JOURNAL OF BACTERIOLOGY, May 2010, p. 2557–2568 Vol. 192, No. 10
0021-9193/10/$12.00 doi:10.1128/JB.01528-09
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Quorum-Sensing Regulation of a Copper Toxicity System in *Pseudomonas aeruginosa*▽

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Received 22 November 2009/Accepted 4 March 2010

The LasR/LasI quorum-sensing system in *Pseudomonas aeruginosa* influences global gene expression and mediates pathogenesis. In this study, we show that the quorum-sensing system activates, via the transcriptional regulator *PA4778*, a copper resistance system composed of 11 genes. The quorum-sensing global regulator LasR was recently shown to directly activate transcription of *PA4778*, a *cueR* homolog and a MerR-type transcriptional regulator. Using molecular genetic methods and bioinformatics, we verify the interaction of LasR with the *PA4778* promoter and further demonstrate the LasR binding site. We also identify a putative PA4778 binding motif and show that the protein directly binds to and activates five promoters controlling the expression of 11 genes—*PA3519* to *-15*, *PA3520*, *mexPQ-opmE*, *PA3574.1*, and *cueA*, a virulence factor in a murine model. Using gene disruptions, we show that *PA4778*, along with 7 of 11 gene targets of PA4778, increases the sensitivity of *P. aeruginosa* to elevated copper concentrations. This work identifies a cellular function for *PA4778* and four other previously unannotated genes (*PA3515*, *PA3516*, *PA3517*, and *PA3518*) and suggests a potential role for copper in the quorum response. We propose to name *PA4778 cueR*.

Pseudomonas aeruginosa is an opportunistic human pathogen and a major cause of infections in persons with cystic fibrosis, burns, and implanted medical devices (44). The pathogenesis of *P. aeruginosa* is mediated in part by the cell density-dependent activation of genes that allow bacterial growth in infected tissues and avoidance of host defense mechanisms. Cell density signaling, or quorum sensing (QS), is the process by which cells sense and respond to the population density of the local environment. The best-understood QS systems in *P. aeruginosa* involve acyl-homoserine lactone (acyl-HSL) signals that accumulate as a bacterial population approaches and enters stationary phase. One such acyl-HSL signal, $3OC_{12}$-HSL, is produced by the enzyme LasI and is recognized by the response regulator protein LasR, a global transcriptional regulator (11, 31, 33). A second acyl-HSL signal, C_4-HSL, is likewise produced by the enzyme RhlI and recognized by its cognate regulator RhlR (35, 62). When the concentrations of $3OC_{12}$-HSL and C_4-HSL reach some critical threshold, their cognate regulators LasR and RhlR, respectively, activate the expression of multiple genes, including those encoding virulence factors.

Genome-wide transcriptional studies have shown that the transcription factors LasR and RhlR, when activated by their respective acyl-homoserine lactone signals, direct the expression of hundreds of genes in *P. aeruginosa* (46, 59). These studies revealed that acyl-HSL-mediated quorum sensing is a global regulatory system that broadly influences gene expression. Genes involved in diverse cellular processes, such as DNA replication, RNA transcription and translation, amino acid synthesis, chemotaxis, biofilm formation, and antibiotic resistance, were shown to be affected by the presence of acyl-HSL signals.

Of particular interest is the quorum-sensing regulation of virulence factors. Many QS-regulated genes are involved in the production or export of exoproducts, and often the secreted products are degradative enzymes or toxic factors. LasR regulates the production of secreted proteases, genes responsible for hydrogen cyanide synthesis, and genes responsible for pyocyanin synthesis (47). RhlR also activates the hydrogen cyanide and pyocyanin genes, as well as those responsible for rhamnolipid synthesis (37, 60, 61). The importance of the two acyl-HSL signals in pathogenesis has been demonstrated in a variety of animal infection models. Quorum-sensing-deficient *P. aeruginosa* strains have shown reduced virulence in a variety of hosts, including both acute and chronic murine infection models (32, 43, 45, 50, 55, 64), *Caenorhabditis elegans* (54), *Arabidopsis thaliana* (40), *Galleria mellonella* (21), and *Dictyostelium discoideum* (9).

Both the Las and Rhl systems are highly integrated into multiple cellular pathways. One such pathway is the oxidative-stress response. Quorum sensing has been shown to trigger gene expression changes, such as the activation of the Mn-cofactored superoxide dismutase (*sodA*), Fe-cofactored superoxide dismutase (*sodB*), and catalase (*katA*) genes, as well as phenotypic changes, such as increased resistance to hydrogen peroxide and phenazine methosulfate (15). The *sodA*, *sodB*, and *katA* genes are also upregulated in response to high levels of copper (57) and in biofilms (14). Biofilms of *Escherichia coli* strains lacking *sodA* and *sodB* genes have been shown to be more sensitive to metals than those of the wild type (WT) (14), suggesting that protection from oxidative stress plays an important role in defense against toxic metal species. Thus, it has been proposed that the activation of oxidative-stress pathways by

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▽ Published ahead of print on 16 March 2010.

QS serves to increase the tolerance of *P. aeruginosa* biofilms for toxic metal species (14).

In this work, we identified an additional metal resistance system that is activated by quorum sensing. The components of the system are encoded by 11 genes and are controlled by the CueR homolog and transcriptional regulator PA4778. LasR was recently shown to directly bind to the *PA4778* promoter (12). We used both *in vivo* and *in vitro* assays to verify that LasR directly activates *PA4778* transcription. Bioinformatics analysis of the *PA4778* promoter revealed a LasR box, and this was confirmed with site-directed mutagenesis of the binding motif. We identified a putative PA4778 binding motif and demonstrated the direct binding of PA4778 to 5 target promoters regulating 11 genes. We then showed that deletion of *PA4778* increased the sensitivity of *P. aeruginosa* to copper and that disruption of 7 of the 11 PA4778-targeted genes influenced the organism's sensitivity to copper. We conclude that *PA4778* controls a copper resistance regulon and propose to name *PA4778 cueR*.

MATERIALS AND METHODS

Plasmids and strains. The plasmids and strains used in this study are shown in Table 1.

Growth conditions, media, and chemicals. Luria-Bertani (LB) broth and agar were used for routine cultivation of bacteria. All cultures were started from a single colony, grown overnight in LB medium plus appropriate antibiotics, and subcultured into fresh medium to an initial optical density at 600 nm (OD_{600}) of 0.08. The following antibiotic concentrations were used: tetracycline (15 μg/ml for *E. coli*; 50 μg/ml for *P. aeruginosa*), carbenicillin (50 μg/ml for *E. coli*; 150 μg/ml for *P. aeruginosa*), gentamicin (5 μg/ml for *E. coli*; 75 μg/ml for *P. aeruginosa*), kanamycin (50 μg/ml for *E. coli*), and mercury chloride (7.5 μg/ml for *P. aeruginosa*). $3OC_{12}$-HSL (2 μM) and C_4-HSL (10 μM) (Cayman Chemical) were added to the initial subculture as indicated.

Construction of mutants. We used the method of gene splicing by overlap extension (SOE) (16) to generate gene deletion mutants in *P. aeruginosa* PAO1. For each deletion mutant, four primers were designed that amplify ca. 1,000 bp of sequence immediately upstream from the gene and 1,000 bp downstream of the gene. The internal primers contained overlapping complementary sequences, as well as an internal restriction enzyme site. The initial round of PCR produced two PCR products that were then purified, combined, and reamplified using the outside primers in order to create a single product. The outside primers were tailed with the appropriate restriction sites so that the PCR product could be cloned into pEXG2. The plasmids were introduced into *P. aeruginosa* by conjugation and selection on gentamicin-containing plates. The cointegrants were resolved by growing the bacteria on plates containing 6% sucrose. The survivors represented either wild-type *P. aeruginosa* or the deletion mutant. Sucrose-resistant colonies were screened by PCR spanning the deleted gene, and the deletion strains were identified by gel analysis of the PCR product, restriction digestion of the PCR product, and sequencing. The *lasI rhlI* double mutant used in Fig. 1B was constructed by introducing the *rhlI* deletion into a PAO1 *lasI* strain.

Transcriptional profiling. Transcriptional-profiling experiments with PAO-MW1 were done in both LB and modified FAB media (59) in the presence of tetracycline and mercury chloride with $3OC_{12}$-HSL and C_4-HSL as indicated. For RNA isolation, aliquots were removed at both mid-logarithmic phase and early stationary phase. For transcriptional-profiling experiments with PAO1 and PAO1 *cueR*, cultures were grown in a Coy anaerobic chamber (0 to 5 ppm oxygen) in LB medium supplemented with 100 mM potassium nitrate. The strains were grown to an OD_{600} of 0.15, at which point each culture was divided into two flasks. One received copper(I) tetrakis (Sigma) (10 μM), and the other received an equivalent volume of water. The cultures were then further incubated in the anaerobic chamber for 3 h before RNA was isolated.

