Pseudomonas aeruginosa AmpR Is a Global Transcriptional Factor That Regulates Expression of AmpC and PoxB β -Lactamases, Proteases, Quorum Sensing, and Other Virulence Factors

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In members of the family *Enterobacteriaceae*, *ampC*, which encodes a β -lactamase, is regulated by an upstream, divergently transcribed gene, *ampR*. However, in *Pseudomonas aeruginosa*, the regulation of *ampC* is not understood. In this study, we compared the characteristics of a *P. aeruginosa ampR* mutant, PAO*ampR*, with that of an isogenic *ampR*⁺ parent. The *ampR* mutation greatly altered AmpC production. In the absence of antibiotic, PAO*ampR* expressed increased basal β -lactamase levels. However, this increase was not followed by a concomitant increase in the *P_{ampC}* promoter activity. The discrepancy in protein and transcription analyses led us to discover the presence of another chromosomal AmpR-regulated β -lactamase, PoxB. We found that the expression of *P. aeruginosa ampR* greatly altered the β -lactamase production from *ampC* and *poxB* in *Escherichia coli*: it up-regulated AmpC but down-regulated PoxB activities. In addition, the constitutive *P_{ampR}* promoter activity in PAO*ampR* indicated that AmpR did not autoregulate in the absence or presence of inducers. We further demonstrated that AmpR is a global regulator because the strain carrying the *ampR* mutation produced higher levels of pyocyanin and LasA protease and lower levels of LasB elastase than the wild-type strain. The increase in LasA levels was positively correlated with the *P_{lasA}*, *P_{lasB}*, and *P_{lasR}* expression. The reduction in the LasB activity was positively correlated with the *P_{rhlR}* expression. Thus, AmpR plays a dual role, positively regulating the *ampC*, *lasB*, and *rhlR* expression levels and negatively regulating the *poxB*, *lasA*, *lasI*, and *lasR* expression levels.

Pseudomonas aeruginosa is an important opportunistic pathogen causing infections in patients suffering from cystic fibrosis, chronic obstructive pulmonary disease, or severe burns and in patients in intensive care units. It poses an overwhelming threat to these individuals, leading to a high risk of morbidity and mortality. The current treatment against *P. aeruginosa* usually includes a combination of β -lactam antibiotics and aminoglycosides (20). However, resistance to these antibiotics is not uncommon, resulting in the failure of chemotherapy. *P. aeruginosa* also possesses various mechanisms to counteract antimicrobial agents. These include inducible AmpC β -lactamase (33), plasmid- and transposon-encoded acquired β -lactamases (12), multidrug efflux pumps (17), low outer membrane permeability (53), the ability to grow in biofilms (7), and hypermutability (41). The rapid emergence of resistance to β -lactam antibiotics in *P. aeruginosa* isolates from cystic fibrosis patients in the early 1990s has been attributed to the in vivo selection of constitutive β -lactamase-producing strains (6, 15, 16). Overproduction of β -lactamase in some of the *P. aeruginosa* isolates has been ascribed to mutations in *ampD* encoding a negative regulator of AmpC (2, 28), but genetic events un-

derlying overproduction of AmpC β -lactamase still remain to be fully elucidated.

Many members of the *Enterobacteriaceae* family have genes that code for a chromosomal Ambler class C β -lactamase (38). The analysis of chromosomally mediated β -lactamases revealed that, in species possessing an *ampR* gene, synthesis of β -lactamase is inducible, whereas in strains without *ampR*, synthesis is constitutive (44, 50). The *ampR* genes in all the species studied thus far are located immediately upstream of *ampC* and are transcribed divergently (18). The short intergenic region of *Citrobacter freundii ampR-ampC* accommodates two overlapping promoters (3, 31). The *P. aeruginosa ampC* and *ampR* genes have similar gene organization (32, 33). However, the mechanism of *ampR* regulation in *P. aeruginosa* is not yet clear.

To address the role of *P. aeruginosa ampR*, we generated an *ampR* mutant in the prototypic, wild-type PAO1 strain. Our analysis of PAO1 and PAO*ampR* showed that a mutation in *ampR* results in a high constitutive expression of β -lactamases and that AmpR is required for AmpC induction. In addition, AmpR modulates expression of yet another chromosomally encoded β -lactamase, extracellular proteases, and pyocyanin. Subsequent analyses suggest that AmpR affects the virulence factors through quorum sensing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains, plasmids, and primers used in this study are shown in Table 1. *Escherichia coli* and *P. aeruginosa*

