

The CpxR/CpxA Two-component System Up-regulates Two Tat-dependent Peptidoglycan Amidases to Confer Bacterial Resistance to Antimicrobial Peptide*

Received for publication, November 3, 2010, and in revised form, December 7, 2010. Published, JBC Papers in Press, December 13, 2010, DOI 10.1074/jbc.M110.200352

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We demonstrate that the twin arginine translocation (Tat) system contributes to bacterial resistance to cationic antimicrobial peptides (CAMPs). Our results show that a deletion at the *tatC* gene, which encodes a subunit of the Tat complex, caused *Salmonella* and *Escherichia coli* to become susceptible to protamine. We screened chromosomal loci that encode known and predicted Tat-dependent proteins and found that two *N*-acetylmuramoyl-L-alanine amidases, encoded by *amiA* and *amiC*, elevated bacterial resistance to protamine and α -helical peptides magainin 2 and melittin but not to β -sheet defensin HNP-1 and lipopeptide polymyxin B. Genetic analysis suggests that transcription of both *amiA* and *amiC* loci in *Salmonella* is up-regulated by the CpxR/CpxA two-component system when *nlpE* is overexpressed. A footprinting analysis reveals that CpxR protein can interact with *amiA* and *amiC* promoters at the CpxR box, which is localized between the predicted –10 and –35 regions but present on different strands in these two genes. In addition, our results show that activation of the CpxR/CpxA system can facilitate protamine resistance because *nlpE* overexpression elevates this resistance in the wild-type strain but not the *cpxR* deletion mutant. Thus, we uncover a new transcriptional regulation pathway in which the Cpx envelope stress response system modulates the integrity of the cell envelope in part by controlling peptidoglycan amidase activity, which confers bacterial resistance to protamine and α -helical CAMPs. Our studies have important implications for understanding transcriptional regulation of peptidoglycan metabolism and also provide new insights into the role of the bacterial envelope in CAMP resistance.

Cationic antimicrobial peptides (CAMPs)³ are short peptides (12–50 amino acids) that carry positive charges in physiological conditions and generally contain abundant hydrophobic residues to allow for interaction with the bacterial membrane as well as other components of the bacterial envelope (1, 2). The specific interaction allows CAMPs to weaken the integrity of the inner and outer membranes and subsequently kill bacterial cells. On the other hand, bacteria have developed a number of mechanisms against CAMPs. For instance, a two-component system in *Salmonella*, PmrA/PmrB, mediates modification of lipopolysaccharide that reduces the negative charges in lipid A moiety, resulting in a decrease of electrostatic interactions between the outer membrane and CAMPs, thus conferring resistance to these peptides (for a recent review, see Ref. 3). Protamine, an arginine-rich 33-residue CAMP isolated from salmon sperm nuclei (4), as well as magainin 2 and melittin, both α -helical peptides from frog skin and honeybee (5, 6), have been used as model CAMPs to select chromosomal loci required for bacterial resistance to antimicrobial peptides. It is suggested that protamine may exert its bactericidal effect by disrupting cytoplasmic membrane energization (7), whereas magainin 2 and melittin induce leakage of cytoplasmic components by forming pores in the bacterial membrane (8–10).

Several independent pathways have been implicated in resistance to these peptides in the Gram-negative species *Salmonella typhimurium* based the following observations. (i) The PhoP/PhoQ system plays a critical role in *Salmonella* resistance to varied CAMPs, including protamine, magainin 2, and melittin (11, 12), mediated in part by the *yqjA* gene encoding an inner membrane protein of unknown function (13). Transcription of the *yqjA* gene is regulated in a PhoP- and CpxR-dependent manner (13, 14). The *yqjA* mutant is susceptible to protamine and α -helical CAMPs, including magainin 2 and melittin (13). In addition, it has been shown that the PhoP-dependent *pgtE* gene contributes to the resistance to several α -helical CAMPs (15). The *Escherichia coli* outer membrane protease, OmpT, which shares significant similar-

* This work was supported by National Institutes of Health Grants U01A152237-05, R01AI07397-01, R01AI039557-11, and R01AI075093-01 (to M. M.) and R21AI083964-01, 1R01AI083646-01, 1R56AI077645, and R01AI075093 (to H. A.-P.). This study was also supported by research funds from the Center for Infectious Diseases and Vaccinology in the Biodesign Institute (to G. Z.) and Arizona State University (to Y. S.) and National Natural Science Foundation of China Grants 30770483 and 30970646 (to Y. K.) and United States Department of Agriculture Grant AFRI CSREES 2009-03579 (to H. A.-P.).

