CysB Negatively Affects the Transcription of *pqsR* and *Pseudomonas* Quinolone Signal Production in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative bacterium that is ubiquitous in the environment, and it is an opportunistic pathogen that can infect a variety of hosts, including humans. During the process of infection, *P. aeruginosa* coordinates the expression of numerous virulence factors through the production of multiple cell-to-cell signaling molecules. The production of these signaling molecules is linked through a regulatory network, with the signal *N*-(3-oxododecanoyl) homoserine lactone and its receptor LasR controlling the induction of a second acyl-homoserine lactone signal and the *Pseudomonas* quinolone signal (PQS). LasR-mediated control of PQS occurs partly by activating the transcription of *pqsR*, a gene that encodes the PQS receptor and is necessary for PQS production. We show that LasR interacts with a single binding site in the *pqsR* promoter region and that it does not influence the transcription of the divergently transcribed gene, *nadA*. Using DNA affinity chromatography, we identified additional proteins that interact with the *pqsR*-*nadA* intergenic region. These include the H-NS family members MvaT and MvaU, and CysB, a transcriptional regulator that controls sulfur uptake and cysteine biosynthesis. We show that CysB interacts with the *pqsR* promoter and that CysB represses *pqsR* transcription and PQS production. Additionally, we provide evidence that CysB can interfere with the activation of *pqsR* transcription by LasR. However, as seen with other CysB-regulated genes, *pqsR* expression was not differentially regulated in response to cysteine levels. These findings demonstrate a novel role for CysB in influencing cell-to-cell signal production by *P. aeruginosa*.

IMPORTANCE

The production of PQS and other 4-hydroxy-2-alkylquinolone (HAQs) compounds is a key component of the *P. aeruginosa* cell-to-cell signaling network, impacts multiple physiological functions, and is required for virulence. PqsR directly regulates the genes necessary for HAQ production, but little is known about the regulation of *pqsR*. We identified CysB as a novel regulator of *pqsR* and PQS production, but, unlike other CysB-controlled genes, it does not appear to regulate *pqsR* in response to cysteine. This implies that CysB functions as both a cysteine-responsive and cysteine-unresponsive regulator in *P. aeruginosa*.

Pseudomonas aeruginosa is a Gram-negative bacterium that is ubiquitous in the environment, due in part to its metabolic versatility that allows it to obtain the nutrients needed for growth from a wide variety of compounds (1). *P. aeruginosa* is also an opportunistic pathogen that can infect plants, nematodes, insects, and mammals (2–5). With regard to humans, it can cause a wide range of diseases and is a major cause of health care-associated illnesses and devastating chronic infections in cystic fibrosis patients (6, 7). In order to thrive under such a diverse range of conditions, *P. aeruginosa* utilizes complex regulatory networks to control gene expression. These networks include global regulatory systems that can direct large-scale changes in gene expression, as well as many systems that allow fine-tuning of gene expression in response to specific environmental stimuli. During infection, *P. aeruginosa* coordinates the expression of many virulence factors through the production of cell-to-cell signaling molecules. There are three main cell-to-cell signaling systems that together are estimated to control the expression of up to 12% of the genes in the *P. aeruginosa* genome (2, 8, 9). The *las* and *rhl* systems, which, respectively, function through the signaling molecules *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C₁₂-HSL) and *N*-butyryl homoserine lactone, and the regulators LasR and RhlR, are similar to homoserine lactone-based systems found in other Gram-negative bacteria. The third system functions primarily through the signaling molecule 2-heptyl-3-hydroxy-4-quinolone, also referred to as the *Pseudomonas* quinolone signal (PQS) (10). These

three systems are linked in a network, with the *las* system positively regulating the components of the PQS and *rhl* systems (10–12). Additionally, the PQS system positively regulates the components of the *rhl* system, while the *rhl* system can negatively impact PQS production (13–17). In spite of all this signaling cross talk, each system can also function independently. In the absence of LasR, the *rhl* system becomes active and can regulate some *las*-controlled functions (18). PQS is also produced in the absence of a functional *las* system (14), and, unlike the other two signals, PQS is not produced maximally until the stationary phase of growth (13). Furthermore, each signaling system can also be affected by environmental conditions, such as the availability of nutrients like

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or source
<i>E. coli</i> DH5 α	λ^- ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U196 <i>recA1 endA1 hsdR17</i> (r_K^- m_K^-) <i>supE44 thi-1 gyrA relA1</i>	66
<i>P. aeruginosa</i> strains		
PAO1	Wild type	67
PJF-MT1	<i>mvaT</i> deletion mutant derived from strain PAO1	This study
PJF-MU1	<i>mvaU</i> deletion mutant derived from strain PAO1	This study
PJF-CB1	<i>cysB</i> deletion mutant derived from strain PAO1	This study
PAO1-P <i>pqsRtc</i>	PAO1 harboring chromosomal <i>pqsR'</i> - <i>lacZ</i>	This study
PJF-CB1-P <i>pqsRtc</i>	PJF-CB1 harboring chromosomal <i>pqsR'</i> - <i>lacZ</i>	This study
Plasmids		
pECP8	<i>ptac-lasR</i> , Amp ^r	30
pLP170	<i>lacZ</i> transcriptional fusion vector	68
pJF-NadA2	<i>nadA'</i> - <i>lacZ</i> transcriptional fusion	This study
pJF-NadA2-LasRmut	<i>nadA'</i> - <i>lacZ</i> fusion with mutated LasR binding site	This study
pMWC1003	<i>pqsR'</i> - <i>lacZ</i> transcriptional fusion	30
pMWC1003-LasRmut	<i>pqsR'</i> - <i>lacZ</i> fusion with mutated LasR binding site	This study
pEX18Ap	Suicide vector for <i>P. aeruginosa</i>	69
p Δ mvaT-suc	<i>mvaT</i> deletion suicide vector	This study
p Δ mvaU-suc	<i>mvaU</i> deletion suicide vector	This study
p Δ cysB-suc	<i>cysB</i> deletion suicide vector	This study
pHERD20T	<i>E. coli</i> - <i>P. aeruginosa</i> expression vector	57
pJF60	P _{BAD} - <i>cysB</i> on pHERD20T	This study
pJF-Tn7T- <i>pqsR'</i> Tc	<i>pqsR'</i> - <i>lacZ</i> transcriptional fusion plasmid for chromosomal integration	70
pJF-Tn7T- <i>cysH'</i> Tc	<i>cysH'</i> - <i>lacZ</i> transcriptional fusion plasmid for chromosomal integration	This study
pJF1003	<i>pqsR'</i> - <i>lacZ tacp-lasR</i>	This study
pACYC184	Cloning vector for <i>E. coli</i>	71
pACYC-P _{BAD}	<i>araC</i> , P _{BAD}	This study
pJF52	<i>araC</i> , P _{BAD} - <i>cysB</i>	This study

iron, phosphate, and certain amino acids (16, 19–22). The interplay between these systems allows *P. aeruginosa* to integrate information from multiple signals and alter gene expression accordingly.

The quinolone signaling system is more complex than either the *las* or *rhl* systems. PQS is synthesized by enzymes encoded in the *pqsABCDE* and *pqsH* operons from the precursors anthranilate, malonyl coenzyme A (malonyl-CoA), and octanoyl-CoA (23–25). The *pqsABCD* genes are also important for the synthesis of 55 other quinolone compounds and the metabolites 2,4-dihydroxyquinoline and 2-aminoacetophenone (25–28). Expression of the *pqsABCDE* operon is controlled by the LysR-type regulator PqsR (also known as MvfR), which is required for virulence (5, 29–31). PQS acts as a coinducer with PqsR to positively regulate *pqsABCDE* expression, forming a positive autoregulatory feedback loop for quinolone synthesis. The direct precursor of PQS, 2-heptyl-4-quinolone, can also act as a coinducer with PqsR but with 100-fold lower activity (31). Transcription of the *pqsABCDE* operon leads to the expression of PqsE, which is not required for PQS biosynthesis but instead independently regulates the expression of a majority of the genes controlled by PQS (5, 17, 32, 33). PqsE is required for virulence, and its function is not known; but it appears to work in conjunction with the *rhl* signaling system to influence gene expression (2, 17, 32, 33). In addition to its role as a signaling molecule, PQS and other 4-hydroxy-2-alkylquinolones produced by *P. aeruginosa* have been linked to other biological activities. These include roles in iron metabolism, redox cycling, membrane vesicle formation, interspecies interactions, and antimicrobial activity (34–36). These findings show that quinolone

production has a major impact on *P. aeruginosa* physiology that extends beyond the ability of this organism to cause disease.