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain or plasmid	Genotype or properties	Reference or source
<i>Escherichia coli</i>		
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (r _K ⁻ m _K ⁺) phoA supE44 λ ⁻ thi-1 gyrA96 relA1	New England Biolabs
TOP10F'	F' [lacI ^q Tn10(Tet ^r)] mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 Δ lacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL (Str ^r) endA1 nupG	Invitrogen
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild type	21
PKM300	ampR::aacCI; Gm ^r	PAOampR; this study
PKM301	attB::P _{ampC} -lacZ; Tc ^r	PAO1 derivative; this study
PKM302	attB::P _{ampR} -lacZ; Tc ^r	PAO1 derivative; this study
PKM303	ampR::aacCI attB::P _{ampC} -lacZ; Gm ^r Tc ^r	PAOampR derivative; this study
PKM304	ampR::aacCI attB::P _{ampR} -lacZ; Gm ^r Tc ^r	PAOampR derivative; this study
PKM305	attB::promoterless lacZ; Tc ^r	PAO1 derivative; this study
PKM306	ampR::aacCI attB::promoterless lacZ; Gm ^r Tc ^r	PAOampR derivative; this study
Plasmids		
pGEMEX-1	Ap ^r ; ColE1ori lacZ α	Promega
pUCGm	Ap ^r Gm ^r ; pUC19 derivative containing gentamicin cassette (aacCI)	47
pEX100T	Ap ^r ; sacB oriT	46
pME6030	Tc ^r ; oriV _{pVS1} oriV _{p15A} oriT	19
pRK2013	Km ^r ; ColE1 ori-Tra (RK2) ⁺	11
Mini-CTX-lacZ	Tc ^r ; integration-proficient vector for single-copy chromosomal lacZ fusion	4
pLP170	Ap ^r ; lacZ transcriptional fusion vector that contains an RNase III splice sequence positioned between the multiple cloning site and lacZ	43
pLPLA	Ap ^r ; pLP170 containing P _{lasA} -lacZ transcriptional fusion	43
pLPLB	Ap ^r ; pLP170 containing P _{lasB} -lacZ transcriptional fusion	43
pPCS1001	Ap ^r ; pLP170 containing P _{lasR} -lacZ transcriptional fusion	42
pPCS1002	Ap ^r ; pLP170 containing P _{rhIR} -lacZ transcriptional fusion	42
pLPR1	Ap ^r ; pLP170 containing P _{rhIR} -lacZ transcriptional fusion	52
pPCS223	Ap ^r ; pLP170 containing P _{lasR} -lacZ transcriptional fusion	52
pQF50	Ap ^r ; broad-host-range vector with promoterless lacZ	10
pSJ01	Ap ^r ; pGEMEX-1 with a 1,220-bp EcoRI-BamHI-flanked fragment containing ampR	This study
pSJ02	Km ^r ; pCRII-TOPO with a 2,150-bp fragment containing ampC	This study
pSJ05	Ap ^r Gm ^r ; pGEMEX derivative with ampR::aacCI	This study
pSJ06	Ap ^r ; pME6030 with a 1,220-bp EcoRI-BamHI-flanked fragment containing ampR	This study
pSJ07	Ap ^r Gm ^r ; pEX100T derivative with ampR::aacCI	This study
pSJ09	Ap ^r ; pGEMEX-1 with a 330-bp EcoRI-BamHI-flanked fragment containing ampC-ampR intergenic region	This study
pSJ10	Tc ^r ; CTX-lacZ fused with ampC promoter, P _{ampC}	This study
pSJ11	Tc ^r ; CTX-lacZ fused with ampR promoter, P _{ampR}	This study
pKKF0675	Km ^r ; pCRII-TOPO with 1,770-bp fragment containing poxAB	This study
pKKF0679	Km ^r ; pCRII-TOPO with DraI-deleted bla	This study
Primers		
SBJ01ampRFor ^a	5'-GGAATTCTGGCGAACAGCAGTGTGGAAGCGG-3'	
SBJ02ampRRev ^a	5'-CGGGAATCCATTCCAATCACAACCCCAACGCC-3'	
SBJ03ampCRFor ^a	5'-GGAATTCTGAGGCCGCGCGGCAGACGCTTGAACA-3'	
SBJ04ampCRRRev ^a	5'-CGGGAATCCCATGAGGATTGGCGTCCTTTG-3'	
SBJ05ampCFor ^a	5'-GGAATTCTGAGGCCGCGCGGCAGACGCTTGAACA-3'	
SBJ06ampCRev ^a	5'-CGGGAATCCAACCCGCGCGGTGGCCAGTCCCGCCAA-3'	
KKF28pox4For	5'-AGCTGCTTGCGCACCTGT-3'	
KKF30pox4Rev	5'-CGGTTTCGAATTCACCGTCA-3'	

^a The italicized portion indicates a restriction site in a PCR product prepared with that primer.

FIG. 1. Physical map of *ampR-ampC* loci (A) and plasmids (B). (A) The restriction map of the region is based on the PAO1 genome sequence with relevant restriction sites. The 5' end of *ampC* (labeled *ampC'*) and the entire *ampR* open reading frames are indicated. The 1.2-kb fragment has the PAO1 genome coordinates 4594088 to 4592869. SBJ03 and SBJ04 show the position where the primers annealed for the promoter amplification. The striped box at positions 197 to 207 is where ligand-bound AmpR is postulated to bind. (B) The horizontal lines below the map show the amount of PAO1 genome DNA present in each plasmid. The restriction sites, EcoRI and BamHI, flanking the ends were introduced in the primers used for PCR (Table 1). Plasmids pSJ01 and pSJ06 are derivatives of pGEMEX (Promega) and pME6030 (19), respectively. The plasmid pSJ06 is used for complementation analysis and is referred to as pAmpR. The gentamycin cassette (▼) was inserted into the SalI site of pSJ01, creating pSJ05. The *ampR::aacCI* (*ampR::Gm*) fragment was subcloned into the suicide vector pEX100T (48) to generate pSJ07, which was introduced into PAO1 to generate PAO*ampR* by homologous recombination. A 342-bp fragment containing the promoters was cloned in both directions, upstream of promoterless *lacZ* (indicated by the striped arrows) in mini-CTX-*lacZ* (4) to generate the transcriptional fusions for *ampC* (pSJ10) and *ampR* (pSJ11) promoters. These fusions were introduced into PAO1 and PAO*ampR* by site-specific recombination using the *attP* site (4).

were routinely cultured in Luria-Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, per liter). *Pseudomonas* isolation agar (Difco) was used in triparental mating experiments. Antibiotics were used at the following concentrations (per milliliter) unless otherwise indicated: ampicillin at 50 μ g, tetracycline at 20 μ g, and gentamicin at 15 μ g for *E. coli* and carbenicillin at 300 μ g, gentamicin at 300 μ g, and tetracycline at 40 μ g for *P. aeruginosa*. For induction, 500 μ g/ml of benzylpenicillin was used.