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³ The abbreviations used are: CAMP, cationic antimicrobial peptide; IPTG, isopropyl β -D-1-thiogalactopyranoside; Tat, twin arginine translocation.

The CpxR/CpxA System Up-regulates the *amiA* and *amiC* Loci

TABLE 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>S. enterica</i> serovar Typhimurium		
14028s	Wild type	ATCC
YS13007	Δ <i>tatC</i>	This work
YS13629	Δ <i>amiA</i>	This work
YS13764	Δ <i>amiB</i>	This work
YS13630	Δ <i>amiC</i>	This work
YS13766	Δ <i>amiA</i> Δ <i>amiC</i>	This work
YS11590	Δ <i>phoP</i>	Ref. 51
YS13644	Δ <i>cpxR</i>	This work
YS13637	Δ <i>amiA-lacZ</i>	This work
YS13640	Δ <i>amiC-lacZ</i>	This work
YS13995	Δ <i>tatC</i> Δ <i>cpxR</i>	This work
<i>E. coli</i>		
DH5 α	F ⁻ <i>supE44</i> Δ <i>lacUI169</i> (ϕ 80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Ref. 52
BW25113	Δ (<i>araD-araB</i>)567 Δ <i>lacZ</i> 4787(:: <i>rrnB-3</i>) λ ⁻ <i>rph-1</i> Δ (<i>rhaD-rhaB</i>)568 <i>hsdR514</i>	Ref. 23
Plasmids		
pKD3	rep _{R6K} γ Ap ^R FRT Cm ^R FRT	Ref. 24
pKD46	rep _{pSC101} ^{ts} Ap ^R P _{araBAD} γ β exo	Ref. 24
pCP20	rep _{pSC101} ^{ts} Ap ^R Cm ^R cl857 λ P _R	Ref. 24
pCE37	rep _{R6K} γ K _m ^R FRT <i>lacZY</i> t _{his}	Ref. 25
pUC19	rep _{pMB1} Ap ^R	Ref. 53
pUHE21-2 <i>lacI</i> ^q	rep _{pMB1} Ap ^R <i>lacI</i> ^q	Ref. 49
pET28a	rep _{CoIE1} K _m ^R <i>lacI</i> P _{T7}	Novagen
pBAD TOPO	rep _{pBR322} Ap ^R <i>araC</i> P _{BAD}	Invitrogen
pBAD- <i>tatC</i>	rep _{pBR322} Ap ^R <i>araC</i> P _{BAD} <i>tatC</i>	This work
pYS2011	rep _{pMB1} Ap ^R <i>lacI</i> ^q <i>amiA</i>	This work
pYS2012	rep _{pMB1} Ap ^R <i>lacI</i> ^q <i>amiC</i>	This work
pYS2131	rep _{pMB1} Ap ^R <i>lacI</i> ^q <i>yaeJ</i>	This work
pYS2132	rep _{pMB1} Ap ^R <i>lacI</i> ^q <i>nlpE</i>	This work
pYS2135	rep _{CoIE1} K _m ^R <i>lacI</i> P _{T7} <i>His6-cpxR</i>	This work

ity with PgtE, has been demonstrated to confer bacterial resistance to protamine by mediating cleavage of this peptide (16). (ii) A set of *sap* loci, encoding factors required for potassium and oligopeptide transport, is essential for *Salmonella* resistance to protamine (17). The *sapABCDF* operon encodes a periplasmic oligopeptide-binding protein and its inner membrane transporter, which is proposed to transport protamine into the cytoplasm to yield resistance (18). (iii) The *yejABEF* operon, which encodes a putative ATP-binding cassette transporter, is suggested to be responsible for resistance to antimicrobial peptides with different structures, including protamine, α -helical melittin, lipopeptide polymyxin B, and β -sheet human defensins (19).

The twin arginine translocation (Tat) complex is a transporter responsible for the export of folded proteins across the cytoplasmic membrane (20). This Sec-independent system consists of three major subunits (TatA, TatB, and TatC), and is known for transporting substrates involved for various cellular activities, such as acquisition of metal ions, energy metabolism, cell wall biogenesis, and virulence (for a review, see Ref. 21). In this study, we used a mutant library containing single deletions at *Salmonella* loci and demonstrated that a Tat-dependent pathway is responsible for resistance to protamine and α -helical CAMPs in *Salmonella*. With genetic analysis of the known and predicted Tat-dependent loci, we can begin to understand a novel regulatory circuit required for CAMP resistance through modulation of the peptidoglycan metabolism in response to the envelope stress.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—All *Salmonella enterica* serovar Typhimurium strains were derived from the

wild-type strain 14028s (Table 1). Phage P22-mediated transductions were performed as described previously (22). The *E. coli* Keio collection was derived from wild-type BW25113 (23). Bacteria were grown at 37 °C in Luria-Bertani (LB) broth. When necessary, antibiotics were added at final concentrations of 50 μ g/ml for ampicillin, 20 μ g/ml for chloramphenicol, or 50 μ g/ml for kanamycin. *E. coli* DH5 α was used as host for the preparation of plasmid DNA. *E. coli* BL21-Gold (Stratagene) was used for protein expression.