We became interested in the regulation of *pqsR* because it directly regulates the *pqsABCDE* operon. The *pqsR* promoter sequence is part of a large intergenic region, with 747 bp separating the coding regions of *pqsR* and the divergently transcribed *nadA* gene. Two transcriptional start sites were identified for *pqsR*, located at bp –190 and –278 relative to the *pqsR* translational start site (TS2 and TS1, respectively) (30). Additionally there is a small open reading frame located upstream from the *pqsR* coding sequence (*uof*) (see Fig. 1A). The function of the peptide encoded by *uof* is not known, but the translation of *uof* is coupled to the translation of *pqsR* (37). It has been shown that the transcription of *pqsR* is positively controlled by LasR and 3-oxo-C₁₂-HSL, which also promotes PQS production by inducing the expression of *pqsH* (9, 38). Previous studies identified a potential consensus sequence for LasR binding in the *pqsR* promoter region and showed that this sequence was important for *pqsR* expression, but they did not fully investigate the interactions of LasR with the *pqsR* promoter (30, 39). In this study, we directly examined the role of LasR in controlling *pqsR* transcription and identified an additional factor that impacts *pqsR* transcription. We report a novel role for CysB, a master regulator of sulfur uptake (40), in controlling *pqsR* expression and found that it can counteract the positive regulatory influence of LasR.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were freshly plated to begin each

experiment. Bacteria were cultured in either lysogeny broth (LB; Lennox formulation) or tryptic soy broth (TSB; Becton Dickinson) as indicated in the text. For experiments to investigate the effects of various sulfur sources, bacteria were grown in a modified form of the sulfur-free salts medium (MSFM) described by Kertesz et al. (41). This medium contained 50 mM Tris, pH 7.3, 25 mM sodium succinate, 20 mM NH_4Cl , 0.5 mM potassium phosphate, 0.5 mM MgCl_2 , 2 μM FeCl_3 , 1 μM MnCl_2 , 1 μM CaCl_2 , 1 μM ZnCl_2 , and 50 $\mu\text{g}/\text{ml}$ of each proteinogenic amino acid except for cysteine and methionine. This medium was supplemented with specific sulfur sources as indicated in the text. Unless indicated otherwise, cultures were incubated at 37°C with shaking at 260 to 280 rpm. When necessary for selection or to maintain plasmids, cultures were supplemented with 200 $\mu\text{g}/\text{ml}$ carbenicillin and/or 30 $\mu\text{g}/\text{ml}$ gentamicin for *P. aeruginosa* and with 100 $\mu\text{g}/\text{ml}$ ampicillin and/or 10 $\mu\text{g}/\text{ml}$ tetracycline for *Escherichia coli*.

Purification of LasR. LasR was purified using the procedure described by Schuster et al. (42). *E. coli* strain DH5 α carrying LasR expression plasmid pECP8 was grown overnight in LB medium with ampicillin. Cells were subcultured in 1 liter of prewarmed LB containing 50 mM morpholinepropanesulfonic acid (MOPS), pH 7, 100 $\mu\text{g}/\text{ml}$ ampicillin, and 5 μM 3-oxo- C_{12} -HSL to an optical density at 600 nm (OD_{600}) of 0.08, and the culture was incubated at 37°C. When the OD_{600} reached 0.05, the culture was chilled to 20°C, supplemented with isopropyl- β -D-thiogalactoside (IPTG) to 0.4 mM, and incubated at 17°C overnight. Cells were then harvested by centrifugation and stored at -70°C. For protein purification, cells from 500 ml of culture were thawed and suspended in 8.5 ml of LasR purification buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM EDTA, 10% glycerol, and 0.05% Tween 20). Cells were disrupted by passing the suspension through a French pressure cell at 16,000 lb/in². The remaining steps in the purification procedure were carried out exactly as described by Schuster et al. (42). Fractions containing LasR were >95% pure as judged by SDS-PAGE. Protein concentrations were determined by the Bradford assay using reagents from Bio-Rad.

EMSA and DNase I protection assays with LasR. Electrophoretic mobility shift assays (EMSA) and DNase I protection assays were performed essentially as described by Schuster et al. (42). DNA probes for EMSA were amplified by PCR using primers listed in Table 2. DNA fragments were purified from an agarose gel and labeled on both ends using [γ -³²P]ATP (PerkinElmer) and T4 polynucleotide kinase. DNA binding reactions were carried out in a buffer containing 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 100 $\mu\text{g}/\text{ml}$ bovine serum albumin (BSA), 15 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA, and 5 μM 3-oxo- C_{12} -HSL. For binding reactions, approximately 0.06 to 0.12 fmol of radiolabeled DNA probe was incubated either with or without the addition of 0.27 pmol of purified LasR in a total volume of 20 μl for 20 min at room temperature. Aliquots of binding reaction mixtures were then electrophoresed on nondenaturing 5% acrylamide gels in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer. Gels were visualized by autoradiography.

To generate probes for DNase I protection assays, oligonucleotide primers PQSR-PRF4 and PQSR-PRR4 (Table 2) were labeled on the 5' end using [γ -³²P]ATP and T4 polynucleotide kinase. A single radiolabeled primer was used in PCRs to generate DNA fragments that were labeled on a single strand, and the resulting fragments were gel purified. Approximately 0.2 pmol of each fragment was incubated with 10 pmol of LasR and 5 μM 3-oxo- C_{12} -HSL in the same buffer used for EMSA but in a final volume of 120 μl . Reaction mixtures were incubated for 20 min at room temperature; then 0.24 U of RQ1 DNase (diluted to 0.04 U/ μl in 25 mM Tris-HCl, pH 7.6, 30 mM MgCl_2 , 30 mM CaCl_2 ; Promega) was added, and reaction mixtures were incubated for another 8 min. Next, 25 μl of stop solution (3 M ammonium acetate, 0.25 M EDTA, 15 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA) was added to each reaction mixture, and then DNA was precipitated by adding 370 μl of ethanol. DNA sequencing ladders were generated with radiolabeled primers PQSR-PRF4 and PQSR-PRR4 using a Thermo Sequenase cycle sequencing kit (USB/Affymetrix). Cycle sequencing reactions were performed using dideoxy-

nucleoside triphosphate (ddNTP) termination mixes that contained dGTP instead of 7-deaza-dGTP. DNase I digestion products and sequencing ladders were separated on denaturing 8% polyacrylamide gels and visualized by autoradiography.

Primer extension analysis. To isolate RNA for primer extension analyses, *P. aeruginosa* strain PAO1 was subcultured in LB medium to an OD_{660} of 0.05 and incubated at 37°C for 3 h. Cells were harvested by centrifugation and treated with RNAlprotect bacteria reagent (Qiagen). Cells were then incubated in lysis buffer (30 mM Tris, pH 8, 1 mM EDTA, 10 mg/ml lysozyme, 2 mg/ml proteinase K) for 5 min at room temperature, and RNA was isolated using TRIzol reagent (Life Technologies). For extension reactions, oligonucleotide primers were labeled on the 5' end using [γ -³²P]ATP and T4 polynucleotide kinase. Approximately 160 fmol of radiolabeled primer was mixed with 80 μg of RNA in 20 μl of a buffer containing 167 mM HEPES, pH 7.5, 1 M NaCl, and 0.33 mM EDTA. Mixtures were heated to 95°C for 1 min and then incubated at 50°C for 30 min. Next, nucleic acids were collected by ethanol precipitation and suspended in diethyl pyrocarbonate (DEPC)-treated water. The entire volume was used for reverse transcription reactions using SuperScript III reverse transcriptase (Life Technologies). Each reaction mixture contained 1 $\times$ first-strand buffer, 5 mM DTT, 5% dimethyl sulfoxide (DMSO), a 0.5 mM concentration of each deoxynucleoside triphosphate (dNTP), 2 U/ μl RNase OUT (Life Technologies), and 125 U of SuperScript III reverse transcriptase in a total volume of 25 μl . Reaction mixtures were incubated at 55°C for 90 min. Then, nucleic acids were isolated by extraction with 1:1 phenol-chloroform, followed by ethanol precipitation. DNA sequencing ladders were generated with the same radiolabeled oligonucleotides used for extension reactions, using a Thermo Sequenase cycle sequencing kit as described for DNase I protection assays. Extension products and sequencing ladders were separated on denaturing 8% polyacrylamide gels and visualized by autoradiography.

Construction of *nadA'*-*lacZ* fusions and β -galactosidase (β -Gal) assays. To generate an *nadA'*-*lacZ* reporter plasmid, a 738-bp fragment from +78 to -661 relative to the *nadA* translational start site was amplified by PCR. For this amplification, the oligonucleotide primers contained XbaI and HindIII restriction sites, and strain PAO1 chromosomal DNA was used as a template. The amplified fragment was digested with XbaI and HindIII, purified from an agarose gel, and ligated into pLP170 digested with the same enzymes to produce reporter plasmid pJF-NadA2. To construct a reporter plasmid that contained mutations in the LasR binding site, plasmids pJF-NadA2 and pMWC1003 were each amplified by PCR with oligonucleotide primers PQSRP mutLasR FWD and PQSRP mutLasR REV. These primers contained alterations from the original nucleotide sequence to generate the desired mutations. The amplified fragments were purified from an agarose gel and ligated to produce reporter plasmids pJF-NadA2-LasRmut and pMWC1003-LasRmut. Plasmids were transformed into *P. aeruginosa* by electroporation.

To assay for β -Gal activity, washed cells from overnight cultures were used to inoculate 10-ml subcultures of LB supplemented with carbenicillin to an OD_{660} of 0.05. Cultures were incubated at 37°C for 6 h, and then aliquots of cultures were diluted 1:10 in fresh LB medium and assayed for β -Gal activity in duplicate (43).

DNA affinity chromatography. DNA affinity chromatography experiments were based on the procedure of Imperi et al. (44) with some modifications. A DNA fragment corresponding to -177 to -570 relative to the *pqsR* transcriptional start site was amplified by PCR using oligonucleotide primers PQSR PRR10B, which was labeled with biotin at the 5' end, and PQSR PRF1. The amplified fragment was purified using a QIAquick PCR purification kit (Qiagen). The purified, biotin-labeled fragment was coupled to Dynabeads M-280 Streptavidin (Life Technologies) as directed by the manufacturer. To prepare cell lysates, washed cells from an overnight culture of *P. aeruginosa* strain PAO1 grown in LB medium were used to inoculate cultures of fresh LB medium to an OD_{660} of 0.05. Cultures were incubated at 37°C for either 2.5 h or 8 h, and then cells from 200 to 300 ml of culture were harvested by centrifugation and stored at -70°C.