DNA manipulations. All molecular techniques were performed according to standard protocols (1).

Cloning of *ampC* and *poxB* genes. Primers were designed to PCR amplify *ampC* and *poxB*. SBJ05*ampC*For and SBJ06*ampC*Rev were used to generate a 1,220-bp fragment with *ampC*, while KKF28*poxB*For and KKF30*poxB*Rev generated a 2,170-bp fragment containing *poxB* (Table 1). These PCR amplicons were electrophoresed and purified according to manufacturer's instructions (QIAGEN, California). The cleaned products were ligated into pCRII-TOPO and transformed into TOP10F' (Invitrogen, California). Because we were concerned that the presence of the *bla* gene in the pCRII-TOPO vector might interfere with data interpretation, an internal 712-bp *DraI* fragment of the *bla* gene was deleted, and the resulting vector was transformed into *E. coli* DH5 α . Selection was carried out using kanamycin, as this resistance marker was also present in pCRII-TOPO. The plasmids pSJ02 and pKKF675 harbored the *ampC* and *poxB* genes, respectively. The negative control, plasmid pKKF679, contained a self-ligated pCRII-TOPO in which the *bla* gene was deleted (Table 1).

Insertional inactivation of the *ampR* gene. A 1,220-bp *ampR* fragment was PCR amplified using SBJ01*ampR*For and SBJ02*ampR*Rev with flanking EcoRI and BamHI sites, respectively (Table 1). The PCR product was ligated to an EcoRI-BamHI-digested pGEMEX-1 (Promega, California), generating pSJ01 (Fig. 1). A gentamycin cassette, *aacCI*, was retrieved from pUCGm (47) and inserted into the PstI site of *ampR* (Fig. 1). This disrupted the reading frame of *ampR* in pSJ01. Subsequently, *ampR::Gm* was subcloned as an EcoRI-HindIII-cut, blunt-ended fragment into the SmaI site of pEX100T (48), which is a

mobilizable suicide plasmid, creating pSJ07. This plasmid was conjugated into *P. aeruginosa* PAO1, with a helper strain harboring pRK2013 (11). The merodiploids, resulting from homologous recombination, were selected with pseudomonas isolation agar containing gentamicin. Gm^r colonies were then screened for gentamicin resistance and carbenicillin sensitivity by replica plating. The insertion was confirmed by PCR and restriction analysis of the PCR product (data not shown). The PAO1 isogenic strain with defective *ampR*, henceforth referred to as PAO*ampR*, was named PKM300.

Construction of a plasmid with intact *ampR* for complementation. Plasmid pSJ06 was generated by subcloning an EcoRI-BamHI-cut fragment from pSJ01 containing *ampR* into an EcoRI-BamHI-cut, broad-host-range, low-copy-number plasmid, pME6030 (19). This plasmid, pSJ06, also referred to as pAmpR, was used in all complementation analyses by conjugation into PAO*ampR*.

Gene fusions with the *lacZ* gene. A 330-bp fragment was PCR amplified with the flanking primers SBJ03*ampC*For and SBJ04*ampC*Rev (Table 1) and then cloned into pGEMEX-1, generating pSJ09 (Fig. 1). This fragment contained the *ampC-ampR* intergenic region with the putative promoters (33). To avoid PCR artifacts, pSJ09 was sequenced by using M13 primers. The fragment containing the promoters was subcloned as an EcoRI-BamHI fragment into an EcoRI-BamHI-cut, promoterless *lacZ*, mini-CTX-*lacZ* reporter plasmid (4), creating pSJ10 (*P_{ampC}-lacZ*) and pSJ11 (*P_{ampR}-lacZ*). This suicide vector contained the integration-proficient *attP* site, which recombines into the chromosomal *attB* site to generate a single-copy reporter fusion (4). The resulting clones were mobilized into PAO1 and PAO*ampR* (Table 1). The presence of the chromosomal insertions was confirmed by PCR and restriction analysis of the product.

