Structural and functional characterization of *Pseudomonas aeruginosa* global regulator AmpR (91 characters with space)

Running title: Structure-function analysis of *P. aeruginosa* AmpR (50 characters with space)

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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 \(\beta\)-lactamase is a common mechanism of \(\beta\)-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 (TSS) of the divergent transcripts were mapped using 5’ 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 the minimal \(P_{ampC}\) extends 98-bp upstream of the TSS. Gel shifts using membrane fractions showed AmpR binds to \(P_{ampC}\) \textit{in vitro} whereas \textit{in vivo} binding was demonstrated using 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 AmpR is likely to be membrane associated. Lastly, \textit{in vivo} cross-linking
study shows AmpR dimerizes. In conclusion, a potential membrane-associated AmpR dimer regulates \emph{ampC} expression by direct binding. (245 words)
INTRODUCTION

*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 bacteria’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 a 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 a 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-
anhydro-MurNAc 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 from either 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 inactive 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
displace the repressor UDP-MurNAc-pentapeptide from AmpR and induce 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 up-regulation mechanism leading to constitutive β-lactamase
hyperexpression (2, 3, 30, 32). Additionally, P. aeruginosa harbors two AmpG
homologs, PA4218 (AmpP) and PA4393 (AmpG) that 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 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 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 AmpR specifically binds to the 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 dimers as previously seen for *C. freundii* and is potentially a peripheral membrane protein.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains, plasmids and primers employed in this study are shown in Table S1. *E. coli* and *P. aeruginosa* were routinely cultured in Luria-Bertani medium (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 gentamycin (Gm) at 15 µg for *E. coli*; Gm and Tc each at 75 µg for *P. aeruginosa*. PA0ΔampC and PA0ΔampR strains used in this work were previously constructed using overlap extension PCR and homologous recombination (40, 42).

**P<sub>ampC</sub> promoter deletions.** To characterize the minimal promoter necessary for full activity, 5'-end deletions of P<sub>ampC</sub> 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: SBJ03ampCRFor-OCP<sub>ampC</sub>RevBc, OCP<sub>ampC</sub>For193-OCP<sub>ampC</sub>RevBc, OCP<sub>ampC</sub>For173-OCP<sub>ampC</sub>RevBc, OCP<sub>ampC</sub>For151-OCP<sub>ampC</sub>RevBc, OCP<sub>ampC</sub>For131-OCP<sub>ampC</sub>RevBc, OCP<sub>ampC</sub>For111-OCP<sub>ampC</sub>RevBc, OCP<sub>ampC</sub>For91-OCP<sub>ampC</sub>RevBc, OCP<sub>ampC</sub>For71-OCP<sub>ampC</sub>RevBc, and OCP<sub>ampC</sub>For51-OCP<sub>ampC</sub>RevBc (Table S1). The fragments were
sequenced, then cloned into the EcoRI-BamHI sites of the integrative vector mini-
CTX-lacZ and integrated into PA01.

Construction of His-tagged AmpR. Primers OCAmpR-His-For and OCAmpR-His-Rev
(Table S1) were used to amplify PA01 genomic ampR. The 933-bp amplicon, carrying
a His6 sequence at the 3’-end was cloned into pBluescriptSK(+) and sequenced (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 E-test (Text S1 and Table S2).

Expression and purification of AmpR-His6. AmpR-His6 was purified according to
standard protocols. Briefly, stationary-phase cultures of PA0ΔampR (pAmpR-His6)
were diluted to an OD600 of 0.02 in 2 liters of LB broth and incubated with shaking at
37°C until the culture density reached an OD600 of 0.2. Cells were then induced with 1
mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for an additional
six hours before harvesting. The cells were recovered by centrifugation at 6,000 X g
for 10 minutes at 4°C and resuspended in 25 ml of lysis buffer (20 mM HEPES pH8,
0.5 M NaCl, 10% glycerol, 1 mM 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 DNase1).
Following disruption of the cells on ice with sonication (20-s pulse ON and 20-s pulse
OFF for 20 minutes, amplitude 50%), the cell lysate was centrifuged at 10,000 X g for
10 minutes at 4°C. The supernatant was further ultracentrifuged at 36, 000 rpm for 1
h at 4°C. Membranes pellets were resuspended in 20 ml of solubilization buffer (20
mM HEPES pH8, 0.5 M NaCl, 10% glycerol, 5 mM imidazole, 1 mM PMSF, 2 pellets of
EDTA-free Protease Inhibitor cocktail tablets complete, and 2 % of CHAPS) and loaded onto a HisTrap FF 1 ml column. AmpR-His$_6$ was eluted with buffer B (20 mM HEPES pH8, 0.5 M NaCl, 10% glycerol, 500 mM imidazole and 0.6% of CHAPS) by using an FPLC chromatograph (Akta, Amersham Biosciences). About 20 ml were 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, AmpRHis39AlaFor-AmpRHis39AlaRev, AmpRLys42AlaFor-AmpRLys42AlaRev, AmpRSer43AlaFor-AmpRSer43AlaRev, AmpRGlu46AlaFor-AmpRGlu46AlaRev, AmpRGly102GluFor-AmpRGly102GluRev, and AmpRAsp135AsnFor-AmpRAsp135AsnRev (Table S1).