TABLE 2 Primers and probes used in this study

Primer or probe function and name	Sequence (5'–3') ^a
EMSA and DNase I protection	
PQSR-PRR1	AAGCGCAAGGGCTGGCGT
PQSR-PRF2	CCGTTCTGGCTCGGCTC
PQSR-PRR4	ACCAAGGGCCTAATACCAAA
PQSR-PRF4	ACTGACGATTGCAGGTTTCG
PQSR-F52	CCGGTAAATATCCGGATT
P cysH F	GCGGGAATGGTAGCAAAGG
P cysH R	CATCAGCACGTCGTAGTCCG
lasIP-F	TTTTGGGGCTGTGTTCTCTC
lasIP-R	CACTTGAGCACGCAACTTGT
kynA promoter fwd	GAGTGAGGGCAAGGACACAT
kynA promoter rev	CGCGAGTGATCCGAAATTCG
Primer extension analysis	
NadA P EXT2	TCTCCTGCTCGCTCAACACACGG
PQSR-PRF4	ACTGACGATTGCAGGTTTCG
Cloning <i>lacZ</i> fusions and mutational analysis	
NadA-F2	AAAAAAGCTTTAGATAGCGCCGAGTGTTT
NadA-R1	AAAAATCTAGACTCCTGCTCGCTCAACAC
PQSRP mutLasR FWD	AG AACAAAAGTCATTAGTTTCGGTCGTTAGACCTACGCGAC
PQSRP mutLasR REV	CGTAGCCACCGGCCAGGCC
LasR tac F	AAAA GAATTCT TAAATCACTGCATAATTCGTGTGC
LasR tac R	GCGATCTGTCTATTTTCGTTTCATCC
CYSH UP	AAAA CTGC AGAGAGAATCACCACCTGGAAGCG
CYSH DOWN	AAAAAAGCTTTCGCGGAATGGTAGCAAAGG
DNA affinity chromatography	
PQSR PRR10B	GTGCGAGGGTGGAATCGG
PQSR PRF1	ATCCCGGCATCCGTACCG
Generation of mutant strains	
MVAT P1.2	AAAA CTGCAGT CGGTACGTAATGGCTGGTCGC
MVAT P2	GGAACTGACTGGTTTAGCCGAGCAGGCGATATTCGTTGATCAGGGACATGTCAGG
MVAT P3	CCTGACATGTCCCTGATCAACGAATATCGCTGCTCGGCTAAACCAGTCAGTTCC
MVAT P4	AAAA CTGCAG CCGCTGGTGATGTC
MVAT SEQ1	CGCGGTTTACTTACAGTTTCGC
MVAT SEQ2	CTATTCGCTGGAGACTTGA
MVAU P1	AAAA CTGC AGAACGACCGGTCACTCGTTGCG
MVAU P2	GGCTTAGCGTTGCAGCCAGGATTGGAACCTCGCAAGTTTGACATTGC
MVAU P3	CGAATGTCCAAACTTGCCGAGTTGGAATCCTGGCTGCAACGCTAAGCC
MVAU P4	AAAA CTGCAGT TCGAGAAGCAAGGGACGATCGC
MVAU SEQ1	ACTTATCCTTTGCCGCGGACAC
MVAU SEQ2	GTGCGAGAATTGATCTGGCGAGG
CYSB P1	TTTTGGATCCCTATGCGGACCAACTGCTG
CYSB P2	GATCAGTAGACCGGCAGTTTCGATAACCTCCAGATATAGCGC
CYSB P3	GCGCTATATCTGGGAGGTTATCGAACTGCCGGTCTACTGATC
CYSB P4	TTTTGGATCCCTATCGTTTCATCGACCAGGTCC
CYSB SEQ2	CAATCTCGACATGCTCTGAGGG
CYSB SEQ3	TATACCTTCGGTTCTTGATCGC
Quantitative real-time PCR	
PQSR RT FWD2	ATTGCGCGGACCCCTTGTGA
PQSR RT REV3	GGACGGCTACAAGGTCGAAC
NADA RT FWD3	GTTTCGGCAACCAGCATCCG
NADA RT REV3	GGCATCAGCACACGCTTCTC
CLPX RT1	CCTGTGCAATGACATCATCC
CLPX RT2	AGGATGGTGCGGATCTCTTT
RPLU RT1	GGTGGCAAGCAGCACAAAGTCACCG
RPLU RT2	GCGGACCTTGTCTGACGGCCGTGG
Cloning CysB expression plasmids and controls	
CYSB EX FWD	AAGCTTCAGCAATTGCGCTATATCTG
CYSB EX REV	AAAA CTGCAG ACACCGTGTTTCATGAGCTTCC
HERD Pbad F	CTACTTCACCTATCCTGC
HERD Pbad R	CCCAGTCACGACGTTG

^a Underlined sequence denotes a restriction site utilized for cloning. Nucleotides shown in bold are altered compared to the original template sequence.

Next, cell pellets were thawed, suspended in 10 ml of lysis buffer (50 mM Tris, pH 8, 100 mM NaCl, 0.5 mM EDTA, 2 mg/ml lysozyme), and incubated at room temperature for 15 min. Cells were then disrupted by sonication, and the resulting cell lysates were cleared by centrifugation at

20,000 × g for 30 min at 4°C. The total protein concentration in the cleared lysates was estimated using the Bradford assay.

For DNA affinity chromatography experiments, aliquots of either uncoupled or DNA-coupled Dynabeads were equilibrated in binding buffer

(50 mM Tris, pH 8, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.05% Triton X-100). The Dynabeads were then incubated for 1 h at room temperature, with gentle mixing, in binding buffer with 1 mM DTT, 15 µg/ml sheared chromosomal DNA from *P. aeruginosa* strain PAO1, and cleared cell lysates (0.6 mg of total protein for every 10 µl of Dynabead suspension used). Next, the Dynabeads were collected by magnetic separation and washed three times with 10 volumes of binding buffer. Bound proteins were then eluted by washing the Dynabeads three times with two volumes of binding buffer containing 2 M NaCl. The eluted proteins were concentrated and exchanged into a buffer containing 50 mM Tris, pH 8, 50 mM NaCl, and 0.5 mM EDTA using Amicon Ultra centrifugal filters (3,000-Da-molecular-mass cutoff). The proteins were then separated by SDS-PAGE on a 15% polyacrylamide gel. Individual protein bands were excised from the gel and identified by mass spectrometry (performed by the Proteomics Core Facility at the University of California, Davis).

Generation of mutant strains. Mutant alleles for *mvaT*, *mvaU*, and *cysB* were generated using PCR as described elsewhere (21). Alleles were constructed to contain in-frame deletions in the coding DNA sequence corresponding to amino acids 9 to 121 for *mvaT* (91% of protein sequence), 8 to 111 for *mvaU* (90% of protein sequence), and 13 to 318 for *cysB* (94% of protein sequence). The mutated fragments were also designed to contain the following at each end: PstI sites for *mvaT* and *mvaU* and BamHI sites for *cysB*. Each fragment and the vector plasmid pEX18Ap were digested with the appropriate restriction enzyme, purified from an agarose gel, and ligated to produce suicide plasmids pΔ*mvaT*-suc, pΔ*mvaU*-suc, and pΔ*cysB*-suc. These plasmids were used to transfer the mutant alleles onto the chromosome of *P. aeruginosa* strain PAO1 as described elsewhere (21). Potential mutants were screened by PCR using appropriate flanking primers, and mutants were further confirmed by determining the DNA sequence of the PCR products.

qRT-PCR analysis. To isolate RNA for quantitative real-time PCR (qRT-PCR) analyses, washed cells from overnight cultures of *P. aeruginosa* were used to inoculate 10-ml cultures of LB medium to an OD₆₆₀ of 0.05. Cultures were incubated at 37°C for 6 h. Then, 400-µl samples of each culture were treated with RNeasy Protect Bacteria reagent (Qiagen), and RNA was isolated from each sample using an RNeasy Midi kit according to the manufacturer's protocol (Qiagen). Isolated RNA samples were extracted twice with 1:1 phenol-chloroform and once with chloroform and then collected by ethanol precipitation. Next, the RNA was suspended in nuclease-free water and treated with RQ1 DNase (Promega) according to the manufacturer's protocol. Following DNase treatment, samples were extracted once with 1:1 phenol-chloroform and once with chloroform and then collected by ethanol precipitation. The purified RNA was suspended in nuclease-free water and stored at -70°C. The concentration of RNA in each sample was determined using a NanoDrop ND-1000 spectrophotometer. The integrity of the RNA in each sample was assessed by electrophoresis on an agarose gel.