RNA was isolated with RNEasy Mini spin columns (Qiagen), along with the on-column DNase treatment (Qiagen). RNA integrity was verified with agarose gel electrophoresis and spectrophotometry. No chromosomal DNA was visible on the agarose gel. RNA (10 μg) was used as a template for cDNA synthesis with Superscript II (Invitrogen). cDNA was purified with QIAquick PCR purification spin columns (Qiagen), and 3 μg cDNA was fragmented with 0.6 unit of DNase (Ambion) to 50 to 200 bp. The labeling, hybridization to the GeneChip *Pseudomonas aeruginosa* Genome Arrays (Affymetrix), and scanning was done according to the GeneChip Expression Analysis Technical Manual (Affymetrix). Raw probe level intensities were normalized to gene expression levels with the robust multiarray average (RMA) method (19).

Construction of strains for assaying transcriptional reporters in a heterologous host. The *in vivo* binding assays in a heterologous host were performed in *E. coli* DH5α. Each strain contained two plasmids. The first plasmid was an expression vector with either *lasR* (pJTT201), *rhlR* (pJTT202), or a negative control (pMMB67EH). The second plasmid contained a promoter-*lacZ* transcriptional fusion. The promoters for *rsaL* (pJTT300), *cueR* (pJTT304), and *mvfR* (pJTT302) were assayed. Strains were prepared by first cloning the entire coding sequence of the transcription factor (*lasR* or *rhlR*) into pMMB67EH and transforming it into *E. coli* DH5α. Individual colonies were isolated on LB-gentamicin plates and screened for the presence of inserts by both PCR and sequencing. Strains containing the recombinant plasmids were then made competent for heat shock transformation in preparation for the additional vector. The three gene promoters were then cloned into pZE21-lacZ, transformed into strains already containing a single plasmid, and isolated on LB-gentamicin-kanamycin plates. Inserts were again verified by PCR and sequencing.

β-Galactosidase measurement. β-Galactosidase assays with PAO1 *lasI rhlI*, along with the integrated mini-ctx-lacZ derivatives, were performed in LB with tetracycline, and $3OC_{12}$-HSL and C_4-HSL were added to the initial subculture as indicated. The cultures were grown to mid-log phase (OD_{600} = 0.6), at which point β-galactosidase was assayed (Fig. 1B). β-Galactosidase assays with PAO1 *lasI* (see Fig. 4), along with the integrated mini-ctx-lacZ derivatives, were performed in LB with tetracycline, and $3OC_{12}$-HSL (5 μM) was added to the initial subculture as indicated. For the β-galactosidase assays with *E. coli* DH5α, cultures were grown in supplemented A medium (36) with gentamicin and kanamycin to an OD_{600} of 0.3, at which point IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 100 μM (see Fig. 2). The cultures were incubated for 1 hour at 37°C, followed by measurements of β-galactosidase activity as described previously (27). β-Galactosidase assays in PAO1 and PAO1 *cueR*, along with the integrated mini-ctx-lacZ vector, were performed in LB with tetracycline (see Fig. 8). The cultures were grown to mid-log phase (OD_{600} = 0.6), and two aliquots were removed. Copper sulfate was added to one of the aliquots to 1 mM final concentration, while the other received an equivalent volume of water. The cultures were incubated at 37°C for 1 hour before β-galactosidase activity was measured.

Protein purification. Plasmid pJTT100 was generated by PCR amplification of the *lasR* gene from the *P. aeruginosa* PAO1 genome, followed by cloning into the EcoRI/HindIII sites in the pPSV35 vector. Plasmid pJTT101 was generated similarly; however, the upstream primer in the *lasR* amplification step was tailed with a sequence coding for a His_6 epitope tag and a single glycine spacer (CACCACCACCACCACCACGGA) after the translational start codon. The expression vectors with wild-type *cueR* (pJTT200) and carboxy-His_6-tagged *cueR* (pJTT203) were generated in a similar manner. The inserts were verified by sequencing. In order to demonstrate that the His_6-LasR construct was biologically active, pJTT100 and pJTT101 were introduced into PAO1 *lasR* by conjugation and tested for elastase activity with the elastin-Congo Red reagent (Sigma). Briefly, bacteria were grown to stationary phase in LB, and following centrifugation, 1 ml of supernatant was added to 2 ml of 100 mM Tris buffer (pH 8.8), along with 10 mg elastin-Congo Red. The reaction mixtures were incubated at 37°C with agitation for 2 h. The bacteria were removed by centrifugation, and the OD_{495} of the supernatant was measured. Elastase activity was then determined by the following equation: elastase activity = ΔOD_{495}/[(ml supernatant) (reaction time)], where ΔOD_{495} is the change in absorption of the sample relative to a cell-free control. In the presence of 0.5 mM IPTG, the His_6-tagged LasR (pJTT101) restored elastase activity to 90.2% of that of wild-type LasR (pJTT100) (0.148 versus 0.164, respectively). In the absence of IPTG induction, pJTT101 restored elastase activity to 59.6% of that of pJTT100 (0.065 versus 0.109, respectively). The CueR-His_6 complementation assay was performed by transferring pJTT203 to PAO1 *cueR*. The presence of the His-tagged gene restored wild-type levels of copper resistance to the *cueR* mutant, as determined by a disk diffusion assay.

His-tagged proteins were purified following cloning of the constructs into pET15b and transforming them into *E. coli* BL21 Rosetta2 (Novagen). A 1-liter culture in LB containing 150 μg/ml carbenicillin (with 10 μM $3OC_{12}$-HSL included for the His_6-LasR purification) was grown to an OD_{600} of 0.5, and IPTG was added to 1 mM. The culture was incubated for an additional 20 h at 15°C with shaking. The cells were collected by centrifugation (10,000 × *g* for 20 min) and resuspended in 5 ml cold lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole). All subsequent steps were performed on ice or at 4°C.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli		
DH5α	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	13
BL21(DE3)/pLysS	F^- *ompT* $hsdS_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysS(Cm^r)	Novagen
SM10	*thi-1 leuB6 supE44 tonA21 lacY1 recA*::RP4-2-Tc::Mu Km^r	56
P. aeruginosa		
PAO1	Wild-type clinical isolate	52
PAO1-MW1	Δ*lasI* Δ*rhlI* derivative of PAO1; *rhlI*::Tn*501 lasI*::*tetA* Hg^r Tc^r	61
PAO1 *lasI rhlI*	Isogenic deletion strain constructed with pJTT1 and pJTT2	This study
PAO1 *lasI*	Isogenic deletion strain constructed with pJTT1	This study
PAO1 *cueR*	Isogenic deletion strain constructed with pJTT3	This study
PAO1 *lasR*	Isogenic deletion strain constructed with pJTT4	This study
PAO1 *PA3520*	Isogenic deletion strain constructed with pJTT5	This study
19045	Δ*PA3515* derivative of PAO1; *PA3515*::*lacZ* Tc^r	20
37652	Δ*PA3516* derivative of PAO1; *PA3516*::*phoA* Tc^r	20
52422	Δ*PA3517* derivative of PAO1; *PA3517*::*phoA* Tc^r	20
39841	Δ*PA3518* derivative of PAO1; *PA3518*::*phoA* Tc^r	20
6803	Δ*PA3519* derivative of PAO1; *PA3519*::*lacZ* Tc^r	20
42105	Δ*PA3521* derivative of PAO1; *PA3521*::*phoA* Tc^r	20
7848	Δ*PA3522* derivative of PAO1; *PA3522*::*lacZ* Tc^r	20
54898	Δ*PA3523* derivative of PAO1; *PA3523*::*phoA* Tc^r	20
55074	Δ*PA3574.1* derivative of PAO1; *PA3574.1*::*phoA* Tc^r	20
361	Δ*PA3920* derivative of PAO1; *PA3920*::*lacZ* Tc^r	20
Plasmids		
pEXG2	ColE1 suicide vector; *mob*$^+$ *sacB*$^+$ Gm^r	42
pJTT1	pEXG2 with Δ*lasI* allele constructed by SOE	This study
pJTT2	pEXG2 with Δ*rhlI* allele constructed by SOE	This study
pJTT3	pEXG2 with Δ*cueR* allele constructed by SOE	This study
pJTT4	pEXG2 with Δ*lasR* allele (nucleotides 1 to 688 deleted) constructed by SOE	This study
pJTT5	pEXG2 with Δ*PA3520* allele constructed by SOE	This study
Mini-ctx-lacZ	Integration vector for single-copy chromosomal *lacZ* fusion; Tc^r	17
pJTT-PA4778	Mini-ctx-lacZ with −1 to −300 *cueR* fragment in HindIII/AvrII site upstream of *lacZ*	This study
pJTT-PA4778B	Mini-ctx-lacZ with −1 to −143 *cueR* fragment in HindIII/AvrII site upstream of *lacZ*	This study
pJTT-PA4778C	Mini-ctx-lacZ with −1 to −59 *cueR* fragment in HindIII/AvrII site upstream of *lacZ*	This study
pJTT-PA4778-mut1	pJTT-PA4778 with T-to-C change at position −75 in *cueR* upstream region	This study
pJTT-PA4778-mut4	pJTT-PA4778 with C-to-T change at position −72 in *cueR* upstream region	This study
pJTT-PA4778-mut5	pJTT-PA4778 with A-to-G change at position −71 in *cueR* upstream region	This study
pJTT-PA4778-mut14	pJTT-PA4778 with A-to-G change at position −62 in *cueR* upstream region	This study
pJTT-PA4778-mut15	pJTT-PA4778 with G-to-A change at position −61 in *cueR* upstream region	This study
pJTT-PA3519	Mini-ctx-lacZ with −1 to −300 *PA3519* fragment in EcoRI/AvrII site upstream of *lacZ*	This study
pJTT-PA3520	Mini-ctx-lacZ with −1 to −300 *PA3520* fragment in HindIII/AvrII site upstream of *lacZ*	This study
pJTT-mexP	Mini-ctx-lacZ with −1 to −402 *mexP* fragment in HindIII/AvrII site upstream of *lacZ*	This study
pJTT-PA3920	Mini-ctx-lacZ with −1 to −300 *PA3920* fragment in HindIII/AvrII site upstream of *lacZ*	This study
pJTT-PA3574.1	Mini-ctx-lacZ with −1 to −329 *PA3574.1* fragment in HindIII/AvrII upstream of *lacZ*	This study
pPSV35	Broad-host-range expression vector from P_{lacUV5}; *lacI*^{q} Gm^r	42
pJTT100	pPSV35 with *lasR* in EcoRI/HindIII site	This study
pJTT101	pPSV35 with His_6-*lasR* in EcoRI/HindIII site	This study
pMMB67EH	Broad-host-range expression vector from P_{tac}; *lacI*^{q} Ap^r	10
pJTT200	pMMB67EH with *cueR* in EcoRI/HindIII site	This study
pJTT201	pMMB67EH with *lasR* in EcoRI/HindIII site	This study
pJTT202	pMMB67EH with *rhlR* in EcoRI/HindIII site	This study
pJTT203	pMMB67EH with *cueR*-His_6 in EcoRI/HindIII site	This study
pZE21-MCS2	ColE1 expression vector from $P_{LtetO-1}$; Kn^r	26
pZE21-lacZ	pZE21-MCS2 with *lacZ* in KpnI/HindIII site	This study
pJTT300	pZE21-lacZ with −1 to −300 *rsaL* fragment in XhoI/KpnI site upstream of *lacZ*	This study
pJTT302	pZE21-lacZ with −1 to −300 *mvfR* fragment in XhoI/KpnI site upstream of *lacZ*	This study
pJTT304	pZE21-lacZ with −1 to −300 *cueR* fragment in XhoI/KpnI site upstream of *lacZ*	This study
pET15b	ColE1 poly-His tag expression vector from P_{T7lac}; Ap^r	Novagen
pJTT400	pET15b with His_6-*lasR* in XbaI/BamHI site	This study
pJTT404	pET15b with *cueR*-His_6 in XbaI/BamHI site	This study