**Membrane fraction purification.** Preliminary studies showed pAmpR-His$_6$ expression and β-lactamase induction were achieved with a 2-hour incubation at a 1 mM-IPTG concentration. Thus, PA0ΔampR (pAmpR-His$_6$) cells at OD$_{600}$ of 0.2 were induced with 1 mM of IPTG and incubated for two hours 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 X g for 10 minutes 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
DNase1). Following disruption of the cells on ice with sonication (15 cycles of 10
second pulse ON and 30 s pulse OFF, amplitude 40%), the cell lysate was centrifuged
at 5,000 rpm for 10 minutes at 4°C. The supernatant was ultracentrifuged at 36,000
rpm (Rotor Ti70) for one hour at 4°C and the pellets were resuspended with 2 ml of
membrane buffer (25% sucrose, 20 mM Tris pH8, and 0.5 mM PMSF). Two-hundred
milliliters of membrane fractions were aliquoted and stored at -80°C.

Electrophoretic mobility shift assay (EMSA). The 193-bp PCR fragment
containing the ampR-ampC intergenic region plus a small part of ampR ORF was used to perform
EMSA. Ten pmol of this fragment were radiolabelled at the 5' end by incubation with
T4 polynucleotide kinase (NEB) and [γ-32P]ATP (3,000 Ci mmol⁻¹; Perkin Elmer). The
labeled fragment was diluted to a final concentration 100 nM and unincorporated
nucleotides were removed by sephadex G-25 (Biorad) spin chromatography. DNA
binding reactions containing 50 fmol of 32P-labeled DNA probe and varying amounts
of total protein membrane fractions were incubated for 20 minutes and loaded
thereafter in a non-denaturing 5% PAGE. 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 32P-labeled probe were mixed
with the unlabeled 193-bp fragment in 10-, 100- and 500-fold molar excess in the
EMSA assay. For nonspecific assays, 50 fmol of the 193-bp 32P-labeled probe were
mixed with a PCR-amplified 233-bp fragment (alg44) in 10-, 100- and 500-fold molar
excess in the EMSA assay.
5′ RACE PCR. The ampC and ampR TSSs were mapped using a classical 5′ RACE-PCR on total mRNAs extracted from PA01, PA0ΔampR and PA0ΔampC (44). Stationary-phase cultures of PA01, PA0ΔampR and PA0ΔampC 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 one hour and subsequently blocked on ice for 15 minutes with 1/5th of the final culture volume in 5% acidic phenol-95% ethanol, pH 4. One milliliter of the cells were recovered by centrifugation and resuspended with 3 mg ml$^{-1}$ of lysozyme (Tris EDTA, pH 8). RNA was then extracted according to the RNeasy mini kit protocol (Qiagen), treated with 10 U of RQ1 DNase (Promega) for one hour, 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 TSS, respectively. In the first round of PCR amplification (Pfu, Stratagen), primers $Q_{o}$, $Q_{b}$, and 5RA-P$_{ampC}$154 were used for ampC TSS determination, while $Q_{o}$, $Q_{b}$, and 5RA-P$_{ampR}$169 were used for determination of the ampR TSS. In the second round of PCR amplification (Pfu, Stratagen), primer pairs $Q_{-}5RA-P_{ampC}113$, and $Q_{-}5RA-P_{ampR}99$ were used for ampC and ampR TSS determination, respectively (Table S1). 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 PA01, PA0ΔrpoN and PA0ΔrpoN (pRpoN) in the presence and absence of the inducer (0.2 µg ml$^{-1}$ imipenem) using the RNeasy mini kit (Qiagen). RNA was reverse transcribed
into cDNA with Superscript III (Invitrogen) and an (NS)₅ random primer using standard methods (45). For 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 conditions tested. Melting curves were generated to ensure primer specificity. Gene expression of each sample was normalized to PA01 uninduced value, to see the effect of induction and mutation at the same time. Primer pairs DBS_QRTAmpRFwd-DBS_QRTAmpRRev and qRT_ampCqRT_ampCR 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 VSV-G tag (YTDIEMNRLGK). The construct was then introduced into PA01 as single copy by mating and clones were selected for gentamycin 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 0.5-1.0 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).
Biosystems) with primers DBS_ChIP_\textit{ampCF} and DBS_ChIP_\textit{ampCR}. Fold enrichment was normalized to clpX (PA1802).