To generate cDNA for qRT-PCR analysis, 2 µg of total RNA was primed with random hexamers (approximately 60% GC content). Superscript III reverse transcriptase (Life Technologies) was used to synthesize cDNA according to the manufacturer's protocol, including the addition of 40 U of RNaseOUT RNase inhibitor (Life Technologies) to each reaction mixture. Reaction mixtures were incubated at 55°C for 1 h as directed by the manufacturer. For real-time PCR analysis, cDNA preparations were diluted either 1:50 or 1:60 in nuclease-free water. PCRs were performed on a Bio-Rad CFX96 real-time system using FastStart SYBR green master mix (Roche) and the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 20 s, with a final step at 95°C for 10 s, followed by a melt curve analysis. The oligonucleotide primers used to detect expression of each gene of interest are listed in Table 2. The efficiency and limits of detection for each primer set were determined by performing real-time PCR with serial dilutions of strain PAO1 chromosomal DNA in 100 µg/ml *Saccharomyces cerevisiae* tRNA (Ambion) as a template (45). Relative gene expression was calculated using the method of Pfaffl (46), with *rplU* as a reference gene for compar-

ison. At least two technical replicates were performed for each cDNA sample analyzed.

PQS extraction and quantification. To analyze PQS production, 10-ml cultures of freshly prepared medium were inoculated with several colonies of freshly grown *P. aeruginosa* cells and incubated at 37°C. Washed cells from starter cultures were used to inoculate 10-ml subcultures to an OD₆₆₀ of 0.05, which were incubated at 37°C for either 6 h or 18 h as indicated in the text. Next, 0.3-ml samples of each culture were extracted with 0.9 ml of acidified ethyl acetate as described elsewhere (47). One half of the resulting organic phase was evaporated to dryness at 37°C and reconstituted in 50 µl of 1:1 acidified ethyl acetate-acetonitrile. Samples of each extract were analyzed by thin-layer chromatography (TLC) as described elsewhere (10). TLC plates were photographed under long-wave UV light, and the amount of PQS present was determined by comparison to known amounts of synthetic PQS using densitometry (47).

Construction of a CysB expression plasmid. In order to generate a plasmid for controlled expression of CysB, a 1,156-bp DNA fragment, which began at the second codon of *cysB* and ended 175 bp past the stop codon, was amplified by PCR. The oligonucleotide primers for this amplification were designed to contain a single PstI site downstream from the stop codon. Vector plasmid pHERD20T was digested with NcoI and then treated with Pfu Turbo DNA polymerase (Agilent Technologies) to generate blunt ends. The plasmid was then digested with PstI. The prepared plasmid DNA was ligated with the *cysB*-containing fragment, which had also been digested with PstI, to produce the CysB expression plasmid pJF60. This construct placed the expression of *cysB* under the control of the inducible P_{BAD} promoter.

Chromosomal *lacZ* fusion strains and β-Gal assays. To construct a *cysH* promoter-*lacZ* fusion, a 448-bp DNA fragment corresponding to the *cysH*-*thrH* intergenic region (from -403 to +26 relative to the *cysH* translational start site) was amplified by PCR. The oligonucleotide primers for this amplification contained a HindIII restriction site downstream and a PstI restriction site upstream from the *cysH* translational start site. The amplified fragment was digested with HindIII and PstI, purified from an agarose gel, and ligated into pUC18T-mini-Tn7T-*lacZ*-Gm, which was digested with the same enzymes, to produce plasmid pJF-Tn7T-*cysH'*tc. To generate *P. aeruginosa* strains carrying either a *pqsR'*-*lacZ* fusion or a *cysH'*-*lacZ* fusion integrated on the bacterial chromosome at the attTn7 site, cells were transformed with plasmid pJF-Tn7T-*pqsR'*tc or pJF-Tn7T-*cysH'*tc as described elsewhere (48). The gentamicin resistance gene carried by the plasmid was also removed from the chromosome as described previously (48).

For assays to assess the effects of CysB overexpression, washed cells from overnight cultures were used to inoculate 10-ml subcultures of TSB, supplemented with either water or 0.1% arabinose, to an OD₆₆₀ of 0.05. Cultures were incubated at 37°C for 6 h, and then aliquots of cultures were diluted 1:10 in fresh TSB medium and assayed for β-Gal activity in duplicate as described elsewhere (43). To test the effects of cysteine or its precursors on gene expression in rich medium, washed cells from overnight cultures were used to inoculate fresh TSB to an OD₆₆₀ of 0.05, and 980-µl aliquots of inoculated medium was transferred to tubes and supplemented with either water, 5 mM L-cysteine, 5 mM Na₂SO₄, or 5 mM O-acetyl-L-serine. Cultures in tubes were incubated at 37°C with agitation as indicated in the text and assayed for β-Gal activity in duplicate. For β-Gal assays from bacteria cultured in defined medium, cells from overnight cultures of MSFM supplemented with 100 µM L-cysteine and 100 µM L-methionine were collected by centrifugation and suspended in unsupplemented MSFM. The washed cells were used to inoculate 10-ml cultures of MSFM supplemented with a 200 µM concentration of sulfur sources (as indicated in the legend to Fig. 6) to an OD₆₆₀ of 0.05. Then, cultures were incubated at 37°C for 6 h and assayed for β-Gal activity. For some experiments washed cells were used to inoculate MSFM supplemented with 400 µM L-cysteine to an OD₆₆₀ of 0.05, and then aliquots of inoculated medium were transferred to tubes and supplemented with

potential CysB ligands. Cultures in tubes were incubated at 37°C as indicated and then assayed for β -Gal activity.

Purification of CysB. To purify CysB, we overexpressed the native *P. aeruginosa* CysB protein in *E. coli*. This approach has also been used to purify the CysB proteins for *Salmonella enterica* serovar Typhimurium and *Klebsiella aerogenes* (49, 50). *E. coli* strain DH5 α (pJF60) was grown overnight in LB medium with ampicillin. Cells were subcultured in 250 ml of LB with ampicillin to an OD₆₀₀ of 0.08, and the culture was incubated at 37°C. When the OD₆₀₀ reached 0.6, arabinose was added to the culture at 0.1% to induce *cysB* expression, and the culture was incubated at 28°C for 4 h. Then the cells were harvested by centrifugation, washed with sterile phosphate-buffered saline, and stored at -70°C .

To prepare a cell lysate, cells were thawed and suspended in 5 ml of ice-cold buffer A [50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 M (NH₄)₂SO₄] containing 10 $\mu\text{g}/\text{ml}$ HALT protease inhibitor cocktail (Thermo Scientific). Cells were disrupted by sonication, and the resulting lysate was centrifuged at $10,000 \times g$ for 30 min at 4°C. The supernatant was collected, 291 mg/ml (NH₄)₂SO₄ was then added, and the solution was incubated on ice with stirring for 30 min. Next, the sample was centrifuged at $10,000 \times g$ for 30 min at 4°C to collect the precipitated material. The precipitate was suspended in 5 ml of 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA and stored at -70°C .

For protein purification, the sample was thawed on ice and diluted 1:1 with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 2 M (NH₄)₂SO₄. A 1-ml HiTrap Phenyl FF (low sub) column (GE Healthcare) was equilibrated with buffer A at room temperature, and then the sample was applied at a flow rate of 0.5 ml/min. Next, the column was washed with approximately 20 volumes of buffer A to remove unbound material. Bound proteins were eluted with a step gradient of 1 M to 0 M (NH₄)₂SO₄ in 50 mM Tris-HCl (pH 8.0)–1 mM EDTA. Each step was eluted with a volume of 1 ml, with the (NH₄)₂SO₄ concentration decreasing in 0.1 M increments. CysB eluted in fractions containing 0.3 to 0.1 M (NH₄)₂SO₄, as determined by SDS-PAGE. These fractions were pooled, and glycerol was added to a final concentration of 10% before storage at -70°C . To complete the purification procedure, the sample was thawed and diluted 1:1 with buffer B (20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT, 10% glycerol). The sample was then applied to a 5-ml HiTrap Heparin column (GE Healthcare), which had been equilibrated with buffer B, at a flow rate of 0.5 ml/min. The column was washed with approximately 3 volumes of buffer B to remove unbound material. Bound proteins were eluted with a 50-ml linear gradient of 0 to 1 M NaCl in buffer B at a flow rate of 1 ml/min, and 3-ml fractions were collected and placed on ice. The CysB protein eluted at approximately 0.7 to 0.8 M NaCl and appeared as a single band on Coomassie blue-stained SDS-PAGE gels (see Fig. S1 in the supplemental material). Protein concentrations in each fraction were determined by the Bradford assay using reagents from Bio-Rad.

EMSA with CysB. DNA fragments for EMSA were amplified by PCR using primers listed in Table 2, and fragments were prepared and labeled with ³²P as described for EMSA with LasR. DNA binding reactions were carried out in a buffer containing 40 mM Tris-HCl, pH 8.0, 90 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mg/ml bovine serum albumin, and 11.25 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA. Each binding reaction mixture contained approximately 1.75 to 3 pmol of the appropriate radiolabeled DNA fragment and amounts of CysB protein as indicated in Fig. 7 and 8 in a final volume of 20 μl . Reaction mixtures were incubated for 10 min at room temperature. Aliquots of binding reactions were then electrophoresed on nondenaturing 5% acrylamide gels in 0.5 $\times$ TBE buffer. Gels were visualized by autoradiography. For EMSA to test the DNA binding activity of both CysB and LasR simultaneously, experiments were performed as described for LasR except that reaction mixtures contained 18 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA and the addition of 25 mM NaCl.

