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# Structural and Functional Characterization of *Pseudomonas aeruginosa* Global Regulator AmpR

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***Pseudomonas aeruginosa* is a dreaded pathogen in many clinical settings. Its inherent and acquired antibiotic resistance thwarts therapy. In particular, derepression of the AmpC β-lactamase is a common mechanism of β-lactam resistance among clinical isolates. The inducible expression of *ampC* is controlled by the global LysR-type transcriptional regulator (LTTR) AmpR. In the present study, we investigated the genetic and structural elements that are important for *ampC* induction. Specifically, the *ampC* ($P_{ampC}$) and *ampR* ($P_{ampR}$) promoters and the AmpR protein were characterized. The transcription start sites (TSSs) of the divergent transcripts were mapped using 5′ rapid amplification of cDNA ends-PCR (RACE-PCR), and strong $\sigma^{54}$ and $\sigma^{70}$ consensus sequences were identified at $P_{ampR}$ and $P_{ampC}$, respectively. Sigma factor RpoN was found to negatively regulate *ampR* expression, possibly through promoter blocking. Deletion mapping revealed that the minimal $P_{ampC}$ extends 98 bp upstream of the TSS. Gel shifts using membrane fractions showed that AmpR binds to $P_{ampC}$ *in vitro* whereas *in vivo* binding was demonstrated using chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR). Additionally, site-directed mutagenesis of the AmpR helix-turn-helix (HTH) motif identified residues critical for binding and function (Ser38 and Lys42) and critical for function but not binding (His39). Amino acids Gly102 and Asp135, previously implicated in the repression state of AmpR in the enterobacteria, were also shown to play a structural role in *P. aeruginosa* AmpR. Alkaline phosphatase fusion and shaving experiments suggest that AmpR is likely to be membrane associated. Lastly, an *in vivo* cross-linking study shows that AmpR dimerizes. In conclusion, a potential membrane-associated AmpR dimer regulates *ampC* expression by direct binding.***

*Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, causes severe and life-threatening infections in susceptible individuals. This pathogen is primarily associated with morbidity and mortality in patients with cystic fibrosis, a deadly genetic disease (1). The bacterium's innate ability to counteract the action of antibiotics often complicates treatment strategies. Intrinsic resistance is conferred by its low membrane permeability, the expression of efflux pumps and hydrolyzing enzymes, the alteration of antimicrobial targets, and the ability to form biofilm (2–4). In particular, resistance to β-lactam antibiotics is mediated by the expression and overproduction of the chromosomally encoded class C β-lactamase AmpC (5–7).

Ambler class C β-lactamases were first described in members of the *Enterobacteriaceae* family where expression is either constitutively low or inducible (8–11). In species where expression is inducible, such as *Citrobacter freundii* and *Enterobacter cloacae*, the induction requires β-lactam challenge and the presence of the transcriptional regulator AmpR (8, 12–14). AmpR is a member of the LysR family of transcriptional regulators and as such is a DNA-binding protein with a predicted helix-turn-helix (HTH) motif at the N terminus and an inducer-binding domain at the C terminus (15, 16). Comprehensive studies in the *Enterobacteriaceae* have established the critical role of AmpR as the regulator of *ampC* expression and the paradigm of β-lactamase induction.

In the *Enterobacteriaceae*, *ampC* inducibility is intimately linked to the recycling of the peptidoglycan (PG) of the murein sacculus (17–20). During normal physiological growth, *N*-acetylglucosaminyl-1,6-anhydromuropeptides (GlcNAc-1,6-anhydroMurNAc tri-, tetra-, and pentapeptides) are continuously being released from the murein sacculus due to remodeling (17, 18). The permease, AmpG, transports the metabolites into the cytoplasm, where the glycosidase NagZ removes the GlcNAc moiety and the amidase AmpD removes the stem peptides either from the incoming GlcNAc-1,6-anhydro-MurNAc peptides or from the NagZ-processed product (18, 21–26). The resulting muramyl peptides are recycled back into the PG biosynthetic pathway to form the PG precursor UDP-MurNAc-pentapeptide (27). It has been proposed that during normal cell growth, the cytosolic concentrations of UDP-MurNAc-pentapeptide maintain AmpR in an inac-

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tive conformation that represses the expression of *ampC* (17, 18). In the presence of β-lactams, however, there is an excessive breakdown of murein leading to accumulation of 1,6-anhydromuropeptides in the cytoplasm, which in turn overwhelm the hydrolytic activity of AmpD (17, 18, 28, 29). The increased number of AmpD-unprocessed muramyl peptides presumably displaces the repressor UDP-MurNAc-pentapeptide from AmpR and induces a conformational change in the protein to promote expression of *ampC* (17, 28, 29).

All *amp* gene homologs (*ampC*, *ampR*, *ampD*, and *ampG*) have been identified and studied in *P. aeruginosa* (30–37). Whether a similar induction mechanism is employed by *P. aeruginosa* is not yet known; however, recent work illustrates significant departures from the classical enterobacterial induction system. In particular, there are three *ampD* homologs in *P. aeruginosa* that are responsible for a stepwise upregulation mechanism leading to constitutive β-lactamase hyperexpression (2, 3, 30, 32). Additionally, *P. aeruginosa* harbors two AmpG homologs, PA4218 (AmpP) and PA4393 (AmpG), which appear to be required for induction of *ampC* (36, 38). Further, our lab has shown that *P. aeruginosa* AmpR is a global transcriptional regulator involved in the control of *amp* and various other genes (31, 39–41).

AmpR exhibits high sequence identity with its counterparts in *C. freundii* and *E. cloacae*, and as in the *Enterobacteriaceae*, *ampR* is located immediately upstream of *ampC* and divergently transcribed (34, 35). Such similarities suggest a common regulatory mechanism among the species; however, the *P. aeruginosa ampR-ampC* intercistronic region bears little resemblance to that of the enterobacteria. *In vitro* studies using crude extracts have shown that *P. aeruginosa* AmpR binds to this region, but the exact binding site and the identity of the amino acids involved in the interaction have not yet been determined (34). In essence, not much is known about the structural elements that are critical to the functioning of *P. aeruginosa* AmpR as a regulator of β-lactamase expression.

In the present work, we define some of the genetic elements in the *ampR-ampC* intergenic region, including the *ampR* and *ampC* transcriptional start sites, as well as the minimal length of the *ampC* promoter needed for induction of the *ampC* system. We further show that AmpR specifically binds to a 193-bp $P_{ampC}$ fragment identified by promoter mapping as being required for induction. We also identify amino acids in the AmpR HTH motif that are critical for the interaction with the promoter. Additionally, we examine the role of two amino acids, Gly102 and Asp135, previously implicated in the repression state of AmpR from the *Enterobacteriaceae*. Lastly, we show that *P. aeruginosa* AmpR likely functions as a dimer, as previously seen for *C. freundii*, and is potentially a membrane protein.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** Bacterial strains, plasmids, and primers employed in this study are shown in Table S1 in the supplemental material. *Escherichia coli* and *P. aeruginosa* were routinely cultured in Luria-Bertani medium (LB; 10 g tryptone, 5 g yeast extract, and 5 g NaCl, per liter). *Pseudomonas* isolation agar (Difco) was used with LB at a 1:1 ratio in triparental mating experiments. Antibiotics were used at the following concentrations (per milliliter): ampicillin (Ap) at 100 μg, tetracycline (Tc) at 15 μg, and gentamicin (Gm) at 15 μg for *E. coli*; Gm and Tc each at 75 μg for *P. aeruginosa*. PA0Δ*ampC* and PA0Δ*ampR* strains used in this work were previously constructed using overlap extension PCR and homologous recombination (40, 42).

**$P_{ampC}$ promoter deletions.** To characterize the minimal promoter necessary for full activity, 5′-end deletions of $P_{ampC}$ were constructed and transcriptionally fused to a promoterless *lacZ*. Briefly, 352-, 193-, 171-, 151-, 130-, 111-, 90-, 70-, and 51-bp fragments were generated by PCR with the following primer pairs, respectively: SBJ03*ampC*RFor-$OCP_{ampC}$RevBc, $OCP_{ampC}$For193-$OCP_{ampC}$RevBc, $OCP_{ampC}$For173-$OCP_{ampC}$RevBc, $OCP_{ampC}$For151-$OCP_{ampC}$RevBc, $OC_{PampC}$For131-$OCP_{ampC}$RevBc, $OCP_{ampC}$For111-$OCP_{ampC}$RevBc, $OCP_{ampC}$For91-$OCP_{ampC}$RevBc, $OCP_{ampC}$For71-$OCP_{ampC}$RevBc, and $OCP_{ampC}$For51-$OCP_{ampC}$RevBc (see Table S1 in the supplemental material). The fragments were sequenced and then cloned into the EcoRI-BamHI sites of the integrative vector mini-CTX-*lacZ* and integrated into PAO1.