Lysozyme was added to 1 mg/ml and incubated for 30 min. Samples were sonicated for 12 cycles of 15 s each on a Branson 450 sonicator at 40% power. The cell lysates were centrifuged at 10,000 × *g* for 30 min, and the supernatant was passed through a 0.2-μM filter; 400 μl of a 50% Ni-nitrilotriacetic acid (NTA) slurry (Qiagen) was added to the filtered supernatant, and the sample was incubated for 1 hour at 4°C with shaking. The subsequent purification steps were performed at 25°C. The cell extract was then passed through a 2-ml empty chromatography column (Bio-Rad) by gravity, and the Ni-NTA beads were

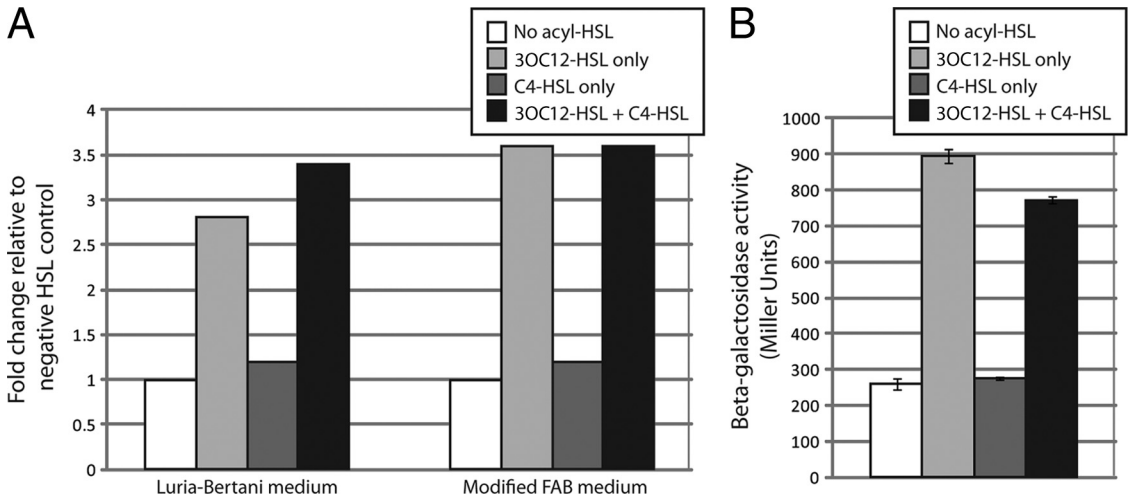

FIG. 1. *cueR* expression changes in response to acyl-HSL signals. (A) Strain PAO1-MW1 was grown in the presence of $3OC_{12}$-HSL, C_4-HSL, or both, and RNA was sampled in mid-log phase. Microarray expression profiles were then generated, and transcript expression levels were normalized to the acyl-HSL-free control cultures to give the fold changes. The experiment was done in both LB and modified FAB media. (B) Plasmid pJTT-PA4778 was integrated into the genome of strain PAO1 *lasI rhlI*, and *cueR* expression was assayed in the presence and absence of exogenous acyl-HSL signals.

washed twice with 2 ml wash buffer 1 (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole) and 3 times with 2 ml wash buffer 2 (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 50 mM imidazole). The sample was eluted by passing 2 ml of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole) through the column. In the case of CueR-His_6, the pH of the elution buffer was adjusted to 7.5. The imidazole was removed by passing the buffer through a PD10 desalting column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.5, 200 mM KCl. The sample was then concentrated with a 10,000-MW Amicon spin column (Millipore) filter, and 100% glycerol was added in a 1:1 ratio to give a final protein buffer of 25 mM HEPES, pH 7.5, 100 mM KCl, and 50% glycerol. The protein was judged to be greater than 95% pure by SDS-PAGE and the ImageJ software package (1). ImageJ was used to determine the signal intensity of the His_6-LasR band as a percentage of the total signal detected per lane. The protein was stored at −20°C.

EMSAs. DNA probes for electrophoretic mobility shift assays (EMSAs) were generated by PCR from a *P. aeruginosa* PAO1 genomic-DNA prep. The *rsaL-lasI*, *cueR*, and *PA2588* probes were each 300 bp long and spanned the region from +50 downstream of the respective start codons to −250 upstream. The *PA3519*, *PA3520*, *mexP*, *PA3574.1*, and *cueA* probes spanned the region from −1 upstream of the translational start codon to −198, −178, −122, −146, and −196, respectively. The nonspecific-competitor probe used in all experiments was a 300-bp fragment from *PA2588* that spanned from +200 to +500 relative to the start codon. The PCR products were purified, and radiolabeled probes were prepared with [γ-^{32}P]ATP (Perkin Elmer) and T4 polynucleotide kinase (NEB). Unincorporated nucleotides were removed by spin chromatography using Sephadex G-25 (Roche). Ten microliters of the protein storage buffer (25 mM HEPES, pH 7.5, 100 mM KCl, 50% glycerol) containing various amounts of the purified protein was mixed with 10 μl of a solution containing 100 fmol of labeled probe, 1 μg poly(dI-dC), 200 μg bovine serum albumin (BSA), 40 mM Tris, pH 7.5, and 100 mM KCl. Unlabeled specific or nonspecific DNA was added as indicated. Samples were incubated at 25°C for 30 min and then loaded onto a 6% acrylamide nondenaturing gel without loading buffer on a vertical gel apparatus and run for 2 h at 200 V. The gel was then removed and visualized with a storage phosphorimager. Binding affinities were calculated by quantifying gel band intensities with ImageQuant software (GE Healthcare) and then estimating the binding constant (K_d) with the following equation: $\log([\text{DNA} \times \text{protein}]/[\text{DNA}]) = \log[\text{protein}] - \log K_d$.