Construction of *Salmonella* Strains with Chromosomal Mutations and Harboring *lac* Gene Fusions—Strains harboring deletions were generated as described previously (24). Deletion of the *amiA* gene was generated using primers 1369 (5'-CTTGAACCTTAATTTTCACAACCTCAGGCCGTCATA-TGAATATCCTCCTTAG-3') and 1370 (5'-CTTTCTGATT-ATCAAACCAGTGAAAATAACGTGTAGGCTGGAGCTGCTTC-3') to amplify the Cm^R cassette from pKD3 and integrate the resulting PCR product into the chromosome. The same strategy was used to construct deletion of other genes: *amiB* with primers 1461 (5'-GTTTAGCCGATTAGC-TATAAAGGTGGCGGGCATATGAATATCCTCCTTAG-3') and 1462 (5'-CCAGCGGCGATTGGTTCGCAAGCTG-CGGGGTGTAGGCTGGAGCTGCTTC-3'); *amiC* with primers 1396 (5'-ATCTCTATTTAGTTTTTGCTCGGGAG-AAGCCATATGAATATCCTCCTTAG-3') and 1397 (5'-CCGCGCAATAAACTCGCCGTCATCTCAGGGTGTAGGCTGGAGCTGCTTC-3'); *cpxR* with primers 1407 (5'-CGTAA-TTTCTGCCTCGGAGGTACGTAAACACATATGAATATCCTCCTTAG-3') and 1408 (5'-TCCTATCATGAAGCGGA-AACCATCAGATAGGTGTAGGCTGGAGCTGCTTC-3'). The Cm^R cassette was removed from resulting mutants using

plasmid pCP20 (24), and the *lac* transcriptional fusion plasmid pCE37 (25) was integrated into the FLP recombination target sequence in the deleted *amiA* and *amiC* loci.

Plasmid Construction—The plasmid pYS2011 was constructed using PCR fragments containing the *amiA* coding region generated with primers 1327 (5'-CGGGATCCTTTT-CACAACTCAGGC-3') and 1328 (5'-CCCAAGCTTTTACC-GTTTCTTCGTG-3') and *Salmonella* wild-type 14028s chromosomal DNA as template, which were digested with BamHI and HindIII and then ligated between the BamHI and HindIII sites of pUHE21-2*lacI*^q. The pYS2012 plasmid was constructed by cloning the *amiC* gene into the HindIII site of pUHE21-2*lacI*^q with primers 1402 (5'-CCCAAGCTTTTGC-TCGGGAGAAGC-3') and 1403 (5'-CCCAAGCTTAACTT-CTTCTCGCCAGCG-3'). The plasmid pYS2131 was constructed by cloning the *yaeJ* gene into BamHI and HindIII sites of pUHE21-2*lacI*^q with primers 1413 (5'-CGGGATCC-TGGCAACAGCCCTCATG-3') and 1414 (5'-CCCAAGCT-TCAATCCAGTGGACGAC-3'). The plasmid pYS2132 was constructed by cloning the *nlpE* gene into BamHI and HindIII sites of pUHE21-2*lacI*^q with primers 1421 (5'-CGGGATCC-ATTTTATAAGGATTTTATGG-3') and 1422 (5'-CCCAAGCTTAGTGAGTGCAATCTTTAC-3'). The plasmid pBAD-*tatC* was constructed by cloning the *tatC* gene into pBAD TOPO (Invitrogen) with primers *tatC*-forward (5'-GGGACCGTAAACATGGCTGTA-3') and *tatC*-reverse (5'-CGGTTGTGTAAGTCTTCAGT-3').