**Construction of His-tagged AmpR.** Primers OCAmpR-His-For and OCAmpR-His-Rev (see Table S1 in the supplemental material) were used to amplify PAO1 genomic *ampR*. The 933-bp amplicon, carrying a $His_6$ sequence at the 3′ end, was cloned into pBluescriptSK(+) and sequenced (see Table S1). The fragment was subsequently subcloned into the EcoRI-BamHI sites of pMMB67EH-Gm, a broad-host-range expression vector (43). His-tagged AmpR was shown to be functional by β-lactamase assay and Etest (see Text S1 and Table S2).

**Expression and purification of AmpR-$His_6$.** AmpR-$His_6$ was purified according to standard protocols. Briefly, stationary-phase cultures of PA0Δ*ampR* (pAmpR-$His_6$) were diluted to an optical density at 600 nm ($OD_{600}$) of 0.02 in 2 liters of LB broth and incubated with shaking at 37°C until the culture density reached an $OD_{600}$ of 0.2. Cells were then induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated for an additional 6 h before harvesting. The cells were recovered by centrifugation at 6,000 × *g* for 10 min at 4°C and resuspended in 25 ml of lysis buffer (20 mM HEPES, pH 8, 0.5 M NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 pellets of EDTA-free protease inhibitor cocktail tablets [complete], 100 μl of 0.1 mg $ml^{-1}$ of lysozyme, and 5 μl of DNase I). Following disruption of the cells on ice with sonication (20-s pulse on and 20-s pulse off for 20 min; amplitude, 50%), the cell lysate was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was further ultracentrifuged at 36,000 rpm for 1 h at 4°C. Membrane pellets were resuspended in 20 ml of solubilization buffer {20 mM HEPES, pH 8, 0.5 M NaCl, 10% glycerol, 5 mM imidazole, 1 mM PMSF, 2 pellets of EDTA-free protease inhibitor cocktail tablets (complete), and 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)} and loaded onto a HisTrap FF 1-ml column. AmpR-$His_6$ was eluted with buffer B (20 mM HEPES, pH 8, 0.5 M NaCl, 10% glycerol, 500 mM imidazole, and 0.6% CHAPS) by using a fast protein liquid chromatograph (FPLC) (Akta; Amersham Biosciences). About 20 ml was recovered and dialyzed to remove imidazole. This AmpR preparation was used to make AmpR-specific antibodies.

**Construction of *P. aeruginosa* AmpR HTH and point mutants.** Site-directed mutagenesis was used to replace various amino acid residues in AmpR. Briefly, Ser38, His39, Lys42, Ser43, and Glu46 were replaced with Ala; Gly102 and Asp135 were replaced with Glu and Asn, respectively. Substitutions were constructed by PCR using the following primer pairs: AmpRSer38AlaFor-AmpRSer38AlaRev, zAmpRHis39AlaFor-AmpRHis39AlaRev, AmpRLys42AlaFor-AmpRLys42AlaRev, AmpRSer43AlaFor-AmpRSer43AlaRev, AmpRGlu46AlaFor-AmpRGlu46AlaRev, AmpRGly102GluFor-AmpRGly102GluRev, and AmpRAsp135AsnFor-AmpRAsp135AsnRev (see Table S1 in the supplemental material).

**Membrane fraction purification.** Preliminary studies showed that pAmpR-$His_6$ expression and β-lactamase induction were achieved with a 2-hour incubation at a 1 mM IPTG concentration. Thus, PA0Δ*ampR* (pAmpR-$His_6$) cells at an $OD_{600}$ of 0.2 were induced with 1 mM IPTG and incubated for 2 h before harvesting for membrane fractionation. For β-lactamase induction, cells were further treated with 200 μg $ml^{-1}$ of penicillin G an hour after IPTG addition. Cells were recovered by centrifugation at 6,000 × *g* for 10 min at 4°C and resuspended in 50 ml of lysis buffer (20 mM HEPES, pH 8, 0.1 M NaCl, 1 mM EDTA, 1 mM PMSF, and 50 μl of DNase I). Following disruption of the cells on ice with sonication (15 cycles of 10-s pulse on and 30-s pulse off; amplitude, 40%), the cell

lysate was centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was ultracentrifuged at 36,000 rpm (Ti70 rotor) for 1 h at 4°C, and the pellets were resuspended with 2 ml of membrane buffer (25% sucrose, 20 mM Tris, pH 8, and 0.5 mM PMSF). Two hundred milliliters of membrane fractions was aliquoted and stored at −80°C.

**EMSA.** The 193-bp PCR fragment containing the *ampR-ampC* intergenic region plus a small part of the *ampR* open reading frame (ORF) was used to perform an electrophoretic mobility shift assay (EMSA). Ten picomoles of this fragment was radiolabeled at the 5′ end by incubation with T4 polynucleotide kinase (NEB) and [γ-$^{32}$P]ATP (3,000 Ci mmol$^{-1}$; PerkinElmer). The labeled fragment was diluted to a final concentration of 100 nM, and unincorporated nucleotides were removed by Sephadex G-25 (Bio-Rad) spin chromatography. DNA-binding reaction mixtures containing 50 fmol of $^{32}$P-labeled DNA probe and various amounts of total protein membrane fractions were incubated for 20 min and loaded thereafter in a nondenaturing 5% PAGE gel. Radioactive signals were detected by scanning a phosphostorage cassette with the GE Healthcare Typhoon 9400 scanner.

For competition assays, 50 fmol of the 193-bp $^{32}$P-labeled probe was mixed with the unlabeled 193-bp fragment in 10-, 100-, and 500-fold molar excess in the EMSA. For nonspecific assays, 50 fmol of the 193-bp $^{32}$P-labeled probe was mixed with a PCR-amplified 233-bp fragment (*alg44*) in 10-, 100-, and 500-fold molar excess in the EMSA.

**5′ RACE-PCR.** The *ampC* and *ampR* transcription start sites (TSSs) were mapped using a classical 5′ rapid amplification of cDNA ends-PCR (RACE-PCR) on total mRNAs extracted from PAO1, PA0Δ*ampR*, and PA0Δ*ampC* (44). Stationary-phase cultures of PAO1, PA0Δ*ampR*, and PA0Δ*ampC* were diluted to an $OD_{600}$ of 0.02 and incubated with shaking at 37°C until the cultures reached an $OD_{600}$ of 0.6. The cultures were then induced with 200 μg ml$^{-1}$ of penicillin G for 1 h and subsequently blocked on ice for 15 min with 1/5 of the final culture volume in 5% acidic phenol-95% ethanol, pH 4. One milliliter of the cells was recovered by centrifugation and resuspended with 3 mg ml$^{-1}$ of lysozyme (Tris-EDTA, pH 8). RNA was then extracted according to the RNeasy minikit protocol (Qiagen), treated with 10 U of RQ1 DNase (Promega) for 1 h, extracted with phenol-chloroform–isoamyl alcohol (25:24:1) and chloroform, precipitated, and dried. Superscript III (Invitrogen) was then used to reverse transcribe 10 μg of RNA as previously described (44) using primers 5RA-$P_{ampC}$233 and 5RA-$P_{ampR}$229 for determination of the *ampC* and *ampR* TSSs, respectively. In the first round of PCR amplification (*Pfu*; Stratagene), primers $Q_t$, $Q_0$, and 5RA-$P_{ampC}$154 were used for *ampC* TSS determination, while $Q_t$, $Q_0$, and 5RA-$P_{ampR}$169 were used for determination of the *ampR* TSS. In the second round of PCR amplification (*Pfu*; Stratagene), primer pairs $Q_i$-5RA-$P_{ampC}$113 and $Q_i$-5RA-$P_{ampR}$99 were used for *ampC* and *ampR* TSS determination, respectively (see Table S1 in the supplemental material). PCR products were cloned into TOPO (Invitrogen), blue colonies were selected for screening, and clones were sequenced.

**qPCR analysis of *ampR* and *ampC* mRNAs.** Total RNA was extracted from PAO1, PA0Δ*rpoN*, and PA0Δ*rpoN* (pRpoN) in the presence and absence of the inducer (0.2 μg ml$^{-1}$ imipenem) using the RNeasy minikit (Qiagen). RNA was reverse transcribed into cDNA with Superscript III (Invitrogen) and an $(NS)_5$ random primer using standard methods (45). For quantitative PCR (qPCR), the ABI 7500 (Applied Biosystems) cycler was used with Power SYBR green PCR master mix with ROX (Applied Biosystems). The reading was normalized to *clpX* (*PA1802*), whose expression remains constant in all the samples and under all the conditions tested. Melting curves were generated to ensure primer specificity. Gene expression of each sample was normalized to the PAO1 uninduced value, to see the effect of induction and mutation at the same time. Primer pairs DBS_QRTAmpRFwd-DBS_QRTAmpRRev and qRT_*ampC*F-qRT_*ampC*R were used for the real-time amplification of *ampR* and *ampC*, respectively.