**Protein cross-linking.** PA0Δ\textit{ampR} (pAmpR-His\textsubscript{6}) was grown to an OD\textsubscript{600} of 0.3 and induced with 1 mM IPTG for 2 hours. Cells were grown for two additional hours in the presence and absence of β-lactam antibiotics (0.1 μg ml\textsuperscript{-1} of imipenem). Cultures were then treated with 0.1% formaldehyde for 20 and 40 minutes 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-σ\textsubscript{70} antibody (NeoClone).

**Polyclonal anti-AmpR-His\textsubscript{6} antibody production.** Purified AmpR-His\textsubscript{6} was used as antigen to raise anti-AmpR-His\textsubscript{6} rabbit polyclonal antibodies (Covance, Princeton, NJ). AmpR-His\textsubscript{6} antibody was affinity-purified as previously described (47).

**Western Blotting and EMSA of HTH mutants.** Concentration of purified AmpR-His\textsubscript{6} was determined by the bicinchoninic acid (BCA) method (48). A calibration Western blot was generated using FujiFilm LAS-3000 imager to correlate intensities with the concentration of purified AmpR-His\textsubscript{6}. Membrane fractions were purified from PA0Δ\textit{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 a 0.4 mg ml\textsuperscript{-1} is sufficient to shift the 193-bp P\textit{ampC} PCR fragment (Data not shown). For a second gel shift, a 0.4 mg ml\textsuperscript{-1} of total membrane fraction (8.44 ng of AmpR), recovered from PA0Δ\textit{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 (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-His6 is equivalent under all conditions and thus in the EMSA experiment (Data not shown). Further, the stability of AmpR-His6 mutants was verified by Western using equal amounts of total protein with AmpR-specific antibodies (Fig. 8). Sigma70 (NeoClone) was used as control to show 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 PVDF membrane (Bio-Rad) and blocked with TBST (TBS 0.1 % Tween) and 5 % non-fat dry milk at 4°C overnight or for four hours, followed by rinsing with the same solution and probing with rabbit anti-AmpR antibody (1:3000). Membranes were subsequently washed with TBST, incubated with goat anti-rabbit IgG (H+L)-horseradish peroxidase-conjugated antibody (1:5000) (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 1220-bp fragment containing ampR was digested at HindIII, HincII and PstI, corresponding to the amino acid positions, Gln15, Val134 and Gln186, respectively.
The resultant fragments were ligated in-frame upstream of \textit{phoA} and \textit{lacZ}-containing plasmids, pTrcphoA and pTrclacZ (Table S1), (50). The \textit{phoA} and \textit{lacZ} activities were qualitatively determined in \textit{E. coli} according to standard protocols (49).

**Protease protection (shaving) assay.** A stationary-phase culture of PA0ΔampR (pAmpR-His6) 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 four hours and chloramphenicol (500 µg ml$^{-1}$) was added 30 minutes 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 minutes 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 X g for 10 minutes and resuspended in 40 mM Tris-Cl pH 8.0, 0.5 M sucrose.

Proteinase K (10 µg ml$^{-1}$) was added to 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 4X SDS PAGE Sample Buffer. Samples were then boiled for 5 minutes and ran in a 4-20% SDS-PAGE (Criterion Biorad). Proteins were transferred to a nitrocellulose membrane (Biorad) and identified using AmpR (Covance), σ$^{70}$ (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 that 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 transcriptional start sites (TSS) were determined using 5’ RACE-PCR on total mRNAs isolated from PA01, PA0ΔampR and PA0ΔampC (Fig. 1). A strong similarity to RpoN (σ54) sigma factor sequences was detected at -12 and -24 positions of the ampR TSS, whereas strong σ70 promoter sequences were observed at the -10 and -35 positions of ampC. Alignment of the *C. freundii*, *E. cloacae* and *P. aeruginosa* intergenic region reveals a fair conservation for the ampC -10 and -35. However, there is a downstream shift in the *P. aeruginosa* ampRTSS that could contribute to the change in sigma factor control observed here (Fig. S1).