Two-plasmid system to analyze *pqsR'*-*lacZ* control by LasR and CysB in *E. coli*. To generate a plasmid that carried both a *pqsR'*-*lacZ* fusion and *tacp-lasR*, a DNA fragment containing *tacp-lasR* was amplified by PCR using pECP8 as a template. The oligonucleotide primers used for

this amplification added an EcoRI site upstream from the *tac* promoter. The amplified fragment was digested with EcoRI and ScaI and then ligated into pMWC1003, which had been digested with the same enzymes, to produce pJF1003. A second set of plasmids was generated for CysB expression and as a control. For these constructs, DNA fragments containing either *araC* and P_{BAD} or *araC* and P_{BAD}-*cysB* were amplified by PCR using either pHERD20T or pJF60 as a template. Vector plasmid pACYC184 was digested with DraI, treated with calf intestinal phosphatase, and purified from an agarose gel. The *araC*-P_{BAD} or the *araC*-P_{BAD}-*cysB* fragment was ligated into the prepared vector to produce control plasmid pACYC-P_{BAD} or CysB expression plasmid pJF52, respectively. These plasmids were transformed into *E. coli* strain DH5 α .

For β -Gal assays, cells from overnight cultures were used to inoculate 10-ml subcultures of LB, supplemented with ampicillin and tetracycline, to an OD₆₀₀ of 0.08. Cultures were incubated at 37°C until the OD₆₀₀ reached 0.2. Then IPTG was added to 1 mM, and 1-ml aliquots of cultures were transferred to tubes in the presence or absence of 20 nM 3-oxo-C₁₂-HSL. Cultures in tubes were additionally supplemented with either water or 1% arabinose and incubated at 37°C with mixing for 2 h. Then samples were taken from each culture and assayed for β -Gal activity in duplicate (43).

RESULTS

LasR controls *pqsR* transcription from a single binding site but does not regulate *nadA*. Previous studies showed that LasR positively regulates the transcription of *pqsR* and that LasR and 3-oxo-C₁₂-HSL could induce *pqsR* expression in *E. coli* (9, 30). In addition, mutation of a consensus sequence for LasR binding, centered at 248 bp upstream from the *pqsR* transcriptional start site 1 (TS1) (Fig. 1A), caused a large decrease in *pqsR* transcription (39). With this in mind, we set out to directly determine the location of LasR binding sites in the *pqsR* promoter region. To investigate this, we expressed LasR in *E. coli* in the presence of 3-oxo-C₁₂-HSL and purified the protein using the method of Schuster et al. (42). We then performed *in vitro* DNA binding experiments to localize LasR binding sites in the *pqsR* promoter. As a positive control, we tested the ability of our purified LasR to bind to the *lasI* promoter. LasR caused a shift in mobility of more than 90% of the labeled *lasI* promoter fragment at the concentration tested (Fig. 1B). There was also a small amount of a higher-molecular-weight complex visible, possibly due to the formation of LasR multimers. LasR did not bind to the promoter region of *kynA*, an unrelated gene (Fig. 1B). When we tested a 516-bp fragment of the *pqsR*-*nadA* intergenic region that included both of the identified *pqsR* transcriptional start sites and extended beyond the *las-rhl* box (Fig. 1A, *pqsR*-P1), we found that LasR did, indeed, interact directly with this fragment (Fig. 1B). Next, we divided this segment into two smaller overlapping DNA fragments (Fig. 1A, *pqsR*-P2 and *pqsR*-P3) and tested the ability of LasR to bind to each. LasR caused a shift in the mobility of fragment *pqsR*-P2, which included the predicted LasR binding consensus sequence but did not affect the mobility of fragment *pqsR*-P3, showing that LasR only binds beyond -471 bp relative to the *pqsR* translational start site (Fig. 1B). Finally, we performed DNase I protection assays to determine the exact site of the interaction between LasR and fragment *pqsR*-P2. This experiment showed that LasR protected a single 27-bp region from DNase I digestion, which completely overlapped the previously identified putative *las-rhl* box (Fig. 1C). When we repeated the experiment with a DNA fragment that was radiolabeled on the opposite strand, we observed a nearly identical region of protection (data not shown). Together, these experiments indicated that

FIG 1 Purified LasR interacts only with a 27-bp region in the *pqsR* promoter centered at -526 relative to the *pqsR* translational start site. (A) A graphical depiction of the *pqsR* promoter region and DNA segments used as probes for EMSA. The bent arrows show the relative positions of identified transcriptional start sites. Numbering indicates the position in base pairs relative to the *pqsR* translational start site. (B) For EMSA, radiolabeled DNA fragments corresponding to either the *lasI* promoter (p-*lasI*, positive control), the *kynA* promoter (p-*kynA*, negative control), or the *pqsR* promoter region were incubated in the presence and absence of 7.2 ng of purified LasR as described in Materials and Methods. 3-oxo- C_{12} -HSL (5 μ M) was included in each reaction mixture. Samples of binding reaction products were electrophoresed on nondenaturing acrylamide gels, and the position of DNA fragments was visualized by autoradiography. (C) DNase I protection assays with purified LasR were performed as described in Materials and Methods. The results shown were generated using the DNA fragment pqsR-P2 that was end labeled on the *pqsR* coding strand. Lanes labeled A, C, G, and T contain DNA sequencing ladders that were generated by cycle sequencing and that terminate at the corresponding nucleotide positions. The DNA sequence that was protected from DNase I digestion by LasR is shown in brackets.

LasR interacts with a single binding site in the *pqsR-nadA* intergenic region.

This binding site placed bound LasR in closer proximity to the divergently transcribed *nadA* gene (centered at bp -221 relative to the *nadA* translational start site) than to the *pqsR* transcriptional start site TS1. The *nadA* gene is predicted to encode a protein involved in the *de novo* biosynthesis of NADH, and it has not been linked to PQS synthesis. We decided to investigate whether LasR binding has any effect on *nadA* transcription since it is known that LasR can regulate divergently transcribed genes from a single binding site (51). First, we mapped the transcriptional start site of *nadA* by primer extension analysis. We identified a single start site that was verified by analyses with two separate primers in independent experiments. This site corresponded to a C residue located at bp -141 relative to the *nadA* translational start site (Fig. 2A). Relative to this transcriptional start site, the *las-rhl* box was centered at bp -81 . To analyze the effects of LasR on both *nadA* and *pqsR* transcription, we constructed *lacZ* fusions to the promoter of each gene and then made five base pair substitutions in the *las-rhl* box on each fusion (Table 2 gives specific mutations). These mutations altered residues that were identified as highly conserved in LasR binding sequences (38), and the binding of purified LasR-3-oxo- C_{12} -HSL to fragment pqsR-P2 was completely abolished when these mutations were present (Fig. 2B). Expression from the *nadA'*-*lacZ* fusion was almost identical with either the wild-type or mutated *las-rhl* box present, indicating that this regulatory element does not control *nadA* (Fig. 2C). In contrast, there was a large decrease in expression from the *pqsR* promoter when the *las-rhl* box was mutated (Fig. 2D). These re-

sults showed that despite the fact that LasR binds in closer proximity to the *nadA* transcriptional start site, it does not regulate the transcription of *nadA* under these conditions. Instead, the primary function of LasR binding to the *pqsR-nadA* intergenic region appears to be to control *pqsR* transcription.

Identification of other proteins that bind to the *pqsR-nadA* intergenic region. Once we had established that LasR interacted with a single site 250 bp upstream from the *pqsR* TS1, we questioned how LasR could affect transcription from this distal binding site. Also, data from other studies showed that *pqsR* expression and PQS production are delayed but still occur in a *lasR* mutant strain (14, 18, 39). Therefore, we hypothesized that additional factors which affect *pqsR* transcription could be binding to the *pqsR-nadA* intergenic region. In an attempt to identify these factors, we utilized DNA affinity chromatography to isolate proteins that interact with this region. In these experiments, a 393-bp DNA fragment, spanning from -177 to -570 relative to the *pqsR* translational start site, was amplified by PCR using a biotinylated oligonucleotide primer and conjugated to streptavidin-coated magnetic beads. These were incubated with *P. aeruginosa* strain PAO1 cell lysates from either the logarithmic or stationary phase of growth. Next, the unbound proteins were washed away, and bound proteins were eluted, separated by SDS-PAGE, and identified by mass spectroscopy. We identified three proteins that were consistently isolated in separate experiments using this technique (Fig. 3). We did not isolate these proteins when we performed the experiment with unconjugated magnetic beads or when we used a different DNA fragment (*pqsL* promoter) (K. A. Tipton and E. C. Pesci, unpublished data). The first of these proteins, CysB, is an

FIG 2 LasR does not affect the transcription of *nadA*. (A) Primer extension analysis of the *nadA* transcript (lane Ext). Lanes labeled A, C, G, and T contain DNA sequencing ladders that were generated by cycle sequencing and that terminate at the corresponding nucleotide positions. Figure 1A shows the relative location of the primer extension product identified by the arrowhead. (B) Radiolabeled DNA fragments corresponding to *pqsR* promoter fragment *pqsR*-P2 or *pqsR*-P2 with a mutated LasR binding site were incubated in the presence or absence of purified LasR and 5 μ M 3-oxo-C₁₂-HSL. Samples of binding reaction products were analyzed on nondenaturing acrylamide gels, and the position of DNA fragments was visualized by autoradiography. (C) *P. aeruginosa* strain PAO1 carrying either a plasmid containing the wild-type *nadA* promoter fused to *lacZ* (open bars) or a plasmid containing an *nadA* promoter with a mutated LasR binding site fused to *lacZ* (striped bars) was grown in LB medium for 6 h, and then cultures were assayed for β -Gal activity. (D) *P. aeruginosa* strain PAO1 carrying either a plasmid containing the wild-type *pqsR* promoter fused to *lacZ* (open bars) or a plasmid containing a *pqsR* promoter with a mutated LasR binding site fused to *lacZ* (striped bars) was grown in LB medium for 6 h, and then cultures were assayed for β -Gal activity. β -Gal data are presented in Miller units as the means $\pm$ standard deviations of results from duplicate assays from at least three separate experiments.