**Construction of VSV-G-tagged AmpR.** A 540-bp fragment corresponding to the 3′ end of *ampR*, minus the stop codon, was amplified using primers DB_ampR3′_F and DB_ampR3′_R. The amplicon was cloned into pP30ΔFRT-MvaT-V (46), a replication-incompetent vector in *P. aeruginosa*, such that the 3′ end of *ampR* was fused in frame with three alanines and the vesicular stomatitis virus G protein (VSV-G) tag (YTDIEMNRLGK). The construct was then was introduced into PAO1 as a single copy by mating, and clones were selected for gentamicin resistance.

**Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR).** Cells harboring the VSV-G-tagged AmpR were harvested after sub-MIC β-lactam exposure (40) and treated with formaldehyde to cross-link proteins with DNA as previously described (46). DNA was sheared by sonication to an average length of 0.5 to 1.0 kb, and AmpR was immunoprecipitated with anti-VSV-G–agarose beads (Bethyl Laboratories, Inc.). ChIP-qPCR was performed using Power SYBR green PCR master mix (Applied Biosystems) with primers DBS_ChIP_*ampC*F and DBS_ChIP_*ampC*R. Fold enrichment was normalized to *clpX* (*PA1802*).

**Protein cross-linking.** PA0Δ*ampR* (pAmpR-$His_6$) was grown to an $OD_{600}$ of 0.3 and induced with 1 mM IPTG for 2 h. Cells were grown for 2 additional hours in the presence and absence of β-lactam antibiotics (0.1 μg ml$^{-1}$ of imipenem). Cultures were then treated with 0.1% formaldehyde for 20 and 40 min at room temperature. Crude extracts containing 10 μg of total protein were separated on an SDS-polyacrylamide gel, and AmpR was visualized using anti-His antibody. The blot was subsequently stripped and reprobed using anti-$\sigma^{70}$ antibody (NeoClone).

**Polyclonal anti-AmpR-$His_6$ antibody production.** Purified AmpR-$His_6$ was used as antigen to raise anti-AmpR-$His_6$ rabbit polyclonal antibodies (Covance, Princeton, NJ). AmpR-$His_6$ antibody was affinity purified as previously described (47).

**Western blotting and EMSA of HTH mutants.** The concentration of purified AmpR-$His_6$ was determined by the bicinchoninic acid (BCA) method (48). A calibration Western blot was generated using a FujiFilm LAS-3000 imager to correlate intensities with the concentration of purified AmpR-$His_6$. Membrane fractions were purified from PA0Δ*ampR* pAmpR HTH mutants, and their concentrations were determined using the BCA method, whereas the exact quantity of AmpR was deduced from Western blotting. Preliminary gel shifts with increasing concentrations of membrane fractions of AmpR HTH mutants showed that 0.4 mg ml$^{-1}$ is sufficient to shift the 193-bp $P_{ampC}$ PCR fragment (data not shown). For a second gel shift, 0.4 mg ml$^{-1}$ of total membrane fraction (8.44 ng of AmpR), recovered from PA0Δ*ampR* overexpressing AmpR HTH mutants in the presence and absence of 200 μg ml$^{-1}$ penicillin G, was hybridized with the 193-bp PCR fragment spanning the *ampC-ampR* intergenic region (see Fig. 7). As this concentration was not enough to visualize AmpR, a higher quantity (33 ng) was used for Western blotting of the HTH mutants to show that the amount of AmpR-$His_6$ is equivalent under all conditions and thus in the EMSA (data not shown). Further, the stability of AmpR-$His_6$ mutants was verified by Western blotting using equal amounts of total protein with AmpR-specific antibodies (see Fig. 8). Sigma$^{70}$ (NeoClone) was used as a control to show that the same amount of total protein was loaded per sample. All Western blots were developed according to standard protocols. Briefly, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and blocked with TBST (Tris-buffered saline–0.1% Tween) and 5% nonfat dry milk at 4°C overnight or for 4 h, followed by rinsing with the same solution and probing with rabbit anti-AmpR antibody (1:3,000). Membranes were subsequently washed with TBST, incubated with goat anti-rabbit IgG (heavy plus light chain [H+L])–horseradish peroxidase-conjugated antibody (1:5,000) (Bio-Rad), rinsed, and developed using enhanced chemiluminescence Western blotting substrate (Pierce).

**β-Galactosidase assay.** β-Galactosidase assays were performed as previously described (49).

**AmpR-LacZ and -PhoA fusion construction and analysis.** The topology of AmpR was investigated using *phoA* and *lacZ* fusions. The plasmid pSJ01 (31), carrying a 1,220-bp fragment containing *ampR*, was digested at HindIII, HincII, and PstI, corresponding to the amino acid positions

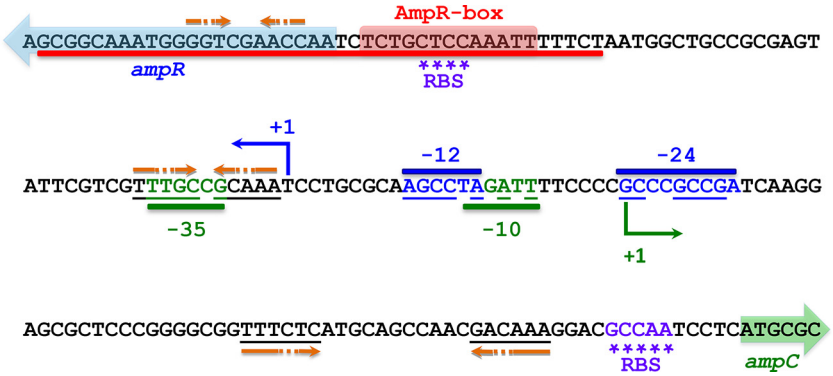


**FIG 1** Intergenic region of *P. aeruginosa ampC* and *ampR*. A 148-bp region separates *ampR* from *ampC*. The *ampR* and *ampC* transcriptional start sites are indicated with a blue and green +1, respectively, whereas the colored arrows designate the beginning of the ORFs. Strong $\sigma^{54}$ (blue) and $\sigma^{70}$ (green) sequences are observed in the *ampR* and *ampC* promoter regions, respectively. The underlined bases correspond to conserved sequences. The Shine-Dalgarno sequences or ribosome binding sites (RBSs) are marked by asterisks. A putative AmpR-binding site was identified based on sequence homology and is indicated by the AmpR box. Orange arrows denote palindromic sequences in close proximity to the *ampC* and *ampR* RBSs indicative of hairpin formation for regulating translation through blocking of ribosome binding.

Gln15, Val134, and Gln186, respectively (see Fig. S2A in the supplemental material). The resultant fragments were ligated in frame upstream of *phoA*- and *lacZ*-containing plasmids, pTrcphoA and pTrclacZ, respectively (see Table S1) (50). The *phoA* and *lacZ* activities were qualitatively determined in *E. coli* according to standard protocols (49).

**Protease protection (shaving) assay.** A stationary-phase culture of PA0Δ*ampR* (pAmpR-$His_6$) was diluted to an $OD_{600}$ of 0.02 and incubated with shaking at 37°C until the culture reached an $OD_{600}$ of 0.4. The cells were then induced with 1 mM IPTG for 4 h, and chloramphenicol (500 μg $ml^{-1}$) was added 30 min prior to harvesting to stop protein synthesis. The cells were harvested by centrifugation and resuspended in 40 mM Tris-Cl, pH 8.0, 0.5 M sucrose. Spheroplasts were obtained by adding 1 mg $ml^{-1}$ of lysozyme and 4 mM EDTA for 10 min in a 30°C water bath followed by the addition of 20 mM $MgCl_2$. The formation of spheroplasts was monitored by light microscopy. Spheroplasts were harvested by centrifugation at 4,000 × *g* for 10 min and resuspended in 40 mM Tris-Cl, pH 8.0, 0.5 M sucrose. Proteinase K (10 μg $ml^{-1}$) was added to a 1-ml aliquot of the spheroplasts and incubated in a 30°C water bath. Samples were taken at different time points and added to 2 mM PMSF and 4× SDS-PAGE sample buffer. Samples were then boiled for 5 min and run in a 4 to 20% SDS-PAGE gel (Criterion; Bio-Rad). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and identified using AmpR (Covance), $\sigma^{70}$ (NeoClone), and His tag (Qiagen) antibodies. The immunoblot was developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

## RESULTS AND DISCUSSION

**Analysis of the *P. aeruginosa ampC-ampR* regulatory region.** *P. aeruginosa* AmpR shares high identity with its homologs in *C. freundii* and *E. cloacae* (58.3 and 62.5%, respectively), as well as the same gene organization, with *ampR* located upstream of *ampC* and divergently transcribed (34, 35). The *P. aeruginosa ampR-ampC* intercistronic region, however, shares no significant similarities with those of *C. freundii* and *E. cloacae*, with the exception of an inverted 38-bp sequence that in *C. freundii* is protected by AmpR (35, 51). The lack of conservation in the promoter region could point to different sigma factor requirements and/or different regulatory mechanisms. To elucidate the transcriptional regulatory elements of *P. aeruginosa ampC* and *ampR*, their 148-bp intergenic region was characterized.