The presence of putative -12 and -24 recognition sequences in the ampR promoter (P_{ampR}) suggested that its expression might be RpoN or σ54-dependent. Quantitative real-time PCR was used to validate this hypothesis. Total RNA was
extracted from β-lactam induced and uninduced PA01, PA0ΔrpoN and PA0ΔrpoN (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 the uninduced samples (normalized to 1) (Fig. 2). A further, significant increase in expression of both genes was observed in PA0ΔrpoN 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 the PA0ΔrpoN that could not be complemented.

In spite of the presence of a probable binding site on PampR, σ^54 is not required for ampR expression. The significant increase in the absence of rpoN and in the presence of the β-lactams suggests σ^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, σ^54 has been shown to repress σ^22-dependent transcription from PalgD 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 σ^54-dependent promoter created a new TSS with σ^70 requirement that exhibited decreased transcriptional activity in the presence of σ^54 (53).

A model of repression by σ^54 through promoter blocking is conceivable, where in the absence of some external stimuli, in this case β-lactams, σ^54 binds PampR and
prevents access and thus transcription by the sigma factor. In the presence of the inducer, however, σ^{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 de-repression of ampR expression in 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 shows 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 σ^{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 \textit{ampC} and \textit{ampR} downregulation by RpoN.

\textbf{Mapping of \textit{P. aeruginosa} \textit{P_{ampC}}}. To map the minimal promoter needed for AmpR-dependent activity, a series of 5'-end deletions of \textit{P_{ampC}} were transcriptionally fused to the promoterless \textit{lacZ} gene of the integrative vector mini-CTX-\textit{lacZ} (54). The activity of each promoter deletion was then analyzed in PA01 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 \textit{P_{ampC}} activity is 193-bp (Fig. 3). This fragment consists of the full \textit{ampR-ampC} intergenic region plus small parts of the \textit{ampR} (22 bp) and \textit{ampC} (23 bp) ORFs. The high activity seen with the 193-bp fragment in the absence of β-lactams as 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 \textit{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 \textit{ampR} ORF seems to be necessary for full induction of \textit{P_{ampC}}. \textit{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). \textbf{Thus, the 42-bp region} (denoted red in Figures 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 \textit{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 **PampC**. Previously, *P. aeruginosa* AmpR has been shown to bind **PampC** using AmpR-overexpressing *E. coli* whole cell extracts (34). Similarly, crude preparations of *C. freundii* AmpR were also shown to retard a radio-labeled **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 section below and Fig. S2), we tested the ability of PA01 membrane fractions to bind **PampC**. EMSA was performed using AmpR-His6 enriched membrane fractions and a [γ-32P] ATP radiolabeled **PampC** 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 100-fold molar excess confirming AmpR binding to **PampC** (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 **PampC** fragment, failed to displace AmpR-His6 from **PampC** illustrating the binding specificity.

To determine if AmpR interacts with **PampC** *in vivo*, ChIP-qPCR was employed (46). A functional VSV-G-tagged AmpR (Table S3) was introduced into PA01 as single copy and then immunoprecipitated with anti-VSV-G antibody. Sequence-specific primers were used to detect the presence of **PampC** DNA with qPCR. Promoter occupancy was detected in the presence and absence of β-lactams as expected of
LTTRs (16) (Fold enrichment over clpX control- Uninduced: 10.6±1.73, Induced: 13.3±4.63; Fold enrichment for negative control target aprX- Uninduced: 1.30±0.02, Induced: 1.34±0.22). AmpR thus binds $P_{amp}$ 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 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 the highest degree of conservation is found in the first two helices, with the most variation in the third helix that provides specificity (Fig. S3). 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 PA01 and PA0ΔampR strains carrying the chromosomal $P_{ampC}$-lacZ fusion (Fig. 6). The $P_{ampC}$ activity observed in PA01 is the result of both ampR
alleles, from the chromosome (ampRchr) and from the plasmid (ampRpls), whereas in PA0ΔampR only ampRpls contributes.