Pyocyanin quantitation assay. The pyocyanin assay is based upon the absorbance of excreted pyocyanin at 520 nm in acidic solution (9). A 5-ml supernatant from a stationary-phase culture (~16 h) in LB broth was mixed with 3 ml of chloroform. The pyocyanin from the chloroform phase was then extracted into 1 ml of 0.2 N HCl, giving it a pink to deep red color, indicating the presence of pyocyanin. The absorbance was measured at 520 nm. Concentrations, expressed

TABLE 2. β -Galactosidase activities of *ampC* and *ampR* promoters

Strain	Relevant properties ^a	β -Galactosidase activity ^b	
		Noninduced	Induced ^c
PKM302	PAO1, P _{<i>ampR</i>} - <i>lacZ</i>	77.1 $\pm$ 8.7	123.0 $\pm$ 1.2
PKM304	PAOampR, P _{<i>ampR</i>} - <i>lacZ</i>	96.3 $\pm$ 15.2	106.0 $\pm$ 16.0
PKM301	PAO1, P _{<i>ampC</i>} - <i>lacZ</i>	124.1 $\pm$ 11.6	1,644.2 $\pm$ 33.7 ^d
PKM303	PAOampR, P _{<i>ampC</i>} - <i>lacZ</i>	113.2 $\pm$ 7.5	122.3 $\pm$ 7.4 ^e
PKM305	PAO1, promoterless <i>lacZ</i>	ND	ND
PKM306	PAOampR, promoterless <i>lacZ</i>	ND	ND

^a The *lacZ* transcriptional reporter fusions were integrated into the *attB* site in the respective strains.

^b β -Galactosidase activities were expressed in Miller units. ND, nondetectable.

^c Induction was carried out using 500 μ g/ml of benzylpenicillin.

^d *P* value of <0.05 compared between noninduced and induced conditions within the strain.

^e *P* value of <0.05 compared between PAO1 and PAOampR under the same condition.

as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the optical density at 520 nm (OD₅₂₀) by 17.072 (9).

LasA staphylolytic assay. LasA protease activity was measured by determining the ability of stationary-phase *P. aeruginosa* culture supernatants to lyse boiled *Staphylococcus aureus* (25). A 30-ml volume of an overnight *S. aureus* culture was boiled for 10 min and then centrifuged for 10 min at 10,000 $\times$ *g*. The resulting pellet was resuspended in 10 mM Na₂PO₄ (pH 4.5) to an OD₆₀₀ of approximately 0.8. A 100- μ l aliquot of bacterial supernatant was added to 900 μ l of *S. aureus* suspension, and the OD₆₀₀ was determined after 0, 5, 10, 15, 20, 30, 45, and 60 min (8, 25).

LasB elastolytic assay. The elastolytic activity of bacterial suspension was determined with the elastin Congo red (ECR; Sigma, St. Louis, MO) assay (40). A 100- μ l aliquot of bacterial supernatant of 16-h culture was added to 900 μ l of ECR buffer (100 mM Tris, 1 mM CaCl₂, pH 7.5) containing 20 mg of ECR and then incubated with shaking at 37°C for 3 h. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control. Activity was expressed as change in OD₄₉₅ per μ g protein.

β -Lactamase assay. The assay of the *P. aeruginosa* chromosomal β -lactamase was modified based on previously published protocols (39). Briefly, stationary-phase cultures were diluted 1:100 into 10 ml of LB broth and incubated with shaking at 37°C until an OD₆₀₀ of 0.6 to 0.8 was attained. For induction (500 μ g/ml benzylpenicillin), the cultures were incubated for an additional 3 hours before harvesting. The cells were washed once with 50 mM sodium phosphate buffer and were then resuspended to a final volume of 2 ml of the same buffer. Following disruption of cells on ice with sonication, the cell lysate was centrifuged at 10,000 $\times$ *g* for 30 min at 4°C and the supernatant was retained. A 2- μ l portion of the β -lactamase-containing supernatant was added to 1 ml (final volume) of assay buffer containing nitrocefin (final concentration, 100 μ M) at room temperature. The reaction was allowed to take place for 20 min before nitrocefin hydrolysis was measured spectrophotometrically at 482 nm. Concurrently, the total protein was determined with the Bradford assay using the same supernatant. One milliliter of β -lactamase is defined as 1 nanomole of nitrocefin hydrolyzed per minute per microgram of protein.

β -Galactosidase assay. Assays for β -galactosidase in *P. aeruginosa* were performed as previously described (34).

Statistical analysis. All data were analyzed with one-way analysis of variance using the statistical software package SPSS (Chicago, IL).

RESULTS AND DISCUSSION

Typically the presence of β -lactam antibiotics in a medium induces the synthesis of chromosome-encoded AmpC β -lactamase. The regulator of *ampC*, the divergently transcribed *ampR* gene product, has been identified in more than 10 species (18). *P. aeruginosa* AmpR has a high sequence identity with the AmpR from *C. freundii* and *Enterobacter cloacae* (58.3 and 62.5% identity, respectively) (33). Because the three species have the same *ampR* and *ampC* gene organization in the chromosome, it has been assumed that they also use the same regulatory mechanism. To date, the role of AmpR is restricted

to the induction of *ampC*. The goal of this study was to examine the role of *P. aeruginosa* AmpR by generating an isogenic *ampR* mutant in the prototypic *P. aeruginosa* strain PAO1 (PAOampR).