The transcription start sites (TSSs) were determined using 5′ RACE-PCR on total mRNAs isolated from PAO1, PA0Δ*ampR*, and PA0Δ*ampC* (Fig. 1). A strong similarity to RpoN ($\sigma^{54}$) sigma factor sequences was detected at the −12 and −24 positions of the *ampR* TSS, whereas strong $\sigma^{70}$ promoter sequences were observed at the −10 and −35 positions of *ampC*. Alignment of the *C. freundii*, *E. cloacae*, and *P. aeruginosa* intergenic regions reveals fair conservation of the *ampC* −10 and −35 sequences. However, there is a downstream shift in the *P. aeruginosa ampR* TSS that could contribute to the change in sigma factor control observed here (see Fig. S1 in the supplemental material).

The presence of putative −12 and −24 recognition sequences in the *ampR* promoter ($P_{ampR}$) suggested that its expression might be RpoN or $\sigma^{54}$ dependent. Quantitative real-time PCR was used to validate this hypothesis. Total RNA was extracted from β-lactam-induced and uninduced PAO1, PA0Δ*rpoN*, and PA0Δ*rpoN* (pRpoN) strains and reverse transcribed into cDNA using standard methods (45). In the wild type, exposure to β-lactams increased *ampR* and *ampC* expression approximately 100-fold over that in the uninduced samples (normalized to 1) (Fig. 2). A further, significant increase in expression of both genes was observed in PA0Δ*rpoN* upon β-lactam challenge that could be rescued by pRpoN. In the absence of β-lactams, a small but significant reduction in expression was observed in PA0Δ*rpoN* that could not be complemented.

In spite of the presence of a probable binding site on $P_{ampR}$, $\sigma^{54}$ is not required for *ampR* expression. The significant increase in the absence of *rpoN* and in the presence of the β-lactams suggests that $\sigma^{54}$ negatively impacts *ampR* expression. RpoN may exert this negative effect indirectly by enabling transcription of a negative regulator or directly by competing for the RNA polymerase holoenzyme with another sigma factor. Alternatively, *P. aeruginosa* RpoN can directly repress transcription by blocking promoter access to a different sigma factor in a phenomenon referred to as σ-factor antagonism, as previously reported (52). Specifically, $\sigma^{54}$ has been shown to repress $\sigma^{22}$-dependent transcription from $P_{algD}$ by directly binding to the overlapping promoter sequences in the absence of an external stimulus (52). Similarly, σ-factor antagonism has also been reported in *E. coli*, where mutagenesis of a $\sigma^{54}$-dependent promoter created a new TSS with a $\sigma^{70}$ require-

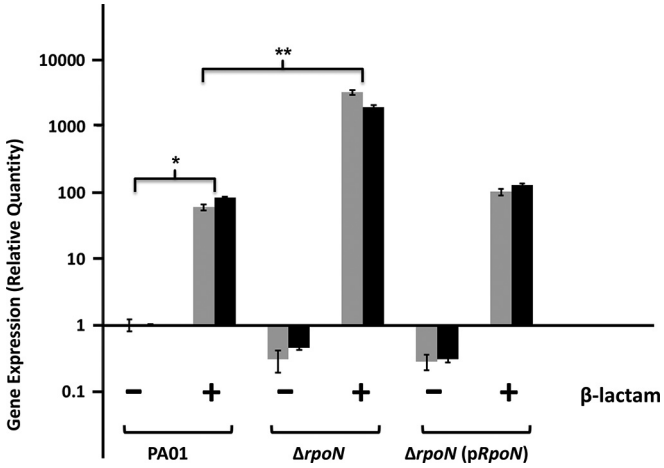


**FIG 2** RpoN downregulates *P. aeruginosa ampR* expression in the presence of β-lactams. RNA was isolated from PAO1, PA0Δ*rpoN*, and PA0Δ*rpoN* (pRpoN) in the presence and absence of β-lactams, reverse transcribed to cDNA, and tested by qPCR with *ampC* (gray bars)- and *ampR* (black bars)-specific primers, as described in Materials and Methods. Values were normalized to the expression of the wild-type uninduced sample and represent the mean (±standard deviation) of two experiments conducted in triplicate. *, *P* value $< 0.001$ for both *ampR* and *ampC* expression in induced PAO1 versus expression in uninduced PAO1; **, *P* value $< 0.005$ for *ampR* and *ampC* expression in induced PA0Δ*rpoN* versus expression in induced PAO1 as determined by unpaired *t* test.

ment that exhibited decreased transcriptional activity in the presence of $\sigma^{54}$ (53).

A model of repression by $\sigma^{54}$ through promoter blocking is conceivable, where in the absence of some external stimuli, in this case β-lactams, $\sigma^{54}$ binds $P_{ampR}$ and prevents access and thus transcription by the sigma factor. In the presence of the inducer, however, $\sigma^{54}$ may be partially or completely displaced by the sigma-RNA polymerase complex to promote *ampR* transcription. Thus, the loss of *rpoN* leads to complete derepression of *ampR* expression under the induced condition.

Lastly and very interestingly, the expression of *ampC* followed a pattern similar to that of *ampR* in all backgrounds. Previously, *ampR* expression in *P. aeruginosa* was shown to be low and not significantly induced upon exposure to the β-lactam benzylpenicillin (31). Similarly, expression from the *C. freundii ampR* promoter in *E. coli* was found to be constitutive in the presence and absence of the inducer (6-aminopenicillanic acid) (51). In *E. cloacae*, induction with cefoxitin significantly increased transcription of *ampC* but had no effect on *ampR* expression (12). Recent work from our lab, however, showed that in the presence of very powerful known inducers, namely, imipenem and meropenem, expression of both *ampC* and *ampR* is equally and very significantly induced in wild-type *P. aeruginosa* (42). In light of such results, it is not surprising that our current data show that wild-type *P. aeruginosa* has similar *ampC* and *ampR* mRNA levels in the presence of imipenem. Similar induction profiles in the absence of *rpoN* suggest that the *ampC* and *ampR* promoters can reach their full induction potential upon removal of the restricting negative effect imposed by RpoN. It is not clear how exactly RpoN, or its absence, can accomplish this, but it is worth noting that in the *ampC* and *ampR* TSSs, the −12 sequence of *ampR* overlaps the −10 sequence of *ampC*. Thus, if $\sigma^{54}$ is in fact blocking promoter access to RNA polymerase, it could be blocking access to both the *ampR* and *ampC* promoters until such time as inducers lead to its partial or complete displacement from the intergenic region. Further studies are needed to elucidate the mechanism of *ampC* and *ampR* downregulation by RpoN.

**Mapping of *P. aeruginosa* $P_{ampC}$.** To map the minimal promoter needed for AmpR-dependent activity, a series of 5′-end deletions of $P_{ampC}$ were transcriptionally fused to the promoterless *lacZ* gene of the integrative vector mini-CTX-*lacZ* (54). The activity of each promoter deletion was then analyzed in PAO1 by assaying β-galactosidase activity in the presence and absence of 200 μg $ml^{-1}$ of penicillin G. The minimum length of the promoter needed for full $P_{ampC}$ activity is 193 bp (Fig. 3). This fragment consists of the full *ampR-ampC* intergenic region plus small parts of the *ampR* (22-bp) and *ampC* (23-bp) ORFs. The high activity seen with the 193-bp fragment in the absence of β-lactams compared to the wild type may be the result of the partial or complete removal of the repressor-binding site. Subsequent loss of a 22-bp *ampR* fragment from the 5′ end of the 193-bp segment resulted in a 2-fold decrease in induction, likely indicating partial removal of the activator-binding site. This 22-bp fragment corresponding to the beginning of the *ampR* ORF seems to be necessary for full induction of $P_{ampC}$. *In silico* analysis reveals that this segment is very well conserved among other species but has no real identifiable features. Induction was abolished with a further 20-bp deletion (151-bp fragment). Thus, the 42-bp region (shown in red in Fig. 1 and 3), present at the 5′ end of the 193-bp fragment but deleted from the 151-bp construct, demarcates the outer bounds of the functional promoter needed for activation of $P_{ampC}$. Since this 42-bp fragment includes the AmpR box, an *in silico*-derived putative AmpR-binding site (Fig. 1), this region could be critical for activator binding.

***P. aeruginosa* AmpR binds to $P_{ampC}$.** Previously, *P. aeruginosa* AmpR has been shown to bind $P_{ampC}$ using AmpR-overexpressing *E. coli* whole-cell extracts (34). Similarly, crude preparations of *C. freundii* AmpR were also shown to retard a radiolabeled *ampR-ampC* intergenic region (51). Since preliminary work from our lab suggested that *P. aeruginosa* AmpR is likely to be a membrane-associated protein (see "Localization studies of *P. aeruginosa* AmpR" below and also Fig. S2 in the supplemental material), we tested the ability of PAO1 membrane fractions to bind $P_{ampC}$. EMSA was performed using AmpR-$His_6$-enriched membrane fractions and a [γ-$^{32}$P]ATP-radiolabeled $P_{ampC}$ fragment. Shift was observed with increasing concentrations of total membrane protein up to 0.4 mg $ml^{-1}$ (Fig. 4). The binding was competed out by mixing labeled DNA with unlabeled promoter DNA in a 100-fold molar excess, confirming AmpR binding to $P_{ampC}$ (Fig. 4). Additionally, competition with a nonspecific, unlabeled fragment (233-bp *alg44* PCR fragment) mixed in 10-, 100-, and 500-fold molar excess with the labeled $P_{ampC}$ fragment failed to displace AmpR-$His_6$ from $P_{ampC}$, illustrating the binding specificity.