Alanine substitutions at Ser43 and Glu46 did not affect the ability of AmpR to activate PampC in PA0ΔampR (Fig. 6). In addition, both AmpR_{Ser43Ala} and AmpR_{Glu46Ala} were able to bind PampC in the presence and absence of β-lactams (Fig. 7). These two findings suggest Ser43 and Glu46 are not critical for AmpR function. However, expression of AmpR_{Ser43Ala} and AmpR_{Glu46Ala} in PA01 that carries AmpR_{chr} significantly increased P_{ampC} activity by more than 2-fold in the presence of inducers suggesting a possible interaction between chromosomal 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 PA01 in the presence of AmpR-\text{His6} further strengthens the idea that AmpR functions as a multimer.

AmpR mutant proteins failed to activate P_{ampC} when Ser38, His39, or Lys42 were substituted 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 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 (Fig. S3).

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, it appears that they, and in particular...
AmpRSer38Ala and AmpRLys42Ala 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<sub>ampC</sub> activity may be due to their inability to bind DNA. Gel-shifts revealed that AmpRSer38Ala failed to bind to P<sub>ampC</sub> while AmpRLys42Ala bound very poorly correlating well with the loss of P<sub>ampC</sub> transcriptional activity (Fig. 7). Surprisingly, the His39Ala substitution did not prevent AmpR from binding to P<sub>ampC</sub> in the presence or absence of β-lactams, although it clearly prevented it from activating transcription from P<sub>ampC</sub> (Fig. 6 and 7). AmpR<sub>His39Ala</sub> 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-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 helix of the same domain in the activator proteins λ cl, 434 cl, and P22 c2 (57-60). AmpR<sub>His39Ala</sub> 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<sub>Asp135Tyr</sub> led to constitutive β-lactamase hyperexpression in an *ampG* mutant background. In *E. cloacae* AmpR Asp135 substitutions 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 PA01 and PA0Δ*ampR* strains carrying P<sub>ampC-lacZ</sub> (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 $\beta$-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 PA01 and PA0$\Delta ampR$ 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 domains 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 $\beta$-lactamase expression and high resistance to $\beta$-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 *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 PA01 and PA0$\Delta ampR$ ($ampR_{pls}$) (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 PA0$\Delta ampR$ (pAmpR-His$_6$) were cross-linked and AmpR was visualized with anti-His antibody. The detection of a 64-kD and a 32 kD species in cross-linked and non-cross-linked samples, respectively, suggests
that AmpR dimerizes in vivo (Fig. 10). Only the monomeric form of σ^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-associated. More specifically, a Kyte-and-Doolittle hydrophobicity plot (75) and the topology prediction softwares 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 this segment is near the protein-protein interface of the dimer and thus unlikely to transverse the membrane (73).

In order to localize AmpR, _phaA_ and _lacZ_ were fused in-frame at amino acid positions Glu15, Val134 and Gln185 (Fig. S2A). Fusions at Glu15 were LacZ-positive and PhoA-negative, whereas fusions at Val134 and Gln185 were PhoA-positive and LacZ-negative suggesting AmpR may traverse the inner membrane with the N- and C-terminals in the cytoplasm and periplasm, respectively. Since this data is 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 (Table S2). Full length AmpR (32 kDa) was detected in whole cell extracts, as well as, in spheroplasts preparations treated with Proteinase K (0-min incubation) that were immediately processed for immunoblotting (Fig. S2B). Incubations with
Proteinase K of 5 minutes 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 σ70 was observed. The evidence provided here is suggestive of AmpR being a peripheral or inner membrane-associated protein. If confirmed, this would be an important finding and could have major implications regarding the regulation of two \textit{ampD} amidase homologs that have now been localized to the periplasm \cite{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 \textit{Rhizobium} species appears to be a peripheral membrane protein associated with the inner leaflet of the cytoplasmic membrane \cite{83}. A few membrane-bound non-LTTRs have also been reported, such as the \textit{Salmonella enterica} serovar Typhimurium acid-sensing CadC, the streptococcal CpsA involved in regulation of capsule production, and the \textit{Vibrio cholera} toxin activator, ToxR \cite{84-86}. Since these proteins act as both signal sensor and response regulator, they form a simple but sophisticated form of transmembrane signaling system.
CONCLUDING REMARKS

The role of AmpR as regulator of \textit{ampC} expression has been clearly established in both the Enterobacteriaceae and \textit{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 \textit{ampC}, however, remains one of its most critical roles, as AmpC derepression is a prevalent mechanism of β-lactam resistance in \textit{P. aeruginosa}.