Site-directed mutagenesis. The region from −1 to −300 upstream of the *cueR* translational start codon in *P. aeruginosa* PAO1 was cloned into the HindIII/KpnI sites in pUC18. For each mutated construct, the plasmid was PCR amplified with *Pfu* Turbo (Stratagene) and two complementary primers, each 35 to 45 bp in length and containing the appropriate nucleotide substitution in the middle. The template vector was then digested with DpnI for 2 h at 37°C, and the remaining DNA was transformed into *E. coli* DH5α. Plasmids were purified from the resultant colonies and were sequenced (Agencourt) to verify the presence of the desired nucleotide substitutions. The mutated promoters were then PCR amplified from the pUC18-derived vectors and cloned into the HindIII/AvrII sites in mini-ctx-lacZ.

Disk diffusion assays. *P. aeruginosa* cultures were grown overnight in LB, along with the appropriate antibiotics. The cultures were grown to an OD_{600} of 2.20, and 20 μl of each culture was then spread onto an LB plate. A paper disk (Laboratory Media Corporation) was placed at the center of each plate, 10 μl of a 1 M copper sulfate solution was added to each disk, and the plates were incubated for 24 h at 37°C.

Bioinformatics analysis of binding motifs. A number of programs at the Regulatory Sequence Analysis Tools website (http://rsat.ulb.ac.be/rsat) were used to find the CueR binding sequences (51). In the first step of our analysis, we used the "dna-pattern" tool to identify *E. coli* CueR-like binding motifs within 300 bp upstream of the *P. aeruginosa* genes listed in Table 2. The results of this analysis were used to construct the CueR binding motif (see Fig. 7B). With the CueR binding motif in hand, we did a genome-wide search in *P. aeruginosa* PAO1 with the "genome-scale dna-pattern" tool in order to find additional promoters that were targeted by CueR. The LasR binding site in the *cueR* promoter was identified with the Fuzzy Search DNA tool in the Sequence Manipulation Suite at http://www.bioinformatics.org. All gene function predictions came from the Pseudomonas Genome Database (63).

RESULTS

LasR directly regulates *cueR* transcription. A previous microarray study showed that *cueR* is activated by the Las quorum-sensing system (46), and recent work showed that LasR binds to the *cueR* promoter *in vitro* (12). The relationship between *cueR* expression and the Rhl quorum-sensing system has not been well described. We used microarray expression arrays to define the transcriptional response of *cueR* to $3OC_{12}$-HSL and C_4-HSL both individually and in tandem. Strain PAO-MW1 (PAO1 *lasI*::*tetA rhlI*::Tn*501*), which cannot generate the acyl-HSL quorum-sensing signals, was grown both in the presence and absence of exogenously supplied acyl-HSL signals. Transcriptional-profiling experiments in both the rich LB medium and the more well-defined modified FAB medium showed that *cueR* expression is positively controlled by $3OC_{12}$-HSL (Fig. 1A). Addition of the QS signal C_4-HSL did not

TABLE 2. Expression changes in the *cueR* mutant both with and without copper

Gene	Fold change[a]		Protein description
	PAO1/ ΔPA4778 + copper	PAO1/ ΔPA4778 − Copper	
hcpC	**2.8**	**7.4**	Secreted protein
prtN	**−2.7**	1.1	Transcriptional regulator
ptrB	**−2.9**	−1.3	Transcriptional regulator
PA0616	**−2.2**	−1.2	Hypothetical protein
PA0619	**−2.0**	−1.2	Probable bacteriophage protein
PA0628	**−2.1**	−1.1	Probable bacteriophage protein
PA0629	**−2.2**	1.0	Predicted chitinase
PA0636	**−2.7**	1.1	Phage-related minor tail protein
rhlC	1.5	2.1	Rhamnosyl transferase
aprA	**2.0**	**−2.0**	Alkaline metalloproteinase precursor
PA1403	**−2.3**	−1.1	Transcriptional regulator
PA1664	**2.1**	**2.5**	Hypothetical protein
PA1666	1.5	**2.1**	Hypothetical protein
PA1667	1.2	**2.3**	Hypothetical protein
pcrH	**2.0**	−1.2	Type III secretion regulatory protein
popB	**2.3**	−1.2	Translocator protein involved in type III secretion
exsE	**2.0**	−1.1	Regulator of type III secretion
pcoB	**17.4**	1.1	Copper resistance protein B precursor
pcoA	**41.1**	1.3	Copper resistance protein A precursor; multicopper oxidase
PA2069	1.7	**2.8**	Probable carbamoyl transferase
PA2405	**2.2**	1.9	Hypothetical protein
opdT	**2.1**	−1.3	Tyrosine porin
PA2807	**23.5**	1.3	Uncharacterized copper-binding protein
ptrA	**18.2**	−1.1	Two-component response repressor
copR	**3.0**	−1.1	Two-component response regulator
ibpA	**−2.3**	−1.4	Heat shock protein
PA3412	**2.3**	−1.1	Hypothetical protein
PA3515	**2.5**	1.1	Hypothetical protein
PA3516	**5.3**	1.1	Probable lyase
PA3519	**5.8**	1.1	Hypothetical protein
PA3520	**7.4**	**2.3**	Probable copper chaperone
exoS	**2.2**	1.0	Type III secretion protein
PA3842	**2.5**	1.2	Probable chaperone
PA3920	**4.0**	1.5	Probable metal binding P-type ATPase
PA4141	1.7	**2.2**	Hypothetical protein
PA4500	−1.3	**−2.1**	Probable binding protein component of ABC transporter
opdD	−1.5	**−2.0**	Glycine-glutamate dipeptide porin
pilA	1.0	**2.4**	Type IV fimbrial precursor
betB	−1.1	**−2.1**	Betaine aldehyde dehydrogenase
PA5481	1.1	**2.0**	Hypothetical protein
PA5482	1.1	**2.2**	Hypothetical protein

[a] Shown are the genes that were differentially expressed in the wild-type strain relative to the *cueR* mutant (change ≥ 2-fold). Transcript expression levels that differ by more than a factor of 2 are shown in boldface.

significantly alter *cueR* expression. The addition of $3OC_{12}$-HSL together with C_4-HSL produced *cueR* expression levels similar to that of $3OC_{12}$-HSL alone. Thus, it appears that *cueR* responds only to $3OC_{12}$-HSL. A promoter-*lacZ* construct was then used to validate the microarray data. We fused *lacZ* to a DNA fragment containing the putative *cueR* promoter in the mini-ctx-lacZ plasmid vectors and used it to integrate the fusion construct into the chromosome of the PAO1 *lasI rhlI* mutant. The acyl-HSL signals $3OC_{12}$-HSL and C_4-HSL were supplied exogenously, and β-galactosidase activity was measured (Fig. 1B). This secondary assay confirmed that *cueR* expression is activated by the Las signal $3OC_{12}$-HSL and is not influenced by the Rhl signal C_4-HSL.

Next, we sought to confirm the findings of Gilbert et al. (12) by showing that LasR directly binds to the *cueR* promoter. Previous studies had used LasR/RhlR-dependent transcriptional reporters in the heterologous host *E. coli* to uncover direct interactions between LasR or RhlR and its target promoters (25, 34). We applied a similar approach. *E. coli* DH5α strains were generated that each contained two vectors—a regulator plasmid with either *lasR* (pJTT201) or *rhlR* (pJTT202) under the control of an IPTG-inducible promoter and a reporter plasmid bearing one of the three promoter-*lacZ* transcriptional fusions: *cueR-lacZ* (pJTT304), *rsaL-lacZ* (pJTT300), or *mvfR-lacZ* (pJTT302). The DNA region upstream of *rsaL* has been shown to directly bind LasR and served as a positive control (41). The *mvfR* gene is known to be regulated by quorum sensing, though the upstream region tested here does not directly bind LasR and functioned as a negative control (65). Constructs were tested for activation of the promoters by either LasR or RhlR in both the presence and absence of the respective cognate autoinducers. Though no promoters were activated by LasR in the absence of $3OC_{12}$-HSL, the addition of LasR, along with $3OC_{12}$-HSL, activated both *cueR* and the positive control *rsaL* (Fig. 2). The presence of RhlR alone did not activate any of the three promoters, and RhlR, along with its cognate autoinducer, resulted in only marginal activation of *cueR* and *rsaL*. The results from the transcriptional reporter assay in *E. coli* again confirmed that *cueR* is activated primarily by LasR/$3OC_{12}$-HSL and further suggest that the interaction between LasR and the *cueR* promoter is direct.