***P. aeruginosa* AmpR does not autoregulate its own promoter.** AmpR is a DNA-binding protein that belongs to the LysR family of regulatory proteins (22, 29, 31). Many LysR-type transcriptional regulators act as transcriptional activators of the target genes but repress their own expression (45). In addition, the *P. aeruginosa ampR-ampC* intergenic region is only 159 bp long, leading to the suggestion that the AmpR expression may be autoregulated (32). To address this, we constructed strains with single copies of the *ampR* promoter upstream of a promoterless reporter gene, *lacZ*. The P_{*ampR*}-*lacZ* fusion was integrated into the PAO1 and the PAOampR chromosomes via *attB-attP* site-specific recombination, thus mimicking the chromosomal regulation (4). As a negative control, the promoterless *lacZ* gene was also integrated in both PAO1 and PAOampR. In the absence of the inducer, PAO1 showed a basal level of expression from the *ampR* promoter, which was not significantly affected by the presence of the inducer (Table 2). This finding concurs with previous *C. freundii ampR* studies showing that the amount of *C. freundii* AmpR expressed in *E. coli* minicells remains constant in the absence and presence of inducer (30), that the constitutive *ampR* expression is low with a short half-life of mRNA (22), and that the *ampR* transcription and the level of AmpR remain unaffected in the absence and presence of antibiotics (31). Expression of P_{*ampR*}-*lacZ* in the PAOampR mutant was not significantly different, suggesting that *ampR* does not regulate its own transcription (Table 2).

***P. aeruginosa* AmpR is a positive regulator of *ampC* expression.** Class C β -lactamase has long been implicated as the culprit in the cephalosporin resistance of *P. aeruginosa* (5). Based on previously published work in other systems (22, 30), we postulated that the mutation in *ampR* would increase the activity of the *ampC* promoter. To test this hypothesis, we generated a P_{*ampC*}-*lacZ* reporter and integrated it as a single-copy fusion into the PAO1 and the PAOampR chromosomes via *attB-attP* site-specific recombination, thus mimicking the chromosomal regulation (4). As a negative control, the promoterless *lacZ* gene was also integrated in both PAO1 and PAOampR. In PAO1, little activity was synthesized from the *ampC* promoter (Table 2). As expected, the presence of ben-

TABLE 3. Expression of β -lactamases in *P. aeruginosa* and *E. coli*

Strain and plasmid	Relevant property(ies)	β -Lactamase activity ^a	
		Noninduced	Induced ^b
<i>P. aeruginosa</i>			
PAO1	Wild type	15.4 $\pm$ 7.8	162.2 $\pm$ 25.5 ^c
PAO <i>ampR</i>	<i>ampR::aacC1</i>	185.0 $\pm$ 8.9 ^d	126.0 $\pm$ 46.0
PAO <i>ampR</i> /pSJ06	<i>ampR::aacC1/ampR</i> ⁺	13.0 $\pm$ 5.0 ^e	169.1 $\pm$ 22.7 ^c
<i>E. coli</i>			
DH5 α /pKKF0679		ND	ND
DH5 α /pSJ06	<i>ampR</i> ⁺	ND	ND
DH5 α /pSJ02	<i>ampC</i> ⁺	1.4 $\pm$ 0.0 ^f	0.2 $\pm$ 0.1 ^f
DH5 α /pSJ02/pSJ06	<i>ampC</i> ⁺ <i>ampR</i> ⁺	5.9 $\pm$ 0.5 ^g	13.0 $\pm$ 1.6 ^{c,g}
DH5 α /pKKF0675	<i>poxAB</i> ⁺	19.5 $\pm$ 0.6 ^f	13.8 $\pm$ 2.1 ^f
DH5 α /pKKF0675/pSJ06	<i>poxAB</i> ⁺ <i>ampR</i> ⁺	6.9 $\pm$ 0.8 ^h	0.5 $\pm$ 0.2 ^{c,h}

^a One milliunit of β -lactamase is defined as 1 nanomole of nitrocefin hydrolyzed per minute per microgram of protein. ND, nondetectable.

^b Induction was carried out using 50 μ g/ml and 500 μ g/ml of benzylpenicillin for *E. coli* and *P. aeruginosa*, respectively.

^c *P* value of <0.05 compared between noninduced and induced.

^d *P* value of <0.05 compared between PAO1 and PAO*ampR*.

^e *P* value of <0.05 compared between PAO*ampR* and PAO*ampR*/pSJ06.

^f *P* value of <0.05 in a comparison of DH5 α /pKKF0679 with DH5 α /pSJ02 and DH5 α /pKKF0675.

^g *P* value of <0.05 compared between DH5 α /pSJ02 and DH5 α /pSJ02/pSJ06.

^h *P* value of <0.05 compared between DH5 α /pKKF0675 and DH5 α /pKKF0675/pSJ06.

zylpenicillin resulted in a significant elevation in the *ampC* promoter activity in PAO1 (Table 2). However, no induction of the *ampC* promoter was detectable in PAO*ampR* in the absence or presence of inducer. The significant loss of transcriptional induction of the *ampC* promoter (Table 2) was likely due to the absence of AmpR, suggesting that AmpR is a positive regulator of *ampC* expression in the presence of an inducer. This is in agreement with previous studies of *C. freundii* and *E. cloacae* demonstrating that AmpR modulates *ampC* expression. The cloned *ampC* genes from *C. freundii* and *E. cloacae* are activated in *E. coli* only if accompanied by their *ampR* gene (22, 30).

AmpR exhibits differential regulation on AmpC and PoxB.

To ascertain the role of AmpR in the expression of β -lactamase, the activities in both PAO1 and the isogenic *ampR* mutant were compared. The parental PAO1 strain showed a low noninduced level of activity, which was increased 11-fold by addition of the β -lactam agent (Table 3). However, PAO*ampR* expressed a high β -lactamase level, 12-fold higher than that of the parental strain in the absence of inducer (Table 3). No further induction was exhibited in the presence of a β -lactam agent. The inducible phenotype was restored in the PAO*ampR* mutant by complementation with the plasmid pSJ06, harboring *ampR*.