In the present work we characterize some of the genetic and structural elements necessary for induction of \textit{ampC} and important for the functioning of AmpR as regulator of AmpC β-lactamase expression. The presence of strong σ\textsuperscript{54} consensus sequences in the \textit{ampR} promoter led us investigate its possible involvement in the regulation of \textit{ampR}. However, contrary to what was expected, RpoN was not required for \textit{ampR} expression in the conditions tested. Instead, RpoN was found to downregulate expression of both \textit{ampR} and \textit{ampC}, although the exact mechanism is yet unknown.

Like other LTTRs, AmpR has two important regions critical for its functioning as activator/repressor of \textit{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 \textit{ampC} promoter activation. A third residue, His39, was shown to be important for function but not for
binding to $P_{\text{ampC}}$ In the effector binding domain, we examine 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 $P. \text{aeruginosa}$, Gly102 appears to be responsible for maintaining a stable structural conformation, while Asp135 is responsible for keeping AmpR in an inactive state that represses $\text{ampC}$ expression.

Additionally, our work suggests AmpR dimerizes and it is likely to be membrane-associated. This is the first comprehensive look at the $P. \text{aeruginosa}$ AmpR and the promoter elements that it regulates.

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**Figure Legends**

**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 σ^{54} (blue) and σ^{70} (green) sequences are observed in the ampR and ampC promoter regions, respectively. The underlined bases correspond to conserved sequences. The Shine-Delgarno sequences or ribosome binding sites (RBS) 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 with the ampC and ampR RBSs indicative of hairpin formation for regulating translation through blocking of ribosome binding.

**Fig. 2.** RpoN downregulates *P. aeruginosa* ampR expression in the presence of β-lactams. RNA was isolated from PA01, PA0ΔrpoN and PA0ΔrpoN(pRpoN) in the presence and absence of β-lactams, reversed transcribed to cDNA and tested by qPCR with ampC (grey bars) and ampR (black bars) specific primers, as described in the Experimental Procedures. Values were normalized to the expression of the wild-type uninduced sample and represent the mean (±SD) of two experiments conducted in triplicates. * p-value< 0.001 for both ampR and ampC expression in induced PA01 versus expression in uninduced PA01, ** p-value< 0.005 for ampR and ampC expression in induced PA0ΔrpoN versus expression in PA01 induced as determined by unpaired t-test.
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-\(\text{lacZ}\) as described in the *Materials and Methods*. Constructs were introduced into PA01 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, that appears to be necessary for activator binding, is depicted in red at the 5′ end of the 193-bp fragment. * \(p\)-value < 0.05 as compared to uninduced PA0\(\text{attB}::P_{ampC352-lacZ}\); ** \(p\)-value < 0.05 versus uninduced PA0\(\text{attB}::P_{ampC171-lacZ}\) as determined by unpaired *t*-test using ANOVA.

Fig. 4. *P. aeruginosa* AmpR binds *P_{ampC}*. Fifty fmol of a 193-bp radio-labeled *P_{ampC}*
fragment (Lane 1) spanning the *ampC-ampR* promoter region were mixed with increasing concentrations of AmpR-His\(_6\) enriched membrane fractions extracted from PA0\(\Delta ampR\) (pAmpR-His\(_6\)) in the presence of 200 \(\mu\)g 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 radio-labeled reaction mix. Lane 13 is the control showing the 193-bp radio-labeled *P_{ampC}*
fragment in the presence of membrane fractions extracted from PA0\(\Delta ampR\) 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 black arrows and denoted as S5, H6, K9, S10 and E13 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.

**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-His6. The mutant proteins were overexpressed in PA01 and PA0ΔampR strains carrying a single copy of the *PampC-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 PA01 vector control; **p*-value < 0.005 versus induced PA01 vector control as determined by unpaired t-test using ANOVA.