In order to further verify that LasR directly binds to the *cueR* promoter, EMSAs were performed with purified LasR and a radiolabeled 300-bp DNA fragment from the *cueR* upstream region (Fig. 3). The *rsaL-lasI* bidirectional promoter interacts strongly with LasR via multiple LasR binding sites and is shown in Fig. 3 as a positive control (41). The *PA2588*

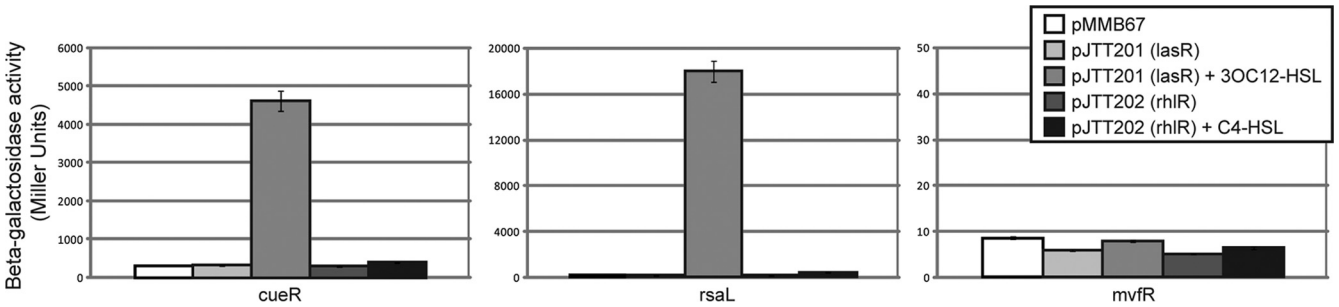

FIG. 2. Direct activation of promoters by LasR and RhlR in a heterologous host. *E. coli* DH5α received two plasmids, a reporter vector containing a promoter-*lacZ* transcriptional fusion and a regulator vector containing either *lasR* (pJTT201), *rhlR* (pJTT202), or a negative control (pMMB67). The acyl-homoserine lactone molecules $3OC_{12}$-HSL and C_4-HSL were added as indicated. The *rsaL* promoter directly binds LasR and is shown here as a positive control. The upstream region of *mvfR* that was tested here has not been shown to bind LasR and was a negative control.

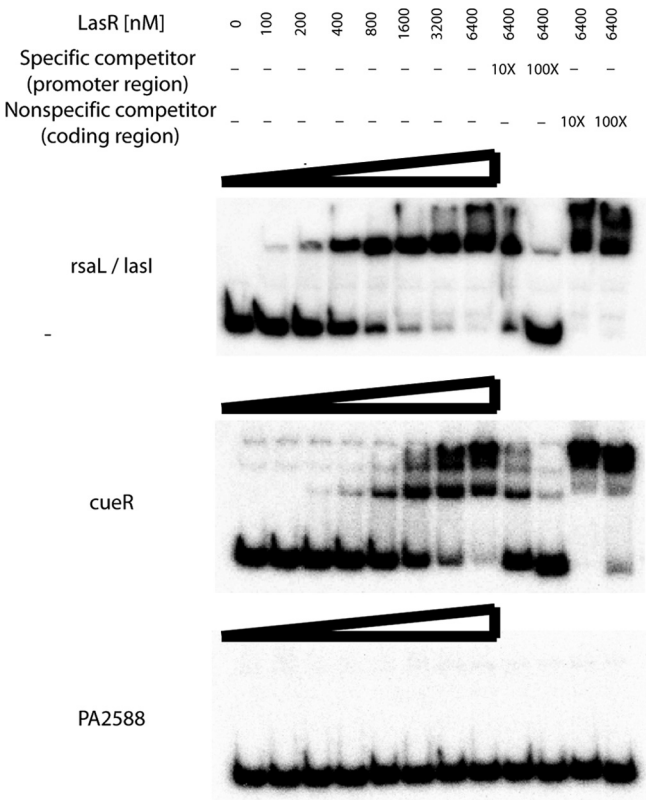

FIG. 3. Electrophoretic mobility shift analysis of the *cueR* promoter. Purified LasR was added to 100 fmol of the labeled promoter DNA. Where indicated, nonlabeled specific or nonspecific competitor DNA was present in the reaction mixture. LasR had previously been shown to bind the *rsaL-lasI* bidirectional promoter region, and this interaction is verified here and shown for reference. The *PA2588* region tested here has never been shown to bind LasR and was a negative control.

promoter is shown as a negative control. In this assay, LasR again bound to the *cueR* promoter. LasR appeared to directly bind to and activate the *cueR* promoter. The binding affinity of this interaction was 1,420 nM, and the binding affinity of LasR with the positive-control promoter *rsaL* was 380 nM. The addition of 10× and 100× concentrations of unlabeled nonspecific competitor DNA had only minor effects on LasR-promoter binding, while identical concentrations of unlabeled specific competitor DNA almost completely abolished the interaction of LasR with the labeled DNA fragment. Thus, it appears that the observed LasR binding is specific to the *cueR* promoter and is not the result of nonspecific DNA binding.

Analysis of the LasR binding motif. A recent study illustrated that LasR activates its target promoters in three distinct ways (47)—multiple LasR proteins may activate the target promoter cooperatively, the protein may bind noncooperatively, or it may interact with RhlR. As illustrated in Fig. 4, the *cueR* promoter possesses the LasR motif (also called the Las box), which corresponds to noncooperative binding of LasR to the promoter (47), though it is difficult to predict the mode of LasR binding based on primary DNA sequence alone (12). We validated the putative LasR binding motif in the *cueR* promoter by generating a series of promoter-*lacZ* constructs with different mutations in the Las box. The modified promoters were cloned into the mini-ctx-lacZ vector and integrated into the genome of PAO1 *lasI* at the *attB* site. The cultures were grown in LB in both the presence and absence of $3OC_{12}$-HSL, and β-galactosidase activity was assayed. Vectors pJTT-PA4778 and pJTT-PA4778B illustrate the activation of *cueR* by $3OC_{12}$-HSL, as expression increased 2- to 3-fold when the autoinducer was included in the growth media (Fig. 4). This was expected, as both vectors contain the complete LasR binding motif. The shorter construct lacking the motif (pJTT-PA4778C) exhibited lower expression in the absence of the autoinducer and was activated only nominally by $3OC_{12}$-HSL. In addition, we mutated several nucleotides that appeared to be well conserved in LasR binding motifs and measured the effect that each had on *cueR* expression. In relation to the LasR motif shown in Fig. 4, nucleotides at positions 1, 4, 5, 14, and 15 were individually altered. In the absence of $3OC_{12}$-HSL, none of the five mutations had a marked effect on *cueR* expression (Fig. 4). When $3OC_{12}$-HSL was added to the medium, however, a reduced level of activation was noted in all cases. The mutation at position 4 had the smallest effect on expression, as *cueR* still increased nearly 2-fold in response to the autoinducer. The mutations at positions 5 and 14 affected *cueR* expression most significantly, as the gene was only weakly activated in the presence of $3OC_{12}$-HSL. It thus appears that the point mutations in the *cueR* promoter do not change the basal transcriptional activity of the gene but inhibit activation in response to $3OC_{12}$-HSL by altering the LasR-promoter interaction.

CueR is a MerR-type transcriptional regulator involved in copper resistance. An alignment of the CueR sequence showed that it is a member of the MerR family of transcriptional regulators. MerR-type regulators have a characteristic N-terminal helix-turn-helix DNA binding region, as well as a C-terminal region that recognizes a particular environmental stimulus. Common environmental triggers include heavy metals, antibiotics, and oxidative stress (5). Among the MerR family members that recognize metals, unique metal binding motifs can be found for those that recognize the monovalent and divalent cations (7) (Fig. 5). While the proteins that recognize the 2+ ions have a metal binding cysteine residue at the N-terminal end of the dimerization helix, the proteins that respond to the 1+ ions have a Ser-Ala-X-(Lys/Arg) signature region. CueR aligns most closely with the proteins that recognize monovalent cations, and in particular with the copper-responsive CueR, a protein shown to mediate resistance to toxic copper levels in *E. coli* (29, 38, 53).