LysR-type transcriptional regulator that controls sulfur uptake and cysteine biosynthesis (40). The second and third proteins, MvaT and MvaU, are considered to be members of the H-NS family of nucleoid-associated proteins. These two proteins are similar to one another, bind to the same regions of the chromosome, and appear to have similar or overlapping functions (52). They can also affect gene expression and primarily act to silence transcription (52). In a previous study, data from experiments using chromatin immunoprecipitation coupled with microarray technology (ChIP-on-chip) suggested that MvaT and MvaU associate with the *pqsR*-*nadA* intergenic region *in vivo* (52). Based on their established regulatory functions, each of the proteins we identified could potentially influence *pqsR* or *nadA* expression.

CysB is a repressor of *pqsR* and PQS production. To investi-

FIG 3 The proteins CysB, MvaT, and MvaU interact with the *pqsR*-*nadA* promoter region. A DNA fragment corresponding to -177 to -570 relative to the *pqsR* translational start site was used to capture proteins by DNA affinity chromatography as described in Materials and Methods. Cleared cell lysates from strain PAO1 grown in LB medium for either 2.5 h or 8 h were used in the procedure. Isolated proteins were separated by SDS-PAGE, and the gel was stained with SYPRO Ruby and visualized on a UV transilluminator. The image shown is a representative of four independent experiments.

gate the role of each of these proteins in the regulation of *pqsR* and *nadA*, we constructed isogenic, in-frame deletion mutants for the *mvaT*, *mvaU*, and *cysB* genes. We also tried to construct an *mvaT* *mvaU* double mutant strain but were unable to after multiple attempts. This result agrees with previous reports, which established that the presence of either MvaT or MvaU is necessary for cell viability in order to silence the expression of prophage genes encoded in the *P. aeruginosa* genome (52, 53). The phenotypes displayed by each mutant strain were similar to what has been described in previous studies (14, 54, 55). The *mvaT* and *mvaU* mutant strains both overproduced the blue-green pigment pyocyanin, and this occurred at a much greater degree in the *mvaT* mutant strain. The *cysB* mutant strain was able to grow on the defined medium MSFM supplemented with cysteine as the sole sulfur source but was unable to grow when supplied with either sulfate or 1-hexyl sulfate as a sulfur source (see Fig. S1 in the supplemental material). All of the mutant strains grew at a rate similar to that of the wild-type strain when cultured on rich medium.

Next, we isolated RNA from each *P. aeruginosa* strain grown to the early stationary phase of growth in LB medium and analyzed *pqsR* and *nadA* expression by qRT-PCR. We observed no changes in the relative expression level of *nadA* in the *mvaT*, *mvaU*, and *cysB* mutant strains compared to the level in the wild-type strain (Fig. 4A). This result indicated that *nadA* transcription is not affected by the removal of each of the respective proteins individually. We also did not observe any significant change in the relative expression of *pqsR* in either the *mvaT* or *mvaU* mutant strain (Fig. 4A). However, there was approximately a 2.5-fold increase in *pqsR* expression in the *cysB* mutant strain (Fig. 4A). We also analyzed PQS production by the *cysB* mutant strain. Somewhat surprisingly, we did not find any significant difference in the levels of PQS

FIG 4 CysB can repress *pqsR* expression and PQS production. (A) The expression of *pqsR* and *nadA* was analyzed by qRT-PCR using total RNA isolated from *P. aeruginosa* strains PAO1 (wild type), PJF-MT1 ($\Delta mvaT$), PJF-MU1 ($\Delta mvaU$), and PJF-CB1 ($\Delta cysB$). RNA was isolated from each strain after 6 h of growth in LB medium. Results are shown as the average fold change, relative to the expression in strain PAO1 $\pm$ standard deviations from at least three separate experiments. (B) PQS production by strains PAO1 (black bar) and PJF-CB1 (white bar) was analyzed after 18 h of growth in TSB medium. Data are presented as the averages $\pm$ standard deviations of results from six independent experiments. *, $P < 0.01$, as determined by Student's *t* test.

produced by the wild-type and *cysB* mutant strains when each was cultured in LB medium (data not shown). However, when we examined PQS levels during growth in TSB medium, we found that the *cysB* mutant produced almost 2-fold more PQS than the wild-type strain (Fig. 4B). We also observed a similar increase in PQS production by the *cysB* mutant in a third growth medium, peptone-tryptic soy broth (56) (data not shown). It is unclear why this phenotype was not observed during growth in LB medium, but perhaps the availability of PQS precursors or differential regulation of PQS biosynthetic genes by factors other than PqsR was able to make the regulatory effects of CysB less apparent. Overall, these data showed that CysB can act as a repressor of both *pqsR* expression and PQS production.

We obtained more support for this conclusion when we complemented the *cysB* mutant strain by supplying an intact *cysB* gene on a multicopy plasmid. We cloned *cysB* onto the *E. coli*-*P. aeruginosa* expression vector pHERD20T (57). This placed *cysB* under the control of the P_{BAD} promoter, allowing *cysB* expression to be induced by supplementing cultures with L-arabinose. To examine the effects on *pqsR* expression, we transformed the vector and *cysB* expression plasmid pJF60 into strains PAO1-*PpqsRtc* (wild type) and PJF-CB1-*PpqsRtc* ($\Delta cysB$), that each harbor a *pqsR'*-*lacZ* transcriptional fusion integrated onto the chromosome at the *attTn7* site. In the strains carrying the vector plasmid, the *cysB*

FIG 5 Overexpression of CysB represses *pqsR* expression and inhibits PQS production. (A) Strains PAO1-*PpqsRtc* (wild type) and PJF-CB1-*PpqsRtc* ($\Delta cysB$), that each contain a *pqsR'*-*lacZ* fusion integrated on the bacterial chromosome, were transformed with either the vector control plasmid pHERD20T or CysB expression plasmid pJF60. Each strain was cultured for 6 h in TSB medium with the addition of either water (open bars) or 0.1% arabinose (black bars) to induce CysB expression, and then cultures were assayed for β -Gal activity. Data are presented in Miller units as the means $\pm$ standard deviations of results from duplicate assays from at least three separate experiments. (B) Organic extracts from *P. aeruginosa* strains carrying either the vector plasmid pHERD20T or CysB expression plasmid pJF60 and grown for 6 h in TSB medium in either the presence or absence of 0.1% arabinose (ind) were analyzed by TLC to detect PQS production. Lanes show results of extracts from the following strains: lanes 1 and 2, PAO1(pHERD20T); lanes 3 and 4, PAO1(pJF60); lanes 5 and 6, PJF-CB1(pHERD20T); lanes 7 and 8, PJF-CB1(pJF60); lane P, 50 ng of synthetic PQS.

mutant strain produced approximately 2.3-fold more β -Gal activity than the wild-type strain [$1,148 \pm 55$ units from strain PJF-CB1-*PpqsRtc*(pHERD20T) versus 491 ± 17 units from strain PAO1-*PpqsRtc*(pHERD20T)] (Fig. 5A). When *cysB* was supplied on pJF60, the β -Gal activity produced by both the wild-type and *cysB* mutant strains decreased (Fig. 5A). Each strain produced approximately an equivalent amount of β -Gal activity in the absence of arabinose (Fig. 5A), and this level of activity was slightly lower than the level of activity produced by the wild-type strain carrying the vector plasmid. This result showed that the *cysB* mutant was fully complemented by *cysB* expression from pJF60 even in the absence of inducer, apparently due to a basal level of expression from the P_{BAD} promoter. When arabinose was added to cultures of strains carrying pJF60, the β -Gal activity decreased further, to approximately 20% of the activity produced by the

FIG 6 Expression of both *pqsR* and *cysH* is controlled by CysB, but only *cysH* expression is altered in response to available sulfur sources. (A and B) *P. aeruginosa* strains carrying either a *cysH'*-*lacZ* or a *pqsR'*-*lacZ* fusion integrated on the bacterial chromosome were grown for 3 h or 6 h, respectively, in TSB medium supplemented with either water, 5 mM L-cysteine, 5 mM Na₂SO₄, or 5 mM O-acetyl-L-serine, and then cultures were assayed for β -Gal activity. (C and D) *P. aeruginosa* strains carrying either a *cysH'*-*lacZ* or a *pqsR'*-*lacZ* fusion integrated on the bacterial chromosome were grown for 6 h in MSFM supplemented with either L-cysteine, Na₂SO₄, or 1-hexyl sulfate as the sole sulfur source, and then cultures were assayed for β -Gal activity. (E) *P. aeruginosa* strains carrying either a *cysH'*-*lacZ* or a *pqsR'*-*lacZ* fusion integrated on the bacterial chromosome were grown for 6 h in MSFM supplemented with 400 μ M L-cysteine as a sulfur source and additionally either water, 5 mM O-acetyl-L-serine, or 5 mM N-acetyl-DL-serine, and then cultures were assayed for β -Gal activity. Data for all β -Gal assays are presented in Miller units as the means $\pm$ standard deviations of results from duplicate assays from at least three separate experiments. (F) PQS production by strain PAO1 was analyzed after 10 h of growth in MSFM supplemented with either L-cysteine, Na₂SO₄, or 1-hexyl sulfate as the sole sulfur source. Data are presented as the means $\pm$ standard deviations of results from four independent experiments.

wild-type strain carrying the vector plasmid (Fig. 5A). These data demonstrated that CysB can repress *pqsR* transcription.