However, based on the *P_{ampC}-lacZ* expression assay (Table 2), we expected the AmpC β -lactamase levels to remain the same in the absence of *ampR* (Table 3). This discrepancy in the biochemical and transcriptional analyses of *ampC* and the characterization of a *P. aeruginosa ampC* mutant led to the discovery of a novel chromosomally encoded class D β -lactamase, encoded by *poxB* (27). This class D β -lactamase was independently reported by Girlich et al., and the enzyme was named OXA-50 (13). However, our analysis of the sequence argues that PoxB is distinct from OXA enzymes (27). This discovery led us to propose that AmpR might also regulate *poxB*. The presence of more than one copy of β -lactamases in *P. aeruginosa* complicated the analysis of the effects of AmpR

on each of these genes in a homologous host model. Therefore, we cloned *ampC* and *poxB* genes into *E. coli* and analyzed their activity in the absence and presence of the *ampR* gene.

In strain *E. coli* DH5 α , AmpC expression was very low (Table 3). This is in contrast to what has been observed with *C. freundii* AmpC activity (30). In the absence of β -lactam antibiotic and *C. freundii ampR*, an increase in *C. freundii* AmpC β -lactamase activity in *E. coli* was observed, suggesting that *C. freundii* AmpR is a negative regulator (30). However, in our analysis, the presence of *ampR* is required for *ampC* expression.

Introduction of *P. aeruginosa ampR* into *E. coli* strain DH5 α failed to induce the endogenous β -lactamase from the chromosomal *ampC* (Table 3). However, when *P. aeruginosa ampR* was introduced along with its cognate *ampC* into *E. coli* strain DH5 α , the β -lactamase activity was significantly elevated both in the absence (fourfold) and in the presence (65-fold) of inducer (Table 3), further supporting the notion that *ampR* is a positive regulator required for *ampC* expression as well as induction.

In contrast to *ampC*, the activity of *poxB* was constitutively high in *E. coli* (Table 3). When AmpR was supplied in *trans*, *poxB* β -lactamase activity significantly dropped 65% and 96% in the absence and presence, respectively, of inducer (Table 3). These data suggest that AmpR suppresses the expression of PoxB.

It has been demonstrated that the increased resistance in clinical isolates of *P. aeruginosa* is also due to the derepression of β -lactamase (5, 14). Since the existence of PoxB was not known, and the biochemical assays used were not sensitive enough to distinguish these two β -lactamases, it was presumed that AmpC was overexpressed in the hyperproducing clinical isolates. A high constitutive expression of β -lactamase seen in PAO*ampR* suggests that the clinical *P. aeruginosa* isolates that are hyperproducing β -lactamase may harbor *poxB* and a mutation in *ampR*. A Southern blot analysis of a few of these clinical *P. aeruginosa* isolates with *ampR*- and *ampC*-specific

TABLE 4. Effect of *ampR* mutation on pyocyanin production and LasA staphylolytic and LasB elastase activity

Strain	Pyocyanin production ^a		LasA activity ^b		Elastase activity ^c	
	Noninduced	Induced ^d	Noninduced	Induced ^d	Noninduced	Induced ^d
PAO1	0.3 ± 0.2	2.3 ± 0.2	0.310 ± 0.065	0.317 ± 0.059	41.7 ± 1.1	105.0 ± 24.0
PAO <i>ampR</i>	2.9 ± 0.8 ^f	3.3 ± 0.6	1.109 ± 0.099 ^f	0.951 ± 0.045 ^f	32.8 ± 2.3	30.7 ± 1.7 ^f
PAO <i>ampR</i> /pSJ06 ^e	0.1 ± 0.0 ^g	1.4 ± 0.3	0.188 ± 0.055 ^g	0.186 ± 0.019 ^g	56.5 ± 1.5	90.5 ± 8.4 ^g

^a Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.

^b LasA activity was expressed as change in OD₆₀₀ per hour per microgram of protein.

^c Elastase activity was expressed as change in OD₄₉₅ per microgram of protein.

^d Induction was carried out using 500 µg/ml of benzylpenicillin.

^e The plasmid pSJ06 is a derivative of low-copy-number plasmid pME6030 containing *ampR*.

^f *P* value of <0.05 compared between PAO1 and PAO*ampR*.

^g *P* value of <0.05 compared between PAO*ampR* and PAO*ampR*/pSJ06.

probes showed no gross changes in the *ampR-ampC* intergenic DNA (5). This led the authors to rule out any changes in *ampR* and *ampC* genes, although this technique is not sensitive enough to detect minor deletions or point mutations (5). In fact, one of these *P. aeruginosa* β-lactamase hyperproducers was shown to have a point mutation in *ampR* (2). It is not clear if this clinical isolate harbors the *poxB* gene, though we have demonstrated that *poxB* is fairly ubiquitous and is found in chromosomes of both clinical and environmental *P. aeruginosa* isolates (27).

Mutation in *ampR* affects pyocyanin production. A change in virulence factor levels was first macroscopically observed when PAO*ampR* produced a highly pigmented culture in mid-log phase, compared to the parent PAO1. Pyocyanin is a typical green pigment produced by *P. aeruginosa* that is involved in microbial competitiveness, the suppression of soilborne plant pathogens, and virulence in human and animal hosts (35). Two seven-gene phenazine biosynthetic operons, designated *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*, are involved in pyocyanin production (35). Besides the presence of a LasR binding site upstream of the *phzA1* operon (35), little is known about the regulation of these operons.