Given that CueR is homologous to known copper regulators, we tested the sensitivity of the *cueR* mutant to high levels of copper, as well as other metals. Both WT and *cueR* mutant strains were grown overnight in LB medium and then plated onto LB agar. A 10-μl aliquot of a 1 M copper sulfate solution was spotted onto a paper disk (5-mm diameter), which was then placed on the center of each plate. The plates were then incubated for 24 h at 37°C. As illustrated in Fig. 6, the mutant was more susceptible to high copper levels than the wild-type PAO1. *cueR* provided in *trans* from pJTT200 (ptac-*cueR*) was able to complement the mutant and restore the wild-type level of copper resistance. We also tested *cueR* sensitivity to silver, nickel, cadmium, iron, mercury, zinc, cobalt, and manganese,

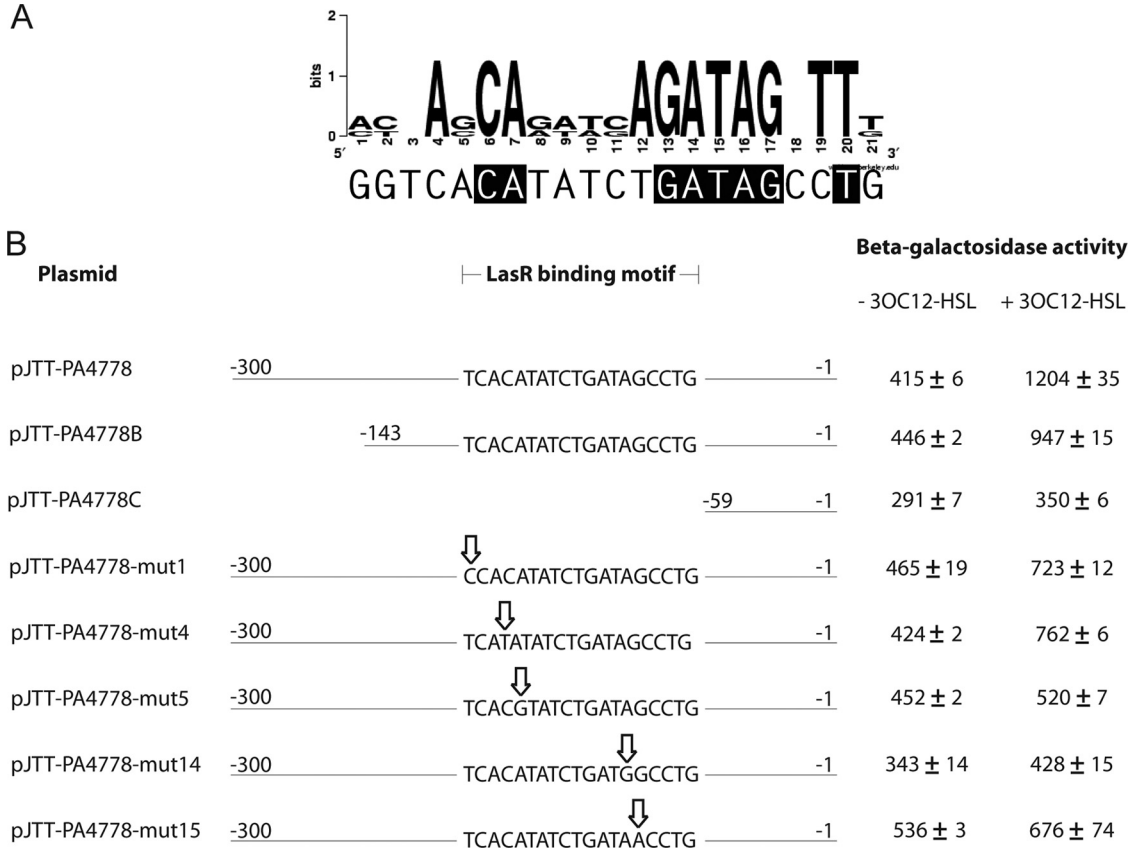

FIG. 4. A LasR binding motif upstream of *cueR*. (A) The weblogo shown here is a LasR binding motif from Schuster et al. (47), and the sequence below it is the *cueR* upstream sequence from −77 to −57 relative to the translational start codon. The nucleotides that perfectly match the binding motif are highlighted in black. (B) Activation of wild-type and mutant *cueR* promoters in PAO1 *lasI*. The extent of the *cueR* upstream region in each construct is indicated relative the *cueR* translational start codon. β-Galactosidase activity is given in Miller units, along with standard deviations. pJTT-PA4778-mut1 through pJTT-PA4778-mut15 each contain a single-base-pair mutation, indicated by an arrow, in the LasR binding motif. The strains were grown both in the presence and absence of $3OC_{12}$-HSL, as shown.

though no changes in resistance relative to the wild type were observed (data not shown).

In order to better understand the role played by CueR in the response to copper, we generated transcriptional profiles of PAO1 and the isogenic *cueR* deletion mutant using Affymetrix *Pseudomonas aeruginosa* Genome Arrays. The strains were grown in Luria-Bertani medium with and without 10 μM copper sulfate. Previous transcriptional studies had shown that a large number of genes were activated in response to elevated levels of copper (6, 57). A subset of these genes, including *pcoAB*, *PA2807*, *ptrA*, *copR*, *PA3412*, *PA3519* to *-15*, *PA3520*, and *PA3920*, appeared to require *cueR* for activation (Table 2). When copper was added to the medium, these genes were highly expressed in the wild-type strain relative to the mutant. In addition, both the number of genes with changed transcript levels and the magnitudes of the changes between the wild-type and mutant strains were greater when copper was present in the medium. This is typical of a MerR-type transcriptional regulator, as a threshold level of the particular metal must be present in order to trigger protein activity.

Direct targets of CueR. Using the list of genes whose transcript levels were affected by the *cueR* mutation, as well as knowledge of the *E. coli* CueR binding motif, we attempted to define the CueR binding motif and identify its direct regulatory targets. There are two distinguishing features of promoters targeted by MerR-type transcription factors (5). The first is an extended spacer region between the −35 and −10 sequence elements. Longer (19- or 20-bp) spacing is necessary for proper regulation by a MerR-type protein. The second feature of the MerR-targeted promoter is a dyad symmetrical region near the spacer that is generally the regulator binding site. The *E. coli* CueR protein, for example, has two known direct targets—*cueA* and *cueO*—and each promoter has the characteristic 19-bp spacer region, as well as a significant dyad symmetrical region (29, 38, 53, 66) (Fig. 7A).

Using the *E. coli* CueR binding motif as a template, we searched for potential CueR binding sites in the *P. aeruginosa* genome. Our initial search included only the upstream regions of genes found in Table 2, and three operons were found to contain a CueR-like binding motif: *PA3519* to *-15*, *PA3520*, and *cueA* (*PA3920*) (Fig. 7B). The initial 9 base pairs of the *E. coli* CueR motif, CTTGACCTT, are nearly identical to the initial 9 base pairs of the *P. aeruginosa* CueR motif, (C/G/A) TTGACCTT. Also present in the predicted CueR motifs are the extended 19-bp spacer regions between the −35 and −10 elements and significant regions of dyad symmetry near the

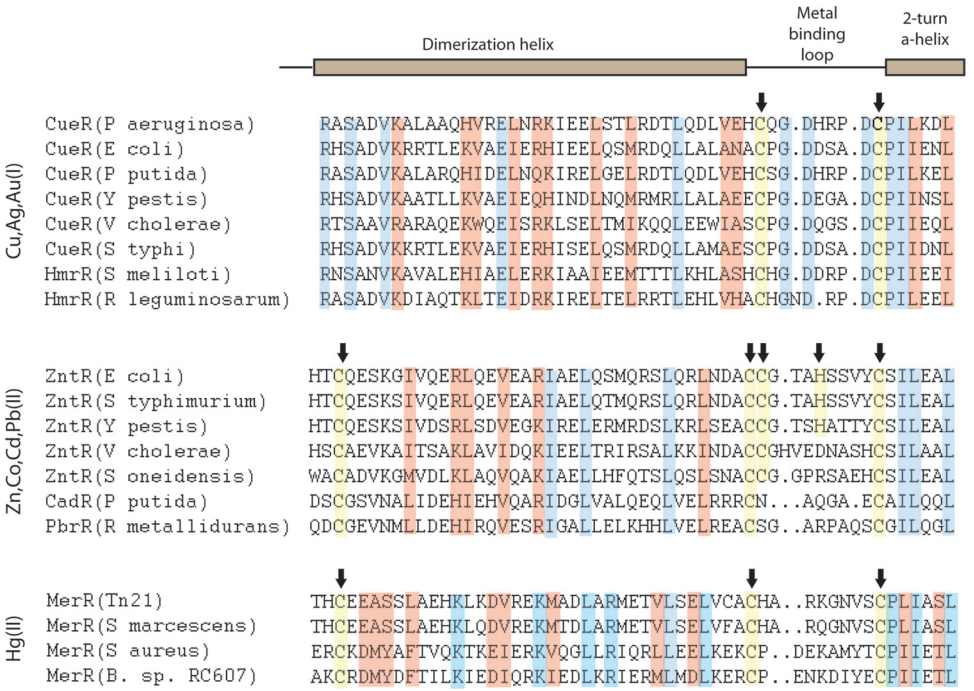

FIG. 5. The MerR family of transcriptional regulators. Residues that are either known or predicted to bind metal are highlighted in yellow and marked with arrows. Identical residues in each subgroup are shown in blue, and conserved residues are shown in red. The figure is modified from reference 7 with permission of the publisher. P putida, *Pseudomonas putida*; Y pestis, *Yersinia pestis*; V cholerae, *Vibrio cholerae*; S typhi, *Salmonella enterica* serovar Typhi; S meliloti, *Sinorhizobium meliloti*; R leguminosarum, *Rhizobium leguminosarum*; S typhimurium, *Salmonella enterica* serovar Typhimurium; S oneidensis, *Shewanella oneidensis*; R metallidurans, *Ralstonia metallidurans*; S marcesens, *Serratia marcesens*.

spacer regions (Fig. 7B). The position of the promoter in relation to the start codon is also highly conserved, as all binding motifs are within 40 bases of one another relative to the respective start codons.