To determine if AmpR interacts with $P_{ampC}$ *in vivo*, ChIP-qPCR was employed (46). A functional VSV-G-tagged AmpR (see Table S3 in the supplemental material) was introduced into PAO1 as a single copy and then immunoprecipitated with anti-VSV-G antibody. Sequence-specific primers were used to detect the presence of $P_{ampC}$ DNA with qPCR. Promoter occupancy was detected in the presence and absence of β-lactams as expected of LysR-type transcriptional regulators (LTTRs) (16) (fold enrichment over *clpX* control: uninduced, 10.6 ± 1.73; induced, 13.3 ± 4.63; fold

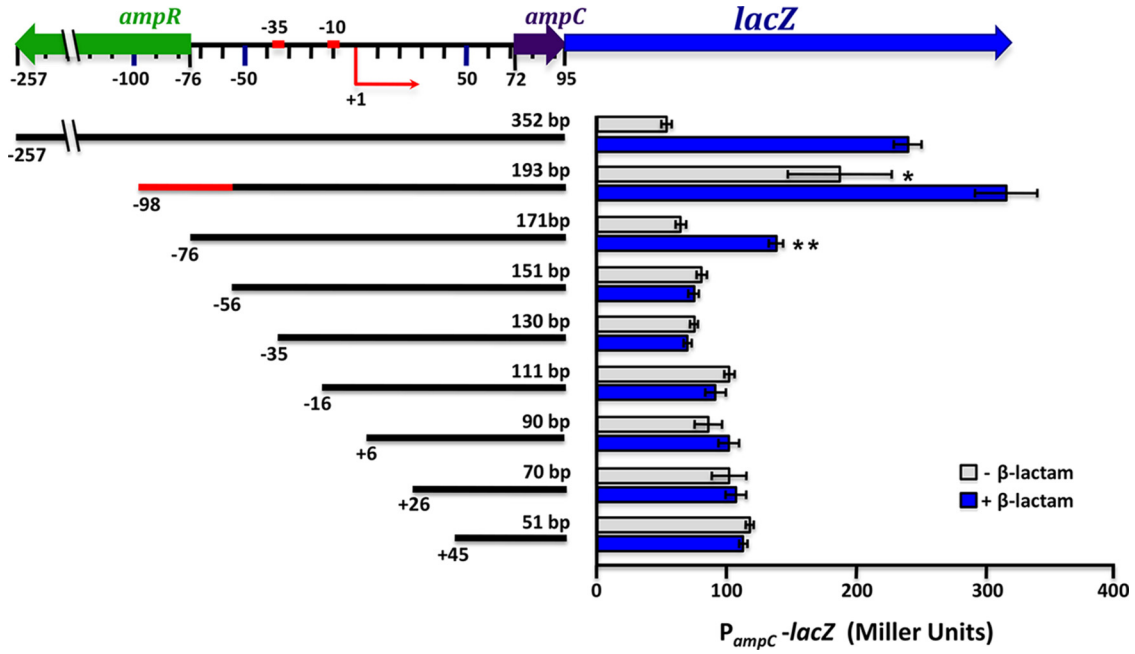


**FIG 3** Mapping of the minimal *P. aeruginosa ampC* promoter. 5′-end deletions of $P_{ampC}$ were constructed and transcriptionally fused to a promoterless *lacZ* gene in the integrative vector mini-CTX-*lacZ* as described in Materials and Methods. Constructs were introduced into PAO1 for integration at the *attB* site to generate single-copy promoter fusions. Promoter activities are expressed in Miller units. The +1 denotes the *ampC* transcriptional start site. The 42-bp segment, missing from the 151-bp construct, which appears to be necessary for activator binding is depicted in red at the 5′ end of the 193-bp fragment. *, *P* value $< 0.05$ compared to uninduced PA0*attB*::$P_{ampC352}$-*lacZ*; **, *P* value $< 0.05$ versus uninduced PA0*attB*::$P_{ampC171}$-*lacZ* as determined by unpaired *t* test using analysis of variance.

enrichment for negative-control target *aprX*: uninduced, 1.30 ± 0.02; induced, 1.34 ± 0.22). AmpR thus binds $P_{ampC}$ *in vivo* in the presence and absence of the inducer.

**HTH is important for AmpR function.** The majority of prokaryotic DNA-binding proteins, including LTTRs, use the HTH motif to interact with DNA (15, 55). In LTTRs, this domain is often found at the N terminus (15, 16). The canonical HTH motif is comprised of three helical bundles, where the second and third

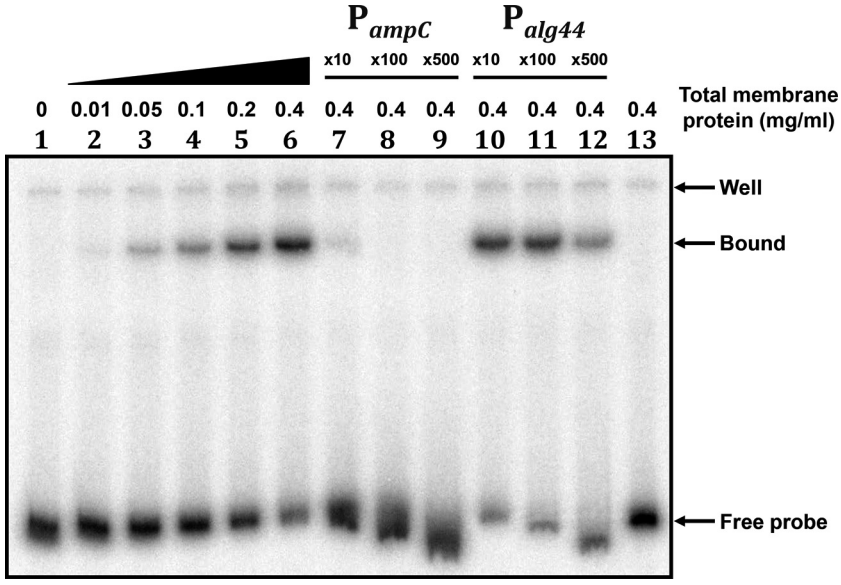


**FIG 4** *P. aeruginosa* AmpR binds $P_{ampC}$. Fifty femtomoles of a 193-bp radiolabeled $P_{ampC}$ fragment (lane 1) spanning the *ampC-ampR* promoter region was mixed with increasing concentrations of AmpR-$His_6$-enriched membrane fractions extracted from PA0Δ*ampR*(pAmpR-$His_6$) in the presence of 200 μg $ml^{-1}$ of penicillin G (lanes 2 to 6). Competition assays were carried out with the cold $P_{ampC}$ fragment in 10- (lane 7), 100- (lane 8), and 500-fold (lane 9) molar excess. To characterize the binding specificity, a nonspecific fragment (233-bp *alg44* PCR fragment) was mixed in 10- (lane 10), 100- (lane 11), and 500-fold (lane 12) molar excess with the radiolabeled reaction mix. Lane 13 is the control showing the 193-bp radiolabeled $P_{ampC}$ fragment in the presence of membrane fractions extracted from PA0Δ*ampR* containing the plasmid backbone alone.