In the parental strain PAO1, pyocyanin production was relatively low and inducible on antibiotic challenge, albeit insignificantly (Table 4). However, pyocyanin production in the *ampR* mutant was significantly higher in the absence and presence of the β-lactam agent (Table 4). The high constitutive pyocyanin level was reduced by *ampR* in *trans* from the low-copy-number plasmid (Table 4). This suggests that AmpR neg-

atively regulates pyocyanin production. It remains to be clarified if AmpR regulates one or both of these *phz* operons.

AmpR regulates staphylolytic protease LasA and LasB elastase. *P. aeruginosa* strains that overproduce pyocyanin are also known to produce other extracellular virulence factors. *P. aeruginosa* secretes a number of proteases, which are believed to play a major role in pathogenesis, especially during acute infections (26, 37). We determined that the mutation in *ampR* also affects the expression of two proteases, the LasB elastase and the LasA staphylolytic protease.

LasA staphylolytic protease is a 20-kDa zinc metalloendopeptidase belonging to the β-lytic endopeptidase family of proteases (24). The PAO*ampR* supernatant lysed the *Staphylococcus aureus* cells at a significantly higher rate than PAO1 (Table 4), indicating an increased production of LasA protease in the absence of *ampR*. This phenotype can also be rescued by *ampR* expressed in *trans*. This suggests that AmpR negatively regulates LasA protease production. Since AmpR is a homolog of LysR, the differential expression of LasA in PAO*ampR* could be a result of the effect of AmpR on the transcription of *lasA*. To address this, we introduced plasmid pLPA containing *P_{lasA}-lacZ* (43) into PAO1 and PAO*ampR* (Table 5). In PAO*ampR*, the *P_{lasA}* expression was significantly increased compared to PAO1, suggesting that the increased transcription may contribute to a higher production of LasA staphylolytic protease. As expected, the induction was not influenced by the presence of inducer. It is interesting that the *lasA* promoter is significantly up-regulated in the presence of inducer.

Elastase, a zinc metalloprotease encoded by *lasB*, is capable

TABLE 5. Transcriptional activity of *lasA*, *lasB*, *lasI*, *lasR*, *rhlI*, and *rhlR* promoters

Promoter fusion ^a	β-Galactosidase (Miller units)			
	Noninduced		Induced ^b	
	PAO1	PAO <i>ampR</i>	PAO1	PAO <i>ampR</i>
<i>lasA</i>	790.0 ± 128.9	1,970.5 ± 312.6 ^c	1,364.7 ± 67.0 ^d	2,842.0 ± 286.4 ^d
<i>lasB</i>	709.3 ± 43.7	676.0 ± 75.1	773.0 ± 112.0	650.5 ± 67.3
<i>lasR</i>	2081.3 ± 63.2	6,904.0 ± 256.5 ^c	783.4 ± 136.9 ^d	6,805.8 ± 173.6 ^c
<i>lasI</i>	7,488.5 ± 193.8	12,985.3 ± 209.7 ^c	8,168.7 ± 405.2	12,413.7 ± 179.1 ^c
<i>rhlR</i>	9,188.8 ± 475.6	6,259.5 ± 26.1 ^c	10,375.5 ± 563.6	8,711.5 ± 301.6 ^c
<i>rhlI</i>	7,880.9 ± 784.1	9,624.4 ± 509.7	7,316.2 ± 796.4	9,245.8 ± 550.2
Vector	353.0 ± 33.7	316.7 ± 37.0	327.0 ± 24.8	327.7 ± 20.5

^a The promoter-*lacZ* fusions are pLP170 derivatives as described in Table 1.

^b Induction was carried out using 500 µg/ml of benzylpenicillin.

^c *P* value of <0.05 compared between PAO1 and PAO*ampR* under the same condition.

^d *P* value of <0.05 compared between noninduced and induced conditions within the same strain.

of inactivating a wide range of biological tissues and immunological agents (43). In the absence of inducer, there is a twofold induction of elastase activity in PAO1. However, in the presence of inducer, the *ampR* mutation failed to induce the production of LasB elastase. This induction could be restored with *ampR* expressed in *trans* (Table 4). This suggests that AmpR positively regulates LasB activity in the presence of inducer. To determine if the effect of AmpR is on the transcription of the *lasB* gene, we introduced plasmid pLPB, containing the P_{lasB} -*lacZ* fusion (43), into PAO1 and PAO*ampR*. The P_{lasB} expression did not differ significantly between PAO1 and PAO*ampR* (Table 5). This suggests that AmpR does not affect P_{lasB} directly and that the noninducibility of LasB protease activity is likely due to an indirect effect of AmpR.

Loss of *ampR* affects the expression of quorum sensing genes. The regulation of *P. aeruginosa lasA* and *lasB* genes has been explored extensively. Both of these genes are regulated by the *las* and *rhl* quorum sensing systems (49). We wanted to determine if AmpR affects the production of protease indirectly via the quorum sensing systems. To address this, plasmids containing P_{lasI} -*lacZ*, P_{lasR} -*lacZ*, P_{rhlI} -*lacZ*, and P_{rhlR} -*lacZ* transcriptional fusions were introduced into PAO1 and PAO*ampR* (42). The analyses of these fusions in PAO*ampR* (p*ampR*) were not investigated. However, based on all the analyses above we predict that p*ampR* will restore the wild-type phenotype in the mutants.