After the putative CueR binding motif was uncovered, we did a genome-wide search in *P. aeruginosa* PAO1 to find additional genes that could be directly regulated by CueR. The PAO1 genome was analyzed for the binding motif shown in Fig. 7B, and an additional operon, *mexPQ-opmE* (*PA3523* to *-21*) was uncovered, as well as a second gene, *PA3574.1*. *mexPQ-opmE* has been shown to be transcriptionally activated in response to copper (57). Our microarray data showed the operon to be upregulated in PAO1 compared to the *cueR* mutant when copper was present, though the magnitude of the change was just below the 2-fold cutoff of Table 2. *PA3574.1* is a small, 198-bp gene that is located between *PA3574* and *PA3575* and partially overlaps *PA3574*. This gene could not be identified by microarray profiling, as it was not represented on the GeneChip *Pseudomonas aeruginosa* Genome Array.

To validate the microarray data, we constructed promoter-*lacZ* transcriptional fusions for the five putative promoter targets of CueR and measured β-galactosidase activity in response to copper (Fig. 8). In the wild-type PAO1 strain, all five promoters were activated by the addition of copper. In the *cueR* deletion mutant, the activation of these promoters was abolished. In fact, each promoter showed a slight decrease in activity when copper was added to the medium. It appears that *cueR* is necessary for copper-induced activation of these five transcriptional units.

We verified the direct targets of CueR with an *in vitro* bind-

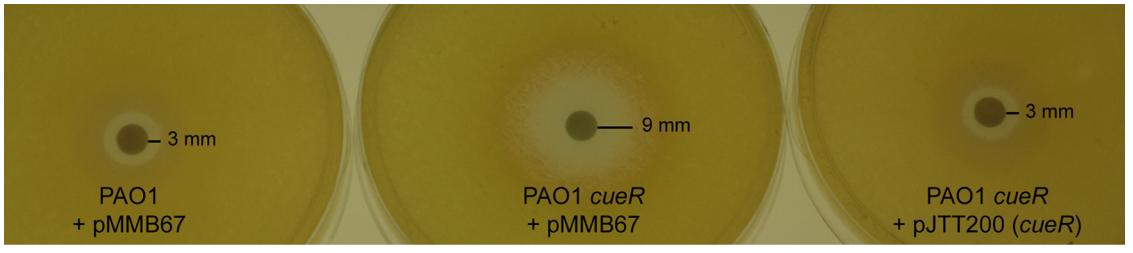

FIG. 6. Copper sensitivity of PAO1 and the isogenic *cueR* mutant. The pJTT200 vector contains the *cueR* gene under the control of an IPTG-inducible promoter, and pMMB67 was the negative-control vector. Twenty microliters of overnight culture was spread onto an LB-gentamicin agar plate. A paper disk containing 10 μl of 1 M copper sulfate was placed on each plate, and the cells were incubated overnight at 37°C. Similar experiments were performed with silver, nickel, cadmium, iron, mercury, zinc, cobalt, and manganese, though no change in metal sensitivity was observed in the *cueR* mutant (data not shown).

FIG. 7. CueR binding sites in *E. coli* (A) and *P. aeruginosa* (B). The weblogos in both panels were generated from the respective *E. coli* CueR and *P. aeruginosa* CueR binding motifs. Putative −35 and −10 sequence elements are shaded, regions of dyad symmetry are indicated by arrows, and the positions of the bases relative to the start codons are displayed above each binding motif.

ing assay. The purified CueR protein was used in an EMSA to test for direct binding of the protein to its promoter targets. As illustrated in Fig. 9, CueR binds to all five promoters, albeit with varying affinities. The dyad symmetry that is present in the target promoters of MerR-type transcription factors is necessary for protein binding (24, 30). This requirement is revealed in the gel shift data, as the four gene promoters with more significant dyad symmetry—*cueA* (binding affinity, 63 nM), *PA3519* to *-15* (binding affinity, 124 nM), *PA3574.1* (binding affinity, 33 nM), and *mexPQ-opmE* (binding affinity, 146 nM)—bind strongly to CueR, while *PA3520* (binding affinity, 2,619 nM) has a smaller degree of symmetry in its promoter and thus binds the protein only weakly. No relationship between the degree of dyad symmetry and binding affinity has yet been established, however, and it is unclear if this result is broadly generalizable. In total, CueR directly activates the expression of 11 genes in response to high levels of copper, while an additional 30 genes appear to be under indirect control (Table 2).

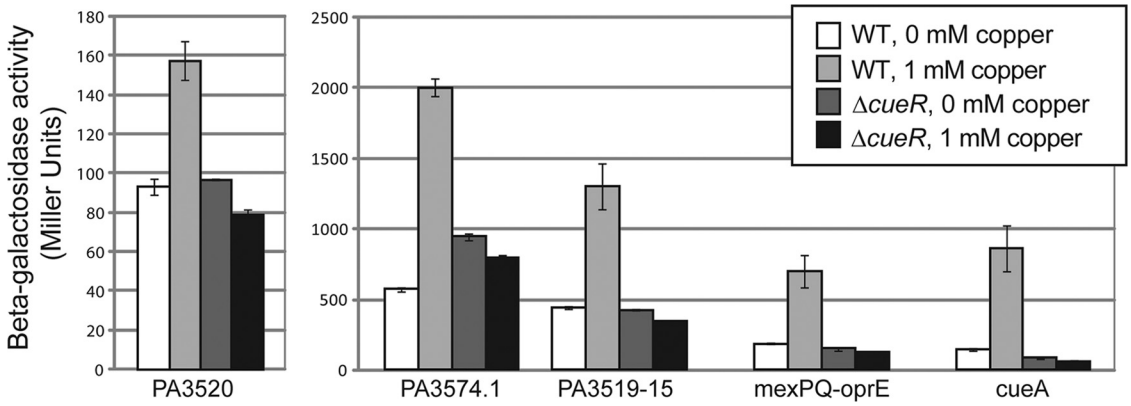

FIG. 8. Promoter activities of CueR-regulated genes in response to copper. Promoter-*lacZ* transcriptional fusions were integrated into the genomes of PAO1 and PAO1 *cueR* with the site-specific integration vector mini-ctx-lacZ. Cultures were grown in LB to log phase and then divided in half, with one half receiving copper sulfate to 1 mM and the other half receiving the equivalent volume of water. After 1 h of incubation at 37°C with shaking, β-galactosidase activity was assayed and is shown here in Miller units with standard deviations.

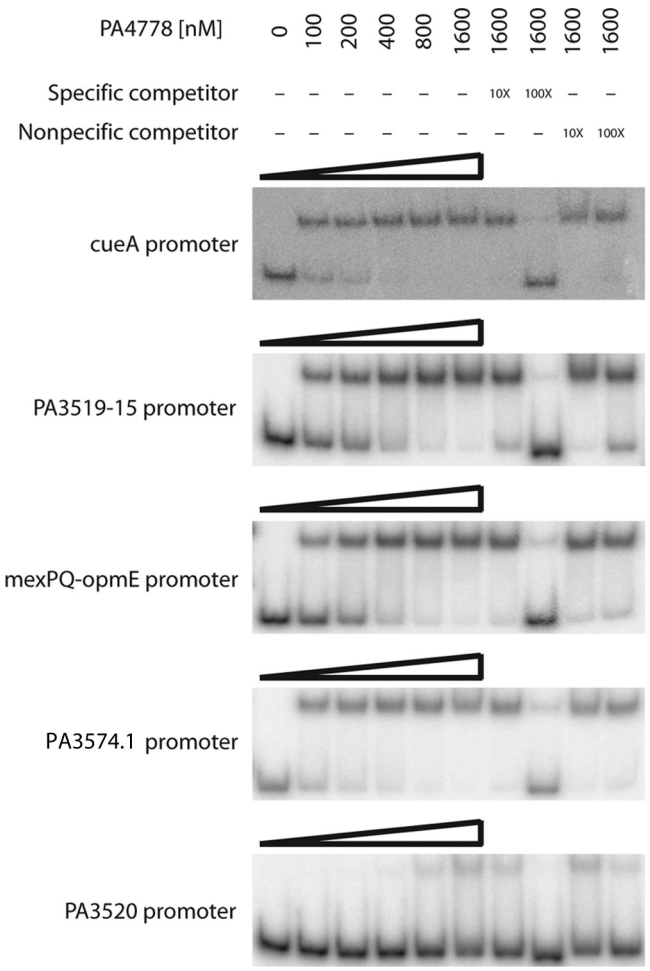

FIG. 9. Gel shift analysis of CueR and its target promoters. Purified CueR was added to 100 fmol of the labeled promoter DNA. Where indicated, nonlabeled specific or nonspecific competitor DNA was present in the reaction mixture.

