCUSSION

In this study we have begun to clarify the role of PqsE in the *P. aeruginosa* quorum-sensing circuitry. Our previous findings showed that *pqsE* is not necessary for the synthesis of PQS but that *pqsE* is required for the expression of virulence factors controlled by PQS (20). This led us to speculate that *pqsE* was involved in the cellular response to PQS (20). In our current study, we used a *pqsE* overexpression plasmid to show that in the absence of the PQS-responsive transcriptional activator PqsR, PqsE can positively regulate expression of the virulence factors pyocyanin, rhamnolipid, and elastase (Fig. 1 and 2 and Table 2). We also showed the same positive effects in a *pqsA* mutant, demonstrating that the regulatory activity of PqsE occurs independently of PQS or any of the other quinolone compounds produced through the action of the enzymes encoded by the *pqsABCDE* operon. These data suggested a model in which PQS and PqsR controlled the expression of *pqsE* and PqsE, in turn, regulated a second set of genes. This indirect regulation is different from that seen in the acyl-HSL-based signaling systems of *P. aeruginosa*, where LasR and RhlR each bind their cognate signals and directly induce the expression of multiple targets. Both PQS and PqsR have been shown to regulate a large number of genes (1, 12), but these data must now be reexamined to determine which genes are directly regulated by PqsR-PQS and which are controlled indirectly through the independent action of PqsE.

As our studies expanded, they led us away from the idea that *pqsE* is involved in the cell's response to PQS and instead pointed us toward investigating the role of *pqsE* in the *rhl* quorum-sensing system. Several previous studies have noted a significant overlap between PQS- and *rhl*-controlled genes (12, 14), but the relationship between these two systems has been unclear. Our experiments to study the effects of *pqsE* expression in various *P. aeruginosa* quorum-sensing mutants showed that the regulatory function of PqsE required the presence of RhlR (Fig. 1 and 2). In addition, we had previously shown that PQS and C_4-HSL could cooperatively induce gene expression in the presence of RhlR (33). Therefore, we examined the involvement of PqsE in the interaction between the PQS and *rhl* signaling systems. Our results showed that PQS itself or in combination with C_4-HSL could activate an *rhlA'-lacZ* fusion in the presence of RhlR (Fig. 3). However, these effects were observed only when a functional copy of *pqsE* was present, thereby providing further evidence that PQS indirectly regulates gene expression by controlling the expression of *pqsE* and establishing PqsE as a link between the quinolone and *rhl* signaling systems.

While these findings provided insight into the relationship between PQS and the *rhl* quorum-sensing system, it was still unclear as to why *pqsE* was required for the full production of numerous *rhl*-controlled virulence factors. This was especially puzzling since *pqsE* does not affect C_4-HSL production (14; also data not shown). When we tested the ability of a *pqsE* mutant to respond to additional exogenous C_4-HSL, we found that a great excess of C_4-HSL restored the induction of *rhl*-controlled virulence factors (Fig. 4). This suggested that the *rhl* quorum-sensing system was still capable of functioning in a *pqsE* mutant but that the regulatory function of PqsE was necessary for the full induction of some genes. We then tested the effects of PqsE on the function of the *rhl* system in *E. coli* and found that the presence of PqsE enhanced the ability of RhlR and C_4-HSL to induce gene expression (Fig. 5). This effect appeared to be specific for the *rhl* system since PqsE did not enhance the induction of *lasB* by LasR and 3-oxo-C_{12}-HSL (Fig. 5). Taken together, our findings suggested that PqsE played a role in the *rhl* quorum-sensing system and that PQS was linked to this through its control of *pqsE*.

Overall, these findings begin to sort out some of the confusion regarding the interactions between the quinolone and *rhl* signaling systems. Previous data showed that PQS was important for the induction of numerous *rhl*-controlled factors and also suggested that PQS can upregulate *rhlR* and *rhlI* themselves (12, 14, 20, 26, 32, 33). Our current results show that PqsE affects the activity of the *rhl* signaling system but does not significantly affect the production of C_4-HSL or the transcription of *rhlR*, implying that the role of PqsE is not to control the components of the *rhl* system. These findings suggest that the induction of *rhlR* and *rhlI* observed in response to PQS by ourselves and others was not through the induction of *pqsE* but may instead be the result of some other activity of PQS and that PQS may influence the *rhl* system in multiple ways. It has also been shown that RhlR and C_4-HSL can negatively impact the production of PQS and other quinolones by influencing the transcription of the *pqsABCDE* operon (32, 59). Our findings showed that PqsE could enhance the ability of RhlR and C_4-HSL to positively regulate gene expression but do not indicate whether it also enhances the negative regulatory function of the *rhl* system as well, although others have reported that a *P. aeruginosa pqsE* mutant and the wild-type strain produced similar amounts of 4-quinolone compounds (13).

The exact mechanism by which PqsE affects the *rhl* quorum-sensing system and achieves its regulatory function remains a mystery. However, our data suggested that PqsE may alter the function of RhlR rather than affecting C_4-HSL. This is supported by our observation that PqsE had some regulatory activity in an *rhlI* mutant but none in an *rhlR* mutant (Fig. 1 and 2). We also saw *pqsE*-dependent regulatory activity in the absence of any acyl-HSL signals (Fig. 3), implying that PqsE may act on the *rhl* quorum-sensing system through a novel form of regulation. It is also interesting that Deziel et al. (12) found that a *pqsE* mutant, but not an *rhlR* mutant, had attenuated virulence in a burned mouse model of infection, suggesting that the influence of *pqsE* may extend beyond the *rhl* system. While the studies presented here do not determine the molecular mechanism through which PqsE acts, they provide a set of interesting data on which to build a model that will explain PqsE's role in the complex scheme that comprises *P. aeruginosa* intercellular signaling.

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