In *P. aeruginosa*, there is a direct association between the production of LasA protease and the LasI-LasR quorumone system (43, 51). Since LasR positively regulates LasA expression, we postulated that the higher production of LasA protease in the PAO*ampR* strain may also be a direct result of the higher transcription of *lasI* and *lasR* genes (Table 5). In fact, there was a significant increase in the expression of the *las* genes, suggesting that AmpR is also a negative regulator of *lasI* and *lasR*, adding yet another layer of complexity to the regulation of these genes (49). Similar elevation was seen in PAO*ampR* with P_{lasI} -*lacZ* and P_{lasR} -*lacZ* fusions in the presence of the inducer (Table 5). This suggests that AmpR negatively regulates *lasI* and *lasR* transcription. In addition, the presence of inducer in PAO1 resulted in a significant ($P < 0.05$) decrease in P_{lasR} -*lacZ* activity. This loss of activity was recovered in the absence of *ampR*, which further supports the idea that AmpR is a negative regulator of *lasR* expression.

The *rhl* quorum sensing is also known to regulate the transcription of *lasA* and *lasB* genes. There was no significant change in the P_{rhlI} -*lacZ* fusion between the PAO1 and PAO*ampR* strains (Table 5). However, in PAO*ampR*, we saw a significant reduction of *rhlR* transcription compared to that in PAO1 either in the absence or in the presence of inducer (Table 5), suggesting that AmpR is a positive regulator of *rhlR* expression.

Though the LasB elastase was slightly reduced in the *ampR* mutant (Table 4), there was no concomitant decrease in the expression of the *lasB* promoter in PAO*ampR* (Table 5). The expression of LasB is more complicated. It involves the coordinate regulation of three quorum sensing systems, including *lasR-lasI*, *rhlR-rhlI*, and *Pseudomonas* quinolone signal (PQS) (36). Thus, AmpR may act indirectly via the *rhlR-rhlI* and *Pseudomonas* quinolone signal (PQS) quorum sensing systems. It appears that the reduction of LasB activity seen in PAO-

ampR is directly correlated with the decrease of *rhlR* expression. These results suggest that AmpR positively regulates *rhlR* expression and LasB activity.

Concluding remarks. (i) *P. aeruginosa* AmpR is a global regulator. *E. coli* naturally lacks *ampR*, and its native *ampC* gene is not inducible by β -lactam antibiotics (22). Thus, most of the studies address the role of *ampR* in *E. coli* using heterologous systems in a multicopy plasmid background. The role of AmpR as a transcriptional activator of β -lactamase production, and also as a global regulator of other virulence factors, was a serendipitous conjecture drawn from the direct observation of PAO*ampR* on LB plates and liquid culture that appeared to synthesize higher levels of pyocyanin. In fact, our studies comparing PAO1 with its isogenic *ampR* mutant revealed that AmpR positively regulates the expression of *ampC* and *rhlR* and the activities of AmpC β -lactamase and LasB. In addition AmpR negatively regulates the expression of *poxB*, *lasA*, *lasI*, and *lasR* and the production of pyocyanin, PoxB β -lactamase, and LasA. In fact this is typical of LysR-type transcriptional regulators that are known for activating divergent transcription of linked target genes or unlinked regulons encoding extremely diverse functions (45). However, this is the first report linking AmpR to many diverse functions.

(ii) Mechanism of AmpR action. As in other members of the LysR family of regulatory proteins, the DNA-binding motif is found in the N terminus of the AmpR protein (22, 29, 31). The binding of *P. aeruginosa* AmpR to the *P. aeruginosa ampC* promoter was suggested by the ability of *P. aeruginosa* AmpR in the *E. coli* extract to immobilize the *ampC* promoter-containing DNA fragment in a gel shift assay (33). The LysR-type proteins generally bind to inverted repeat motifs, including the T-N(11)-A motif (45). However, the binding motifs of *C. freundii* and *E. cloacae* AmpR have been determined in the *ampR-ampC* intergenic regions (31). Using this consensus sequence (5'-TCTGCTGCTAAATTT), we found a strong conserved sequence (5'-TCTGCTCCAAATTT-3') within the *ampR-ampC* intergenic region of PAO1.

Since AmpR appears to regulate also other genes, we analyzed the intergenic regions of all the AmpR-regulated genes for the presence of its binding site. Since the transcription start sites for many of these genes have not been determined, we searched for a potential AmpR binding sequence 200 bp immediately upstream from their open reading frames, both manually and using computer software such as AlignACE (23). No AmpR binding site was found upstream of *phzA1*, *rhlI*, and *rhlR*. However, the putative sites in other genes are relatively weak. A detailed promoter analysis, followed by footprinting with purified protein, is necessary to further elucidate the significance of these sequences and the mechanism of regulation.

Although much is known about the ubiquity of *ampC-ampR*, little is understood about the regulation mechanism of β -lactamase. The study presented here provides new insights into the role of *P. aeruginosa* AmpR as a global regulator involved in antibiotic resistance and the expression of many virulence factors. The combination of increased resistance to antibiotics and hyperproduction of pyocyanin and LasA protease would provide a mechanism of protection and survival in the harsh lung environment.

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