Copper sensitivity of the CueR downstream target genes. We have shown that CueR is a transcriptional regulator involved in copper tolerance and that it directly regulates the expression of 11 genes in a copper-dependent manner. In order to determine which particular downstream genes mediate copper sensitivity, we tested the 11 mutants, *PA3519* to *-15*, *PA3520*, *mexPQ-opmE*, *cueA*, and *PA3574.1*—for sensitivity to copper relative to the wild-type *P. aeruginosa* PAO1 strain (Table 3). Ten of the 11 mutants were taken from the University of Washington Genome Center's PAO1 mutant collection (20). The one exception was the *PA3520* mutant, which was not present in the University of Washington collection. A *PA3520* mutant was generated as described in Materials and Methods. As described previously, the strains were grown overnight in LB medium and then plated onto LB agar. A paper disk with 10 μl of a 1 M copper sulfate solution was then placed on the center of each plate. The plates were then incubated for 24 h at 37°C. Mutations in 7 genes—*PA3515*, *PA3516*, *PA3517*, *PA3518*, *mexP*, *mexQ*, and *cueA*—all increased the sensitivity to copper. The most dramatic result was seen with *cueA*, which had a zone of inhibition that was 5 times that of the wild type.

DISCUSSION

In this work, we showed that the *P. aeruginosa* quorum-sensing system directly activates a regulatory pathway involved in resistance to copper toxicity. LasR, when triggered by its cognate autoinducer, $3OC_{12}$-HSL, upregulates the expression of *cueR*, encoding a transcriptional regulator that responds to copper levels within the cell. The LasR binding site was identified by sequence analysis and confirmed by expression studies involving *cueR* promoters with single-base-pair substitutions in the LasR binding motif. Though the sequence analysis suggested that the LasR binding site in the *cueR* promoter is a noncooperative binding motif, the EMSA data in Fig. 3 show a pattern that represents either cooperative binding of LasR to the *cueR* promoter or multiple LasR binding sites. The EMSA data of Gilbert et al. (12) show a similar binding pattern. The discrepancy between predicted and observed binding patterns likely stems from our incomplete understanding of the specific base pairs that differentiate cooperative from noncooperative binding motifs. In a 2004 study, Schuster et al. (47) appeared to show discrete cooperative and noncooperative binding motifs that could be easily differentiated. More recent data from Gilbert et al. (12), however, have shown that the LasR cooperative binding motif is more similar to the noncooperative motif than previously recognized. This more recent work indicates that it is not yet possible to differentiate the mode of binding based on primary DNA sequence alone. There may be a second, more degenerate sequence within the cooperative motif that binds additional copies of LasR after the consensus sequence has been bound (12), although this secondary sequence element has thus far escaped detection.

We further showed that copper-activated CueR directly triggers the expression of 11 genes that are grouped into 5 transcriptional units. Analysis of the CueR-targeted genes revealed that 7 of the 11 genes, when disrupted individually, increased the sensitivity to copper. The most dramatic results were seen with *cueA*, a gene for a P-type ATPase. Among the 11 genes

TABLE 3. Copper sensitivities of CueR downstream target genes

PAO1 ORF[a]	Cu disk assay[b] (mm)	Identifier in PAO1 library	Annotation
Wild type	3		
PA3515	5	19045	Hypothetical protein
PA3516	5	37652	Probable lyase
PA3517	5	52422	Probable lyase
PA3518	5	39841	Hypothetical protein
PA3519	3	6803	Hypothetical protein
PA3520	3	NA[c]	Hypothetical protein
PA3521 (*opmE*)	3	42105	Probable OMP precursor
PA3522 (*mexQ*)	6	7848	Probable RND efflux transporter
PA3523 (*mexP*)	4	54898	Probable RND efflux transporter
PA3574.1	3	55074	Putative periplasmic metal-binding protein
PA3920 (*cueA*)	15	361	P-type ATPase

[a] Mutants of the CueR downstream target genes were taken from the University of Washington Genome Center's PAO1 mutant collection (20). ORF, open reading frame.

[b] Twenty microliters of the overnight cultures was spread onto an LB-tetracycline agar plate. A paper disk containing 10 μl of 1 M copper sulfate was placed on each plate, and cells were incubated overnight at 37°C.

[c] NA, not applicable. The University of Washington collection did not contain a *PA3520* mutant, so an isogenic mutant was generated and tested.

directly activated by CueR, several have been studied previously. The product of *cueA* is a P-type ATPase that has been shown to reduce sensitivity to copper (49, 57). It is believed to be associated with the cytoplasmic membrane, where it transfers copper to the periplasm. In addition, the gene has been shown to be a virulence factor in a murine model (49). The *mexPQ-opmE* operon constitutes an RND efflux pump. The genes have previously been shown to marginally affect copper resistance (57) and have also been shown to influence resistance to several antibiotics (28). The remaining seven CueR target genes have not yet been studied. *PA3574.1* is a 198-bp gene that is homologous to the PA14_18070 locus in *P. aeruginosa* PA14. A BLAST analysis of PA3574.1 indicated it is a putative periplasmic metal binding protein with heavy-metal binding sites near the amino terminus (2). PA3520 contains a heavy-metal-associated domain and may also be a copper chaperone. The *PA3519* to *-15* operon consists of three putative enzymes and two unclassified genes. PA3516 and PA3517 are probable lyases, and PA3515 is a probable methyltransferase. Both PA3518 and PA3519 are unclassified proteins. Though disruption of *PA3515*, *PA3516*, *PA3517*, or *PA3518* was sufficient to increase the sensitivity to copper (Table 3), it is unclear how these genes mediate resistance to the metal.

CueR is a MerR-type transcriptional regulator, and this family of proteins interact with their target promoters in a characteristic way. A typical MerR target promoter has a spacer region of 19 or 20 bp between the −35 and −10 sequence elements. This extended spacer region is greater than the optimal 16 to 18 bp of a σ^{70}-dependent promoter (5). The MerR target promoter also contains a region of dyad symmetry that appears to be critical for MerR recognition (24, 30). In the absence of an appropriate stimulus, such as Cu(I) in the case of CueR, the transcription factor binds to the promoter and recruits the RNA polymerase, though the extended spacer region prevents open-complex formation. The binding of the appropriate metal causes a conformational change in the transcription factor, and the tight association between protein and DNA results in a distortion of the DNA and reorientation of the −35 and −10 sequences. This new, active conformation permits the −35 and −10 elements to interact productively with the σ^{70} subunit and allows an open transcriptional complex to form (3, 4). The CueR copper resistance system thus remains primed for activation and can upregulate its transcriptional targets after Cu(I) enters the cytoplasm. This system is exquisitely sensitive, as studies of *E. coli* CueR have shown that the protein is capable of responding to zeptomolar concentrations of free Cu(I) (7).

One of the surprising aspects of this study is the finding that LasR directly regulates a transcription factor that is involved in copper homeostasis. The importance of copper in cellular metabolism has been well documented, as the metal is a cofactor in proteins involved in electron transport (8, 58) and iron trafficking (18). Proper regulation of intracellular levels of the metal is vital, as excess copper can result in cell damage or death through a number of mechanisms (57). The metal can disrupt protein structure by binding free thiol groups (23), can displace essential metal cofactors in key proteins, or may promote the generation of reactive oxygen species (39, 48). Indeed, the importance of systemic copper regulation was illustrated by a recent study showing that a mutant in the *cueA* gene, encoding a P-type ATPase involved in copper transport and shown here to be CueR regulated, was less virulent than the wild type in a murine model (49). Still, it is not clear why copper regulation would be so closely linked to quorum sensing and high cell density. Quorum sensing indirectly activates additional genes that are likely to play roles in protection from metal toxicity, including Mn-cofactored superoxide dismutase (*sodA*), Fe-cofactored superoxide dismutase (*sodB*), and catalase (*katA*), and it has been hypothesized that activation of these enzymes may be responsible in part for the metal resistance seen in biofilms (14). Perhaps the activation of the CueR-mediated copper toxicity resistance system has a similar role in enhancing the long-term fitness of *P. aeruginosa*, though additional work is needed to fully understand the role of this copper toxicity system in *P. aeruginosa* physiology.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant GM078987 to T.S.G. Work in S.L.'s laboratory was supported by NIH grant AI021451.

We thank E. Peter Greenberg for providing strains and Anja Brencic, Massimo Merighi, and Mauricia Matewish for technical advice regarding experiments.

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