Selection for Genes Required for Protamine Resistance—A *Salmonella* collection containing more than 1,400 single mutant strains (26) were used to screen for genes required for resistance to protamine. Strains were cultured overnight, reinoculated (1:100) in LB broth, and grown for 4 h at 37 °C. Each was diluted 10⁵ times, and 5 μl (i.e. 10² to 10³ cells, depending on growth of individual strains) were dropped onto LB agar plates containing varying concentrations (0.8–1.5 mg/ml) of protamine sulfate (MP Biomedicals) and incubated overnight to screen for sensitivity. Those strains displaying a sensitive phenotype, in comparison with an isogenic wild type, were selected and streaked onto protamine plates to confirm initial findings. A similar approach was used when screening *E. coli* mutants from the Keio collection, except 0.6–1.2 mg/ml protamine was used to confirm susceptible mutants.

Antimicrobial Peptide Killing Assay—Strains cultured in LB overnight were inoculated 1:100 in fresh medium and grown for 4 h. Cultures were diluted with LB broth to 1–2 × 10⁵ bacteria/ml. Ampicillin (50 μg/ml), L-arabinose (10 mM), and IPTG (0.25 mM) were supplemented when necessary. Susceptibility to antimicrobial peptides was determined as described previously (13) with the following modifications. For protamine, 5 μl of cell suspension was plated onto LB agar plates with determined concentrations of protamine. Plates were incubated overnight at 37 °C, and the number of colony-forming units (cfu) were counted. For magainin 2 (Bachem), melittin (Bachem), and polymyxin B (Sigma), peptides were dissolved and diluted with autoclaved distilled water. 5 μl of peptide solution (concentration 10 times higher than the final concentration) were placed in wells of a microtiter 96-well

plate, and 45 μl of the diluted bacterial culture (see above) was added. After a 1-h incubation at 37 °C with aeration, 20 μl was mixed with 180 μl of LB broth, and 50 μl of this solution was plated on to LB agar plates. For defensin HNP-1 (Bachem), bacterial strains were grown as described above, harvested, washed once with TSB medium-sodium phosphate buffer, pH 7.4, and diluted to 1–2 × 10⁵ bacteria/ml in the same buffer. HNP-1 was dissolved in autoclaved distilled water and diluted in 0.01% acetic acid to a concentration 10 times higher than the final concentration. An aliquot of 5 μl of HNP-1 solution was placed in wells in a 96-well plate, and 45 μl of bacterial solution was added. After incubation for 90 min at 37 °C with aeration, 20 μl was mixed with 180 μl of LB broth, and 50 μl was plated on an LB agar plate. The percentage survival was calculated by the formula, (cfu from a plate with a given CAMP concentration/cfu from the LB plate) × 100.

Vancomycin Killing Assay—*Salmonella* survival after a vancomycin challenge was determined as follows. Overnight cultures were inoculated 1:5 in fresh LB broth. An appropriate inducer IPTG (0.25 mM) or L-arabinose (10 mM) was added when necessary. Strains were shaken for 4 h at 37 °C to allow for induction. Cells were diluted to ~10⁵ bacteria/ml and added to microtiter wells containing 0 and 0.5 mg/ml (final concentration) vancomycin (Sigma). Strains were challenged overnight with aeration at 37 °C, and survival was determined by measuring the optical density. Survival percentage was calculated as described previously (27) (i.e. A_{600 nm} of culture with vancomycin/A_{600 nm} of cultures without vancomycin) × 100.

Screening for the Regulator That Up-regulates *amiA* and *amiC* Transcription—Chromosomal DNA prepared from wild-type strain 14028s was digested with Sau3AI (1 unit; New England Biolabs) for 15, 30, or 45 min. The digested DNA was separated on 0.8% agarose gel, and 2–5-kb fragments were recovered and ligated to BamHI-digested pUC19 plasmid DNA. The ligation mixture was transformed into *E. coli* DH5α selecting for ampicillin-resistant transformants. Plasmid DNA was isolated from a pool of about 20,000 transformants (~95% of which carried an inserted chromosomal fragment) and introduced into strains, YS13637 and YS13640, which harbored a chromosomal *lac* transcriptional fusion at the *amiA* and *amiC* loci, respectively. Ampicillin-resistant transformants were selected on an LB ampicillin agar plate containing X-Gal (40 μg/ml). Plasmid DNA was purified from those colonies that were darker blue than others and reintroduced into YS13637 and YS13640 by electroporation. The resulting strains were used to measure β-galactosidase activity and to compare with those that received a control plasmid pUC19. To determine the inserted fragments, the plasmids were sequenced using primers 232 (5'-GAAACAGCTATGACCATG-3') and 233 (5'-TTCCCAGTCACGACGTTG-3').

β-Galactosidase Assay—β-galactosidase assays were carried out in triplicate (28), and the activity (Miller units) was determined using a VERSAmax plate reader (Molecular Device). Data correspond to three independent assays conducted in duplicate, and all values are mean ± S.D.