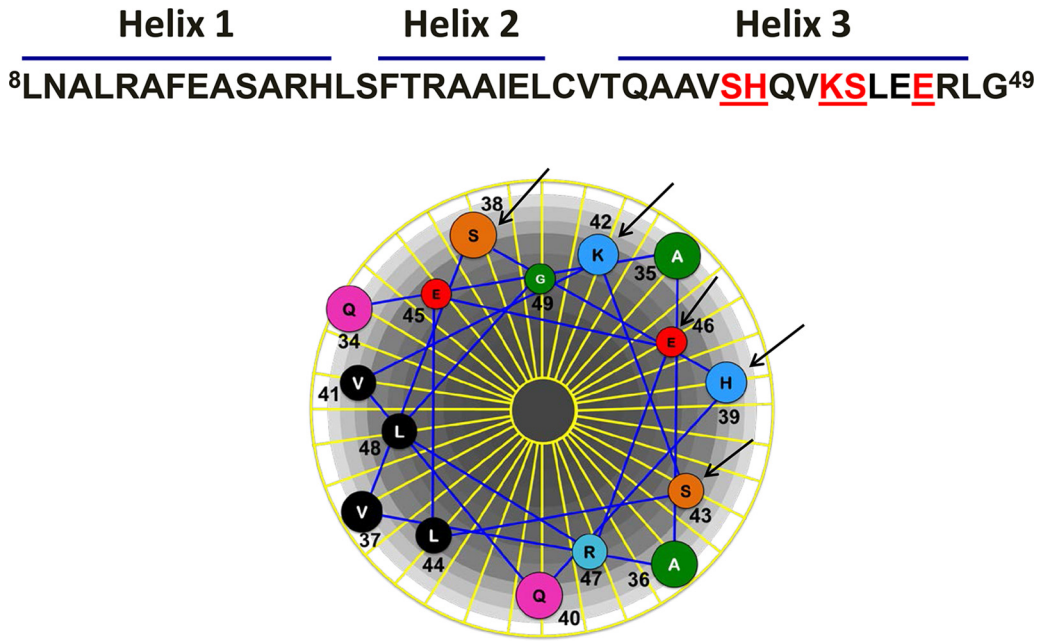


**FIG 5** Analysis of the third helix of the *P. aeruginosa* AmpR HTH motif. An amphipathic wheel of the third helix (residues Q34 to L48) was generated using DNAStar Protean in order to identify polar and charged amino acids likely facing the major groove of the DNA. The AmpR residues Ser38, His39, Lys42, Ser43, and Glu46 were identified as amino acids likely to interact with the DNA and were thus targeted for mutagenesis. They are indicated by the arrows and denoted as S38, H39, K42, S43, and E46 in the helical wheel, respectively. The N-terminal sequence of *P. aeruginosa* AmpR illustrates the HTH motif and the location of the above amino acids (in red) in the third helix of AmpR.

helices interact with the DNA and the third makes the essential contacts with the major groove to provide recognition (15, 55, 56). A multiple alignment of the AmpR family HTH motif shows that the highest degree of conservation is found in the first two helices, with the most variation in the third helix that provides specificity (see Fig. S3 in the supplemental material). Although *P. aeruginosa* AmpR has been shown to bind the *ampR-ampC* intercistronic region (34), the amino acids involved in the interaction have not been identified.

An amphipathic wheel of the third helix (residues Gln34 to Leu48), generated using DNAStar Protean, identified polar and charged amino acids potentially facing the major groove of the DNA (Fig. 5). Point mutations corresponding to these residues were generated by site-directed mutagenesis of AmpR-$His_6$. Residues Ser38, His39, Lys42, Ser43, and Glu46 were thus replaced with alanine, and the mutants were overexpressed in PAO1 and PA0Δ*ampR* strains carrying the chromosomal $P_{ampC}$-*lacZ* fusion (Fig. 6). The $P_{ampC}$ activity observed in PAO1 is the result of both *ampR* alleles, from the chromosome ($ampR_{chr}$) and from the plasmid ($ampR_{pls}$), whereas in PA0Δ*ampR* only $ampR_{pls}$ contributes.

Alanine substitutions at Ser43 and Glu46 did not affect the ability of AmpR to activate $P_{ampC}$ in PA0Δ*ampR* (Fig. 6). In addition, both $AmpR_{Ser43Ala}$ and $AmpR_{Glu46Ala}$ were able to bind $P_{ampC}$ in the presence and absence of β-lactams (Fig. 7). These two findings suggest that Ser43 and Glu46 are not critical for AmpR function. However, expression of $AmpR_{Ser43Ala}$ and $AmpR_{Glu46Ala}$ in PAO1 that carries $AmpR_{chr}$ significantly increased $P_{ampC}$ activity by more than 2-fold in the presence of inducers, suggesting a possible interaction between chromosome-encoded $AmpR_{chr}$ and the variants (Fig. 6). In particular, the Ser43Ala substitution increased basal levels in the absence of inducers while leading to hyperinduction in the presence of β-lactams. Similarly, significant activation of $P_{ampC}$ in PAO1 in the presence of AmpR-$His_6$ further strengthens the idea that AmpR functions as a multimer.

AmpR mutant proteins failed to activate $P_{ampC}$ when Ser38, His39, or Lys42 was replaced with alanine. These three residues are thus essential for AmpR activity and are presumably involved in the binding to $P_{ampC}$. A multiple alignment reveals that Ser38 and Lys42 are well conserved in members of the AmpR family, as expected of amino acids that play a critical role in the functionality of a protein (see Fig. S3 in the supplemental material).

The loss of $P_{ampC}$ activity in $AmpR_{Ser38Ala}$, $AmpR_{His39Ala}$, and $AmpR_{Lys42Ala}$ could be attributed to the destabilization of the proteins. Their expression was thus analyzed using Western blotting with anti-AmpR antibody (Fig. 8). Interestingly, not only are these three mutant AmpR proteins made, but it appears that they, and in particular $AmpR_{Ser38Ala}$ and $AmpR_{Lys42Ala}$, are made in large quantities. These amino acid substitutions, therefore, appear to stabilize rather than destabilize the proteins. Thus, we argued that the loss of $P_{ampC}$ activity may be due to their inability to bind DNA. Gel shifts revealed that $AmpR_{Ser38Ala}$ failed to bind to $P_{ampC}$ while $AmpR_{Lys42Ala}$ bound very poorly, correlating well with the loss of $P_{ampC}$ transcriptional activity (Fig. 7). Surprisingly, the His39Ala substitution did not prevent AmpR from binding to $P_{ampC}$ in the presence or absence of β-lactams, although it clearly prevented it from activating transcription from $P_{ampC}$ (Fig. 6 and 7). $AmpR_{His39Ala}$ is thus a positive-control mutant that can bind DNA but cannot activate transcription from the promoter to which it binds.

Positive control (*pc*) mutants are proteins that are defective in transcriptional activation but retain the ability to bind DNA. The *pc* phenotype is caused by the disruption of favorable protein-

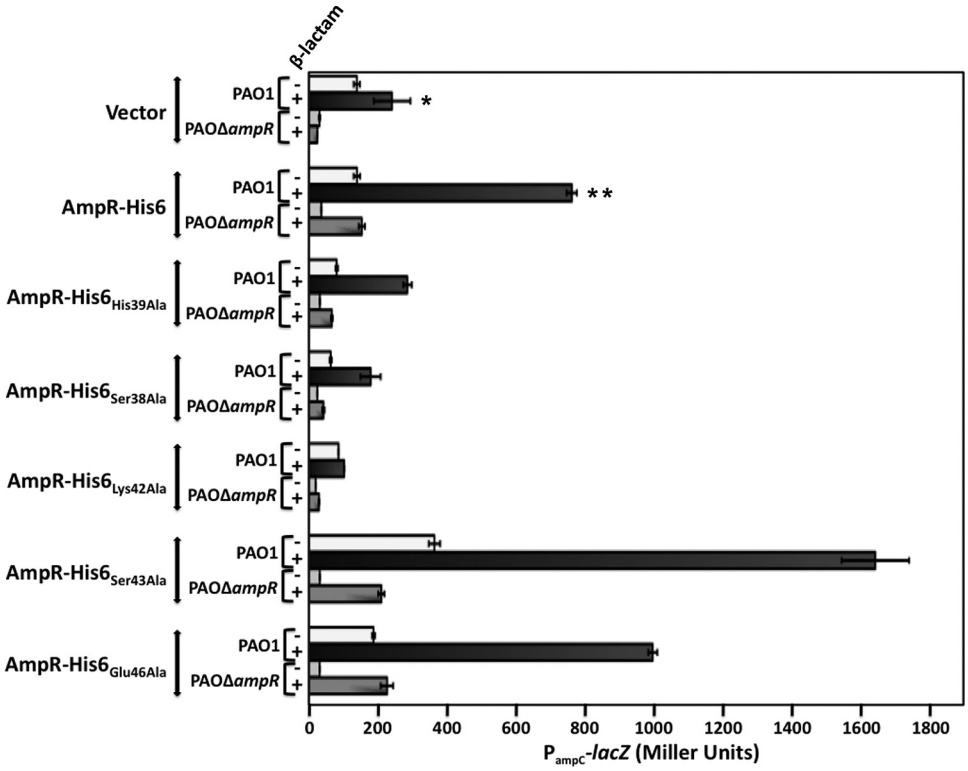


**FIG 6** Functional analysis of the *P. aeruginosa* AmpR HTH motif. Site-directed mutagenesis was used to replace Ser38, His39, Lys42, Ser43, and Glu46 with Ala in AmpR-$His_6$. The mutant proteins were overexpressed in PAO1 and PA0Δ*ampR* strains carrying a single copy of the $P_{ampC}$-*lacZ* fusion integrated at the *attB* site. β-Galactosidase activity was quantified in the presence and absence of β-lactams. *, *P* value < 0.05 versus uninduced PAO1 vector control; **, *P* value < 0.005 versus induced PAO1 vector control as determined by unpaired *t* test using analysis of variance.

protein interactions between the activator protein and the RNA polymerase (57–59). Several *pc* mutants of other proteins have been characterized with mutations in or near the DNA-binding domain (57–62). Mutations away from this region have also been reported (63). In particular, mutations in the DNA-binding region have been mapped to the second helix of the HTH motif and to the junction between the second and third helices of the same domain in the activator proteins λ cI, 434 cI, and P22 c2 (57–60). $AmpR_{His39Ala}$ is different from previously reported *pc* mutants in that its mutation is found in the helix of the DNA-binding domain that is thought to directly interact with the major groove of the DNA (helix 3) and not in the helix which usually lies across the major groove (helix 2) and makes contacts with the DNA backbone. Although it is not clear whether this third helix can contact the RNA polymerase, it may interact with other sites in the nearby helix to indirectly affect transcription. Our work here does not reveal how the disruption of His39 affects activation of transcription but merely that it is required for it.