We observed a similar but more striking trend when we analyzed PQS production in the presence of the CysB expression plasmid. The *cysB* mutant PJF-CB1(pHERD20T) appeared to produce a slightly increased amount of PQS (Fig. 5B, lanes 5 and 6) compared to the wild-type PAO1(pHERD20T) (Fig. 5B, lanes 1 and 2), and this was not affected when cultures were supplemented with arabinose. However, when we transformed either strain with the CysB expression plasmid, PQS production was completely abolished, even in the absence of inducer (Fig. 5B, wild type, lanes 3 and 4, and *cysB* mutant, lanes 7 and 8). These results showed that even the basal level of CysB expression from pJF60 could significantly impact PQS production. We should also note that we obtained nearly identical results when we analyzed *pqsR'*-*lacZ* activity and PQS production in either LB medium (data not shown) or TSB medium (Fig. 5). Taken together, these data provide clear evidence that CysB can repress both *pqsR* expression and PQS production.

Expression of *pqsR* is not affected by cysteine availability. Since CysB has an established regulatory role in controlling cysteine biosynthesis, we next investigated the role of cysteine and sulfur source availability on *pqsR* expression and PQS production. Studies on CysB in several enteric bacteria showed that the primary role of CysB was to activate the expression of genes necessary for sulfur uptake in response to cysteine starvation (40). This regulatory activity occurs in response to the ligand N-acetylserine

(NAS). NAS is derived from the cysteine precursor O-acetylserine (OAS), which accumulates when there is not enough sulfur available for cysteine biosynthesis, and acts as a coinducer with CysB to stimulate sulfate uptake and cysteine production (40, 50). Similarly, in *P. aeruginosa* and *Pseudomonas putida*, CysB appears to act as a master regulator of sulfur assimilation pathways although its exact regulatory functions are not clearly defined (58, 59). So to examine the influence of cysteine availability, we compared the expression of *pqsR'*-*lacZ* and *cysH'*-*lacZ* fusions in response to various sulfur sources as well as OAS and NAS. The *cysH* gene encodes a 5'-adenylylsulfate reductase, an enzyme that is involved in sulfate assimilation and cysteine biosynthesis (60). It is expected to be part of the CysB regulon based on studies in other Gram-negative bacteria (40, 60). In addition, *cysH* is located downstream from the *cysB* gene in *P. aeruginosa* but on the opposite strand.

First, we analyzed *pqsR* and *cysH* expression in TSB medium supplemented with either excess cysteine or cysteine precursors. We observed that the activity produced by the *cysH'*-*lacZ* fusion in the wild-type strain decreased when cultures were supplemented with cysteine but not when the cultures were supplemented with sulfate or OAS (Fig. 6A). Additionally, *cysH'*-*lacZ* expression was much lower in the *cysB* mutant strain, indicating that CysB regulates *cysH*, and the β -Gal activity in this strain remained the same in the presence of excess cysteine. In contrast, expression of the *pqsR'*-*lacZ* fusion was not affected by the addition of cysteine to cultures of either the wild-type or *cysB* mutant strain (Fig. 6B). We

also examined *cysH* and *pqsR* expression in the wild-type strain during growth on a chemically defined growth medium with various sulfur sources. There was a 1.5-fold increase in β -Gal activity produced by the *cysH'*-*lacZ* fusion during growth on sulfate compared to the activity produced during growth on cysteine and a 2-fold increase in activity during growth on an alternative sulfur source, the organic sulfur compound 1-hexyl sulfate (Fig. 6C). However, we observed only minor changes in *pqsR'*-*lacZ* expression and PQS production during growth on different sulfur sources (Fig. 6D and F). These experiments showed that CysB positively regulates *cysH*, and in keeping with its predicted role in cysteine biosynthesis, *cysH* was expressed at lower levels in the presence of excess cysteine and at higher levels in the absence of cysteine. Unlike what has been described in enteric bacteria, these regulatory effects do not appear to depend on the cysteine precursor OAS or the potential CysB ligand NAS since neither compound was able to stimulate increased *cysH'*-*lacZ* expression (Fig. 6A and E). Somewhat surprisingly, *pqsR* expression and PQS production did not change in response to cysteine availability, implying that CysB does not regulate *pqsR* expression in the same manner as sulfur acquisition and cysteine biosynthetic genes.

CysB directly interacts with the *pqsR* promoter region. Having established that CysB can regulate both *pqsR* and *cysH*, we wanted to confirm that CysB could directly interact with the *pqsR* and *cysH* promoter regions. To do this, we overexpressed the native *P. aeruginosa* CysB in *E. coli* and purified the protein as described in Materials and Methods (see also Fig. S2 in the supplemental material). We then tested the ability of the purified *P. aeruginosa* CysB protein to interact with promoter DNA fragments using EMSA. First, we examined the ability of CysB to bind to the *cysH* promoter region. As expected, CysB caused a shift in the mobility of the *cysH* promoter fragment, with 20 ng of CysB causing a shift of at least 95% of the labeled probe (Fig. 7A). As a control, we found that 40 ng of CysB did not cause any change in the mobility of the *kynA* promoter (Fig. 7B). This indicated that CysB was interacting specifically with the *cysH* promoter fragment. Most interestingly, the *pqsR* promoter fragment *pqsR*-P1 (Fig. 7A) also exhibited a shift in mobility when CysB was added (Fig. 7B). In this case, 20 ng of CysB caused a shift of only approximately 50% of the labeled *pqsR* promoter fragment, suggesting that CysB has a lower affinity for the *pqsR* promoter than the *cysH* promoter. Additionally, we observed a second, higher-molecular-weight complex formed when larger amounts of CysB were incubated with the *pqsR* promoter (Fig. 7B). To begin to more clearly define the site or sites where CysB interacts with the *pqsR* promoter, we tested two smaller, nonoverlapping fragments of the *pqsR* promoter region (Fig. 1A shows relative positions). CysB interacted with *pqsR* promoter fragment *pqsR*-P2, causing the formation of a single, higher-molecular-weight complex (Fig. 7C). CysB also formed a single complex with the *pqsR*-P4 fragment, but this occurred only when more than 10 ng of CysB was included in the binding reaction mixtures (Fig. 7D). These results indicated that CysB can interact with two separate sites in the *pqsR* promoter region *in vitro* and provided an explanation for the two distinct CysB-DNA complexes formed with the *pqsR*-P1 fragment (Fig. 7B). However, because CysB appeared to bind to the *pqsR*-P4 fragment with low affinity, it is unclear whether this interaction is relevant *in vivo* or is simply due to nonspecific interactions between CysB and DNA sequences close to the *pqsR* transcriptional start sites. Aside from a preference for A-T-rich DNA sequences,

FIG 7 Purified CysB interacts with the *pqsR* and *cysH* promoter regions. Radiolabeled DNA fragments were incubated with purified CysB as described in Materials and Methods. Figure 1A shows the relative location of the *pqsR* promoter fragments. Binding reaction mixtures contained between 0.5 and 1 fmol of labeled DNA for each fragment tested. Equal aliquots of binding reaction mixtures were electrophoresed on nondenaturing 5% acrylamide gels, and the position of DNA fragments was visualized by autoradiography. The data presented are representative of at least three independent experiments.

CysB binding sites lack a strong consensus sequence (59, 61), and we were unable to clearly identify specific sequences in the *pqsR* or *cysH* promoter that might be recognized by CysB. Nevertheless, these data provide evidence that both *cysH* and *pqsR* are directly regulated by CysB in *P. aeruginosa*.

Activation of *pqsR* can be blocked by CysB. Since part of our original goal was to investigate LasR-mediated control of *pqsR* and since we found that LasR and CysB interact with similar regions of the *pqsR* promoter, we wondered if CysB could interfere with the activation of *pqsR* transcription by LasR and 3-oxo-C₁₂-HSL. First, we tested whether both proteins could interact with the *pqsR* promoter region using EMSAs. We incubated the *pqsR*-P2 DNA

FIG 8 Purified CysB competes with LasR for binding to the *pqsR* promoter during *in vitro* DNA binding assays. (A and C) Radiolabeled DNA fragments were incubated with purified CysB and LasR as described in Materials and Methods. Binding reaction mixtures contained between 0.02 and 0.05 fmol of labeled DNA for each fragment tested. Equal aliquots of binding reaction mixtures were electrophoresed on nondenaturing 5% acrylamide gels, and the position of DNA fragments was visualized by autoradiography. The amount of each protein included in each binding reaction mixture and the molar ratio of CysB monomers to LasR monomers are shown above each lane. Black arrowheads show the position of CysB-DNA complexes, and white arrowheads show the position of LasR-DNA complexes. (B and D) The results from panels A and C were analyzed by densitometry using Bio-Rad ImageQuant software.

fragment with either LasR and 3-oxo- C_{12} -HSL, CysB, or both proteins and analyzed the DNA-protein complexes formed in each reaction. We observed a single DNA-protein complex formed when the *pqsR*-P2 fragment was incubated with only LasR and a single complex of even higher molecular weight when the fragment was incubated with only CysB (Fig. 8A). In reaction mixtures that contained both proteins, we observed complexes that migrated at the same rate as each of the individual LasR- or CysB-DNA complexes, but we did not detect the formation of any larger-molecular-weight complexes (Fig. 8A) even after X-ray film was exposed to the gels for extended periods of time, suggesting that the protein-DNA complexes we observed contained either LasR or CysB exclusively. Furthermore, as an increasing ratio of CysB to LasR was included in each binding reaction mixture, the amount of the *pqsR*-P2 fragment bound by LasR decreased from 36% to 16% (Fig. 8B). We also performed these experiments using a DNA fragment corresponding to the *lasI* promoter. CysB formed only a small amount of a complex when incubated with the *lasI* promoter alone, most likely due to nonspecific interactions with this DNA fragment, and this complex could not be detected when LasR was present (Fig. 8C). Instead, we observed a single complex formed in all reactions when the reaction mixture contained LasR, and the addition of CysB had little effect on the formation of this complex (Fig. 8C and D). This result showed that CysB does not adversely affect the ability of LasR to bind DNA, and together these experiments suggest that LasR and CysB compete for similar or overlapping binding sites at the *pqsR* promoter.