**Fig. 7.** Electromobility shift assay of *P. aeruginosa* AmpR HTH mutants. A 50 fmol of the 193-bp radio-labeled *PampC* fragment (Lane 1) were mixed with membrane fractions recovered from PA0ΔampR in the absence (Lanes 2 to 8) and presence of β-lactams (Lanes 9 to 15) and carrying pMMB67EH-Gm (Lanes 3 & 10), pAmpR-His6 (Lanes 2 & 9), pAmpR-His6(His39Ala) (Lanes 4 & 11), pAmpR-His6(Ser38Ala) (Lanes 5 &
pAmpR-His6 (Lys42Ala) (Lanes 6 & 13), pAmpR-His6 (Ser43Ala) (Lanes 7 & 14), and pAmpR-His6 (Glu46Ala) (Lanes 8 & 15).

**Fig. 8.** Stability of *P. aeruginosa* AmpR mutant proteins. Total protein extracts were recovered from AmpR HTH and point mutants after a 1½-hour incubation with 1 mM of 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; σ70 was used as a loading control and detected with anti-σ70 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 PA01 and PA0ΔampR strains carrying *P. aeruginosa* 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 PA0ΔampR pAmpR-His6; ** p-value<0.01 versus induced PA0attB::mini-CTX-lacZ; *** p-value<0.001 versus induced PA0ΔampR pAmpR-His6 as determined by unpaired t-test using ANOVA.

**Fig. 10.** *P. aeruginosa* AmpR appears to dimerize in vivo. A fresh culture of PA0ΔampR strain harboring pAmpR-His6 was treated with 0.1% formaldehyde for 20 and 40 minutes 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-σ^70 antibody. Monomeric AmpR is detected in non-cross-linked samples at zero time point, while AmpR dimeric entities (64KDa) are detected 20 and 40 minutes after protein cross-linking.
Figure 1

AmpR-box

AGCGGCAAATGGGGTCGAACCAATCTCTGCTCCAAATTTTTCTAATGGCTGCCGCGAGT

ampR

+1

****

RBS

ATTCGTCGTTTGC

CGCAAA

TCCTGCGCAAGCC

TA

GA

TT

TTCCCCGC

CCGCCG

ATCAAGG

ampC

*****

RBS

AGCGCTCCCGGGGCGGTTTCTCATGCAGCCAACGACAAAGGACGCCAATCCTCATGCGC

ampC

Figure 1

AGCGCTCCCGGGGCGGTTTCTCATGCAGCCAACGACAAAGGACGCCAATCCTCATGCGC

ampC

*****

RBS

AGCGCTCCCGGGGCGGTTTCTCATGCAGCCAACGACAAAGGACGCCAATCCTCATGCGC
Figure 2

Gene Expression (Relative Quantity)

PA01

ΔrpoN

ΔrpoN (pRpoN)

β-lactam

Figure 2
Figure 3
lacZ
+1
95-50-100 50-257 72-76
-10-35 ampCampR
352 bp
-98
-76
-257
352 bp
193 bp 
171 bp 
151 bp 
130 bp 
111 bp 
90 bp 
70 bp 
51 bp 
+26
+45
β-lactam
β-lactam-
+
+45
51 bp
0 100 200 300 400
P_{ampC-lacZ} (Miller Units)
Figure 4

- **P**<sub>ampC</sub> and **P**<sub>alg44</sub>
- Total membrane protein (mg/ml)
- Bound and Free probe

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Downloaded from http://jb.asm.org on August 15, 2017 by guest
Helix 1  Helix 2  Helix 3
8LNALRAFEASARHLSFTRAAIELCVTQAAVSHQVKSLEERLG49
Figure 6

![Graph showing the effect of various alleles on beta-lactamase activity.](image_url)
Figure 7

β-lactam + β-lactam

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

- β-lactam

+ β-lactam

Probe
AmpR-His6
Vector
AmpR-His6His39Ala
AmpR-His6Ser38Ala
AmpR-His6Lys42Ala
AmpR-His6Ser43Ala
AmpR-His6Glu46Ala
AmpR-His6
Vector
AmpR-His6His39Ala
AmpR-His6Ser38Ala
AmpR-His6Lys42Ala
AmpR-His6Ser43Ala
AmpR-His6Glu46Ala
Figure 9
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-
+
+
Vector
wt
∆ampR
AmpR-His6 wt
∆ampR
-
-
+
+
p
200 800 400 600
-
-
+wt
∆ampR
**

P_lacZ (Miller units)
+∆ampR
2000 4000 6000 8000

***
PampC-lacZ (Miller units)
Figure 10

Crosslink (min)

32 kDa

64 kDa

σ

70

0 20 40