In the present work, we show that the highly conserved residues Ser38 and Lys42 (not conserved in *Klebsiella pneumoniae*) in the HTH motif are critical for binding and function of AmpR. The less conserved His39 is also necessary for promoter activation but not for binding to the DNA.

**Gly102 and Asp135 are critical for AmpR function.** Previous work identified *C. freundii ampR* mutants that constitutively express β-lactamase (64, 65). Specifically, a change in Gly102 to Glu (Gly102Glu) resulted in high constitutive β-lactamase expression in an inducer-independent manner, while a Gly102Asp substitution yielded a similar but less pronounced phenotype (64, 65). In addition, Asp135 was also found to play a role in the function of *C. freundii* and *E. cloacae* AmpR (64, 66). The expression of *C. freundii* $AmpR_{Asp135Tyr}$ led to constitutive β-lactamase hyperexpression in an *ampG* mutant background. In *E. cloacae* AmpR, Asp135 mutations to Val or Asn resulted in higher β-lactamase activity in the presence and absence of inducers and contributed to increased β-lactam resistance in two different *E. coli* backgrounds (64, 66). Gly102 and Asp135 thus appear to play important roles in the activation/repression state of AmpR in, at least, the *Enterobacteriaceae*.

To investigate the role of these amino acids, *P. aeruginosa* AmpR Gly102 and Asp135 were replaced with Glu and Asn, respectively. The two mutant AmpR proteins were overexpressed in PAO1 and PA0Δ*ampR* strains carrying $P_{ampC}$-*lacZ* (Fig. 9). Unlike in *C. freundii*, in *P. aeruginosa* the Gly102Glu substitution resulted in the loss of $P_{ampC}$ activity in the presence and absence of β-lactams. The loss of activity is due to destabilization of the protein (Fig. 8), suggesting a structural role for Gly102 in *P. aeruginosa* AmpR. On the other hand, the Asp135Asn substitution led to an inducer-independent increase in $P_{ampC}$ transcriptional activity in both PAO1 and PA0Δ*ampR* with no concomitant increase in the amount of protein being made (Fig. 8 and 9). Thus, we postulate that the Asp135Asn substitution in the effector binding domain appears to stabilize the active conformation, effectively turning AmpR into a constitutive activator of *ampC* transcription. The Asp135Asn substitution has also been reported in AmpR from a *P. aeruginosa* clinical variant that exhibited hyperconstitutive β-lac-

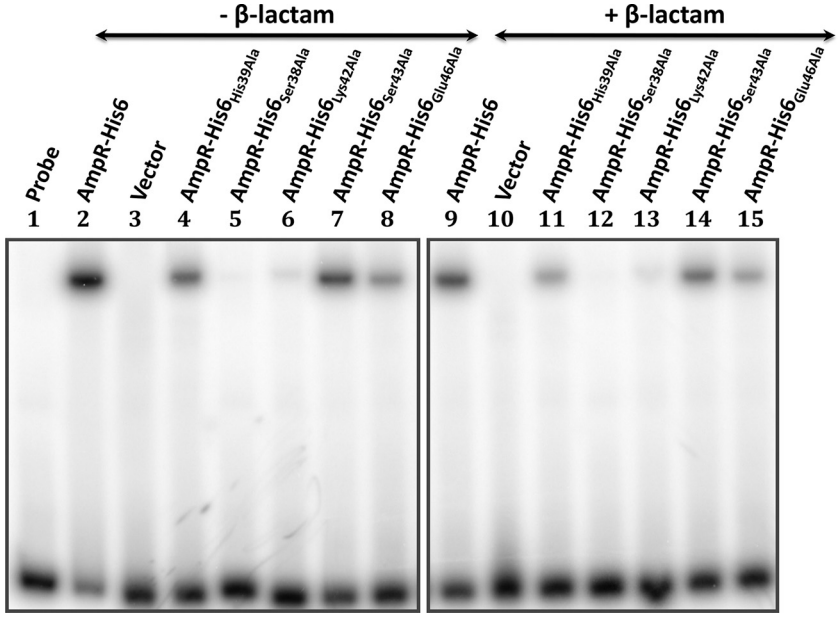


**FIG 7** Electrophoretic mobility shift assay of *P. aeruginosa* AmpR HTH mutants. A 50-fmol amount of the 193-bp radiolabeled $P_{ampC}$ fragment (lane 1) was mixed with membrane fractions recovered from PA0Δ*ampR* in the absence (lanes 2 to 8) and presence (lanes 9 to 15) of β-lactams and carrying pMMB67EH-Gm (lanes 3 and 10), pAmpR-His6 (lanes 2 and 9), pAmpR-His6$_{(His39Ala)}$ (lanes 4 and 11), pAmpR-His6$_{(Ser38Ala)}$ (lanes 5 and 12), pAmpR-His6$_{(Lys42Ala)}$ (lanes 6 and 13), pAmpR-His6$_{(Ser43Ala)}$ (lanes 7 and 14), and pAmpR-His6$_{(Glu46Ala)}$ (lanes 8 and 15).

tamase expression and high resistance to β-lactams (67). The importance of Asp135 corroborates the previous work in *E. cloacae* and *C. freundii* (64, 66). Gly102, on the other hand, clearly plays different roles in the *P. aeruginosa* and *C. freundii* AmpR proteins.

**Cross-linking studies suggest that *P. aeruginosa* AmpR dimerizes.** The $P_{ampC}$ activity in the presence of both $ampR_{chr}$ and $ampR_{pls}$ is always considerably higher than that in PAO1 and PA0Δ*ampR* ($ampR_{pls}$) (Fig. 6). These findings suggest a possible interaction between the wild-type and His-tagged AmpR. This is not surprising as LTTRs exist and/or function as dimers or tetramers (15, 16, 68–72). To determine if in fact *P. aeruginosa* AmpR can dimerize, proteins from PA0Δ*ampR* (pAmpR-$His_6$) were cross-linked and AmpR was visualized with anti-His antibody. The detection of a 64-kDa and a 32-kDa species in cross-linked and non-cross-linked samples, respectively, suggests that AmpR dimerizes *in vivo* (Fig. 10). Only the monomeric form of $\sigma^{70}$ was detected after stripping and reprobing the blot. Our findings corroborate previous work in *C. freundii* where both AmpR and its effector binding domain were shown to dimerize in solution and in the crystallized form, respectively (73, 74).

**Localization studies of *P. aeruginosa* AmpR.** Although it is generally accepted that AmpR is a cytoplasmic protein, our bioinformatics analyses suggested that AmpR may be membrane asso-

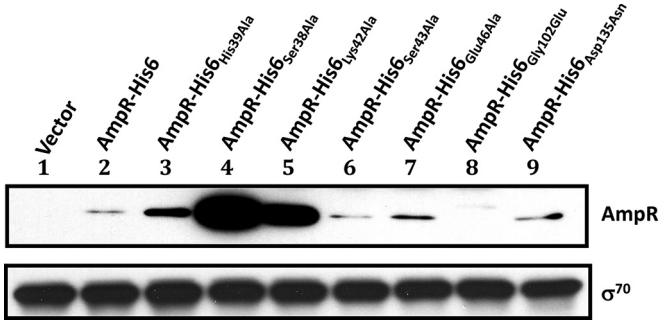


**FIG 8** Stability of *P. aeruginosa* AmpR mutant proteins. Total protein extracts were recovered from AmpR HTH and point mutants after a 1.5-hour incubation with 1 mM IPTG. The stability of each mutant was verified by Western blotting using AmpR-specific antibodies. Equal amounts of total membrane protein were loaded per well; $\sigma^{70}$ was used as a loading control and detected with anti-$\sigma^{70}$ antibody.