In order to test the effects of these two proteins *in vivo*, but in the absence of other *P. aeruginosa*-encoded factors, we expressed each protein in *E. coli* and analyzed the effects on the activation of a *pqsR'*-*lacZ* transcriptional fusion. To do this, we transformed *E. coli* strain DH5 α with a plasmid carrying either a *pqsR'*-*lacZ* fusion or a *pqsR'*-*lacZ* fusion and *tacp-lasR*. Then, we added a second plasmid carrying either *araC* and P_{BAD} -*cysB* so that we could control CysB expression or *araC* and P_{BAD} without any coding sequence cloned downstream from the promoter as a control. In the absence of LasR, CysB expression caused no significant change in the basal level of β -Gal activity produced by the *pqsR'*-*lacZ* fusion in *E. coli* (Fig. 9). With the expression of LasR and only the control plasmid present, we found that 354 ± 14 units of β -Gal activity was produced, and this activity increased to 837 ± 21 units when 3-oxo- C_{12} -HSL was added to cultures (Fig. 9). This showed that LasR and 3-oxo- C_{12} -HSL can activate *pqsR* in *E. coli*. However, when CysB was expressed along with LasR, the addition of 3-oxo- C_{12} -HSL caused only a minor increase in β -Gal activity (Fig. 9), indicating that CysB blocked the induction of *pqsR'*-*lacZ* by LasR and 3-oxo- C_{12} -HSL. This also implies that CysB may modulate *pqsR* expression in *P. aeruginosa* by interfering with the LasR-mediated activation of *pqsR* transcription.

DISCUSSION

In *P. aeruginosa*, PqsR plays a key role in the induction of 4-quinolone biosynthesis. We set out to investigate the regulation of *pqsR* transcription since it seemed likely to involve the integration of both cell-to-cell signaling systems and environmental signals. We

FIG 9 CysB can block the activation of *pqsR'*-*lacZ* transcription by LasR and 3-oxo-C₁₂-HSL in *E. coli*. *E. coli* strain DH5α was transformed with two plasmids: one carrying either a *pqsR'*-*lacZ* fusion (pMWC1003) or a *pqsR'*-*lacZ* fusion and *tacp-lasR* (pJF1003) and a second one carrying either a vector control (pACYC-P_{BAD}) or P_{BAD}-*cysB* (pJF52). Bacteria were grown for 2 h in LB medium supplemented with 1 mM IPTG and 1% arabinose either in the absence or presence of 20 nM 3-oxo-C₁₂-HSL. The β-Gal activity produced in each culture was assayed, and data are presented in Miller units as the means ± standard deviations of results from duplicate assays from at least three separate experiments.

began by focusing on LasR, the only factor that has been shown to positively regulate *pqsR* directly. Our experiments showed that LasR interacts with a single binding site in the *pqsR-nadA* intergenic region (Fig. 1), and this interaction only affects *pqsR* transcription (Fig. 2). The LasR binding site is centered 248 bp upstream from the *pqsR* TS1, and it is unclear how LasR activates the transcription of *pqsR* from this distal binding site. Based on the spacing, it seems unlikely that LasR could make direct contact with RNA polymerase without additional DNA binding proteins present to facilitate bending in the *pqsR* promoter region. LasR may instead act indirectly, possibly through recruiting additional factors to the *pqsR* promoter or by affecting DNA supercoiling in the region (62). A genome-wide identification of LasR binding sites showed that the spacing between these sites and established transcriptional start sites varied widely (38), suggesting that LasR can use diverse mechanisms to affect gene expression.

To identify additional factors that could bind to the *pqsR* promoter region and affect *pqsR* transcription, we used DNA affinity chromatography and found that the MvaT, MvaU, and CysB proteins could associate with the *pqsR-nadA* intergenic region. This experiment did not isolate either LasR or PmpR, a regulatory protein reported to bind the *pqsR* promoter and repress transcription (63). This could have been due to low abundance of these proteins, poor solubility, poor DNA binding under the conditions tested, or a combination of these issues, which illustrates some of the limitations of this technique. Our observation that MvaT and MvaU associated with the *pqsR-nadA* intergenic region was supported by a previous genome-wide identification of MvaT and MvaU binding sites (52). We did not observe any effect on either *pqsR* or *nadA* expression in *mvaT* or *mvaU* single mutant strains, and we were unable to construct an *mvaT mvaU* double mutant. However, Li et al. reported the successful construction of an *mvaT mvaU* mutant and additionally reported that PQS production was greatly reduced in this strain (54). While this finding might sug-

gest a potential role for MvaT and MvaU in positively regulating *pqsR*, it is inconsistent with the established functions of MvaT and MvaU, which like other H-NS family members appear to oligomerize along A-T-rich regions of DNA to silence gene expression (64). Additionally, depletion of both MvaT and MvaU from *P. aeruginosa* cells appears to cause wide-scale changes in gene expression (52), so it is difficult to tell whether the phenotype reported by Li et al. was due to direct or indirect regulatory effects. Therefore, the role of MvaT and MvaU in controlling *pqsR* expression, if any, remains unclear.

With all of this in mind, we decided to focus on CysB since no previous studies had provided any connection between CysB and PQS biosynthesis. CysB was first characterized in enteric bacteria as a master regulator of sulfur assimilation through the cysteine biosynthetic pathway, which is the predominant route of sulfur acquisition in bacteria (40). CysB also appears to have this role in *P. aeruginosa* based on the growth phenotypes displayed by *cysB* mutants. Additionally, we found evidence that CysB directly regulates *cysH* in *P. aeruginosa* (Fig. 6 and 7), and others showed that CysB regulates *cysP*, which encodes the periplasmic sulfate binding protein (44). In enteric bacteria the genes for sulfate uptake and reduction to sulfide are activated by CysB when it binds the ligand NAS, which is derived from the direct cysteine precursor OAS. However, although *cysH* was differentially regulated in response to cysteine levels, we found that neither NAS nor OAS was able to stimulate the expression of *cysH* (Fig. 6). Furthermore, previous studies showed that the *in vitro* DNA binding activity of recombinant CysB proteins from *P. aeruginosa* and *P. putida* was not affected by NAS or OAS (44, 59). So while CysB seems to fulfill the same regulatory functions in *P. aeruginosa*, it appears to respond to different signals than those in enteric bacteria. Instead, CysB may interact with other intermediates in the cysteine and methionine biosynthetic pathway, or it may regulate in response to sulfide, which binds to CysB and acts as an anti-inducer in *Salmonella* Typhimurium (65).

We confirmed that CysB can directly interact with the *pqsR* promoter region (Fig. 6), and found that CysB can repress *pqsR* transcription and PQS production (Fig. 4 and 5). We also provided evidence that CysB can compete for binding with LasR at the *pqsR* promoter (Fig. 8) and showed that CysB can block LasR and 3-oxo-C₁₂-HSL from activating a *pqsR'*-*lacZ* fusion in *E. coli* (Fig. 9), providing a potential mechanism for these regulatory effects. But unexpectedly and in contrast to *cysH* expression, *pqsR* expression was not altered in response to cysteine levels (Fig. 6). This finding suggests that CysB may regulate *pqsR* in response to other signals. Alternatively, CysB may interact with the *pqsR* promoter independent from ligands, and the regulatory effects may be due instead to the amounts of CysB that are present to compete with LasR for binding at the *pqsR* promoter. In enteric bacteria CysB appears to be autoregulatory (40), but *cysB* expression has not been investigated in *P. aeruginosa*. Interestingly, CysB also regulates other genes in *P. aeruginosa* that are not directly linked to sulfur metabolism or cysteine biosynthesis. CysB positively regulates the expression of *algD*, which encodes an enzyme that is essential for synthesis of the polysaccharide alginate (55). It also positively regulates the *pvdS* gene, which encodes an extracytoplasmic function sigma factor that controls numerous genes involved in iron metabolism and virulence, including genes for the biosynthesis of the siderophore pyoverdine (44). Since CysB can modulate the expression of several genes that affect host coloniza-

tion and virulence factor production, it is interesting to speculate that perhaps CysB could play a role in the transition from a free-living to host-associated growth environment, which would also include changes in available sulfur sources. Overall, CysB appears to have multiple functions in *P. aeruginosa* that extend beyond sulfur uptake and cysteine biosynthesis.

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