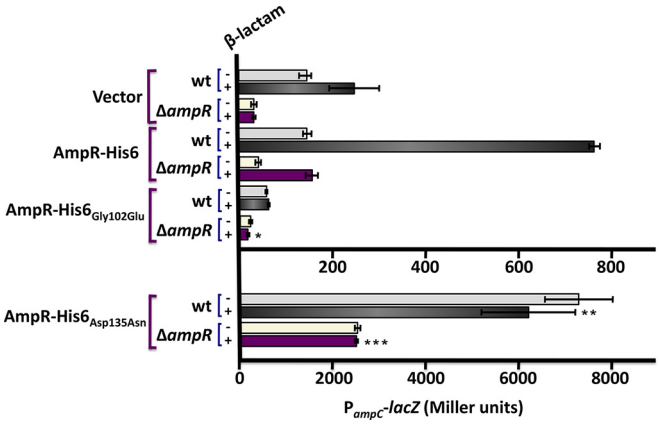


**FIG 9** Activity of the *P. aeruginosa ampC* promoter in the presence of AmpR-His6$_{(Gly102Glu)}$ and AmpR-His6$_{(Asp135Asn)}$ mutants. Site-directed mutagenesis was used to replace Gly102 and Asp135 of *P. aeruginosa* AmpR with Glu and Asn, respectively. Mutant AmpRs were expressed in wild-type PAO1 and PA0Δ*ampR* strains carrying $P_{ampC}$-*lacZ*. β-Galactosidase activity was quantified in the presence and absence of β-lactams and is represented in Miller units. *, *P* value < 0.01 versus induced PA0Δ*ampR* pAmpR-$His_6$; **, *P* value < 0.01 versus induced PA0*attB*::mini-CTX-*lacZ*; ***, *P* value < 0.001 versus induced PA0Δ*ampR* pAmpR-$His_6$ as determined by unpaired *t* test using analysis of variance.

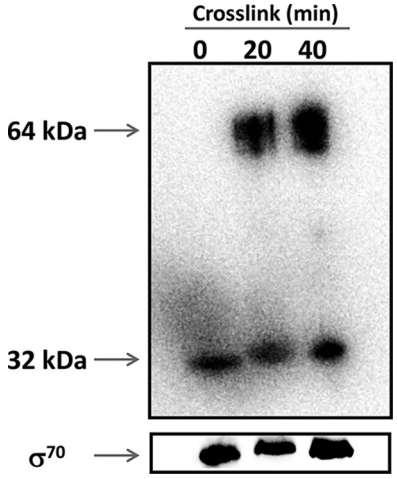


**FIG 10** *P. aeruginosa* AmpR appears to dimerize *in vivo*. A fresh culture of the PA0Δ*ampR* strain harboring pAmpR-His$_6$ was treated with 0.1% formaldehyde for 20 and 40 min at room temperature to achieve protein cross-linking. Crude extracts containing 10 μg of total protein were separated on an SDS-polyacrylamide gel, and AmpR was visualized using anti-His antibody. The blot was later stripped and reprobed using anti-$\sigma^{70}$ antibody. Monomeric AmpR is detected in non-cross-linked samples at the zero time point, while AmpR dimeric entities (64 kDa) are detected 20 and 40 min after protein cross-linking.

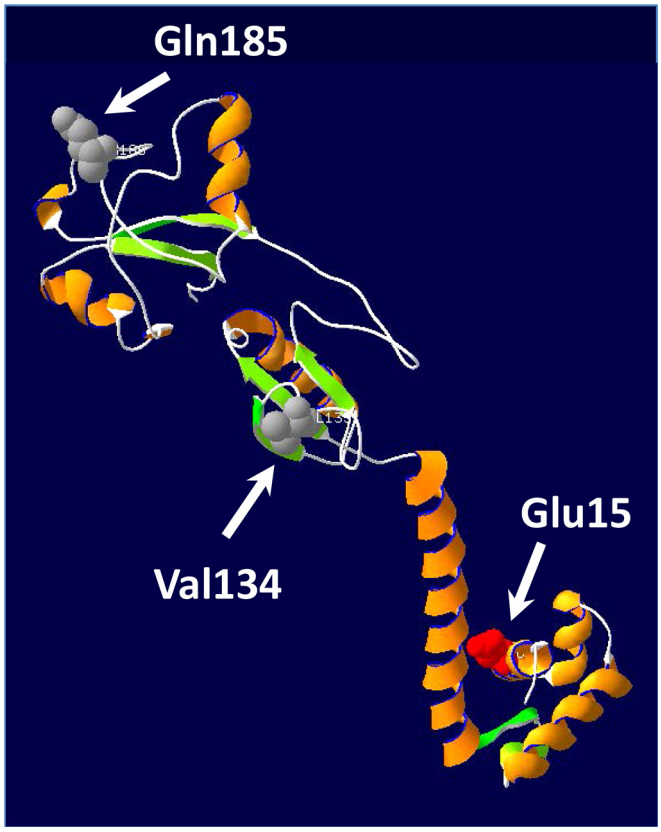


**FIG 11** AmpR model. A three-dimensional structure of AmpR was generated using RasMol (http://www.umass.edu/microbio/rasmol/). The topology of AmpR was investigated by introducing *phoA* and *lacZ* fusions at amino acid positions Glu15, Val134, and Gln185. Fusions at Glu15 were LacZ positive but PhoA negative, whereas fusions at Val134 and Gln185 were PhoA positive and LacZ negative.

ciated. More specifically, a Kyte and Doolittle hydrophobicity plot (75) and the topology prediction software TopPred2 (76), DAS (77), MEMSAT (78), TMpred (79), and SCAMPI (80) suggested the presence of a transmembrane domain somewhere between amino acids 92 and 114 of *P. aeruginosa* AmpR. The crystal structure of *C. freundii* AmpR, however, reveals that this segment is near the protein-protein interface of the dimer and thus unlikely to traverse the membrane (73).

In order to localize AmpR, *phoA* and *lacZ* were fused in frame at amino acid positions Glu15, Val134, and Gln185 (see Fig. S2A in the supplemental material). Fusions at Glu15 were LacZ positive and PhoA negative, whereas fusions at Val134 and Gln185 were PhoA positive and LacZ negative, suggesting that AmpR may traverse the inner membrane with the N and C termini in the cytoplasm and periplasm, respectively (Fig. 11). Since these data are only qualitative, localization of *P. aeruginosa* AmpR was further investigated with a protease protection assay using a C-terminal His-tagged AmpR that was shown to be functional (see Table S2). Full-length AmpR (32 kDa) was detected in whole-cell extracts, as well as in spheroplast preparations treated with proteinase K (0-min incubation) that were immediately processed for immunoblotting (see Fig. S2B). Incubations with proteinase K of 5 min or longer resulted in the visible reduction of full-length AmpR and the appearance of the degradation product (10-kDa fragment). However, slight degradation of $\sigma^{70}$ was observed. The evidence provided here is suggestive of AmpR being an inner membrane-associated protein. If confirmed, this would be an important finding and could have major implications regarding the regulation of two *ampD* amidase homologs that have now been localized to the periplasm (81, 82). The identity of the muramyl peptides that are important for regulating AmpR will further confirm its localization and is the subject of ongoing work in the lab.

Although the majority of purified LTTRs appear to be soluble cytoplasmic proteins, the nodulation factor, NodD, from *Rhizobium* species appears to be a peripheral membrane protein associated with the inner leaflet of the cytoplasmic membrane (83). A few membrane-bound non-LTTRs have also been reported, such as the *Salmonella enterica* serovar Typhimurium acid-sensing CadC, the streptococcal CpsA involved in regulation of capsule production, and the *Vibrio cholerae* toxin activator ToxR (84–86). Since these proteins act as both signal sensor and response regulator, they form a simple but sophisticated type of transmembrane signaling system.

**Concluding remarks.** The role of AmpR as regulator of *ampC* expression has been clearly established in both the *Enterobacteriaceae* and *P. aeruginosa*. Our recent work has further redefined AmpR as a major global regulator, playing an important role in acute infections through its regulation of virulence, biofilm formation, quorum sensing, and non-β-lactam resistance (31, 39–41, 87). Regulation of *ampC*, however, remains one of its most critical roles, as AmpC derepression is a prevalent mechanism of β-lactam resistance in *P. aeruginosa*.

In the present work, we characterize some of the genetic and structural elements necessary for induction of *ampC* and important for the functioning of AmpR as a regulator of AmpC β-lactamase expression. The presence of strong $\sigma^{54}$ consensus sequences in the *ampR* promoter led us to investigate its possible involve-

ment in the regulation of *ampR*. However, contrary to what was expected, RpoN was not required for *ampR* expression under the conditions tested. Instead, RpoN was found to downregulate expression of both *ampR* and *ampC*, although the exact mechanism is yet unknown.

Like other LTTRs, AmpR has two important regions critical for its functioning as an activator/repressor of *ampC* expression: the HTH motif for binding to DNA and the effector binding domain for ligand interaction. Analysis of polar and charged amino acids on the AmpR HTH revealed two residues, Ser38 and Lys42, important for binding of AmpR to the promoter region and consequently for *ampC* promoter activation. A third residue, His39, was shown to be important for function but not for binding to $P_{ampC}$. In the effector binding domain, we examined the role of two amino acids, Gly102 and Asp135, previously shown to be important for maintaining AmpR in an inactive conformation in the enterobacteria. In *P. aeruginosa*, Gly102 appears to be responsible for maintaining a stable structural conformation, while Asp135 is responsible for keeping AmpR in an inactive state that represses *ampC* expression. Additionally, our work suggests that AmpR dimerizes and that it is likely to be membrane associated. This is the first comprehensive look at the *P. aeruginosa* AmpR protein and the promoter elements that it regulates.

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We declare no conflict of interest.

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