The CpxR/CpxA System Up-regulates the *amiA* and *amiC* Loci

Primer Extension—The primer extension assay was performed using primers 1472 (5'-TTAGGAGTTTAAAAGTGCTCAT-3') for *amiA* and 1482 (5'-ATAAAATTTACGCTTGCACAGA-3') for *amiC* as described previously (29). Total RNA was isolated from bacterial cells grown in 5 ml of LB medium containing IPTG (0.25 mM) to $A_{600\text{nm}}$ 0.6 with RNazol (Molecular Research Center) by following the manufacturer's instructions. Samples were analyzed by 6% denaturing polyacrylamide electrophoresis by comparison with DNA sequences amplified from chromosome with primers ^{32}P -1472 and 1567 (5'-ACCAAGATTATGGCGCAAACATC-3') for *amiA*, or ^{32}P -1482 and 1484 (5'-CTTCGCCGCGGAGCAT-3') for *amiC* and generated using Maxam and Gilbert A + G reactions.

Purification of His₆-CpxR Protein—The *Salmonella* CpxR protein was fused to a His₆ tag at its N terminus by PCR amplification with the primers 1512 (5'-GTTTCATATGAATAAATCCTG-3') and 1513 (5'-ACGCGTGCAGCTCATGAA-GCGGAAACCAT-3') and wild-type 14028s chromosomal DNA as template. The PCR product was digested with NdeI and Sall and then ligated between the NdeI and Sall sites of pET28a. The His₆-CpxR protein was purified from *E. coli* BL21-Gold with His-Select nickel affinity gel (Sigma) according to the manufacturer's instructions. After purification, the fractions containing His₆-CpxR protein were desalted and concentrated using an Amicon Ultra centrifugal filter (Millipore).

Electrophoretic Mobility Shift Assay (EMSA)—Primers were labeled using T4 polynucleotide kinase (New England Biolabs) and [γ - ^{32}P]ATP (PerkinElmer Life Sciences). 10 nmol of ^{32}P -labeled DNA fragments containing 206-bp *amiA* and 188-bp *amiC* promoter regions, amplified by PCR from *Salmonella* chromosomes with primers 1472 and ^{32}P -labeled 1567 and primers 1482 and ^{32}P -labeled 1484, respectively, were incubated at room temperature for 30 min with 0, 25, or 50 pmol of His₆-CpxR protein in 20 μl of an EMSA buffer consisting of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, 10 mM NaCl, 1 mM MgCl₂, and 5% glycerol. After the addition of the DNA dye solution (40% glycerol, 0.05% bromphenol blue, 0.05% xylene cyanol), the mixture was directly subjected to 4% polyacrylamide electrophoresis. Signals were detected by autoradiography.

DNase I Footprinting Assay—DNase I footprinting assays were carried out using the 206-bp *amiA* promoter region amplified from *Salmonella* chromosome with primers ^{32}P -1567 and 1472 for the coding strand and primers 1567 and ^{32}P -1472 for the non-coding strand and using the 188-bp *amiC* promoter region amplified with primers ^{32}P -1484 and 1482 for the coding strand and primers 1484 and ^{32}P -1482 for the non-coding strand. Approximately 25 pmol of ^{32}P -labeled DNA and 0, 50, or 100 pmol of His₆-CpxR protein were mixed in a 100- μl reaction containing 20 mM HEPES, pH 8.0, 10 mM KCl, 1 mM DTT, and 0.1 mg/ml BSA. The reaction mixture was incubated at room temperature for 20 min. Then 1 μl of 100 mM CaCl₂, 1 μl of 100 mM MgCl₂, and 0.005 units of DNase I (Fermentas) were added, and the mixture was incubated at room temperature for 2 min. The DNase I digestion was stopped by phenol treatment, and the DNA was pre-

cipitated. Samples were analyzed by 6% polyacrylamide electrophoresis by comparison with a DNA sequence ladder generated with the same primers using a Maxam and Gilbert A + G reaction. The site-directed mutagenesis of DNA fragments was performed by following a two-step PCR method described previously (30). The first step used the mutagenic primers and the reverse universal primer 1472 or 1482 flanking the 3' end of the *amiA* or *amiC* promoter region. The mutagenic primers were as follows: for the CpxR box 1 mutation of *amiA*, 1595 (5'-ATAATGGCGATGTGTACGATTCA-CATGAAAACACATACAATTCTCATCACCAC-3'); for the CpxR box mutation of *amiC*, 1598 (5'-CTTCTACCAGT-TCGGTATGTGGTTCCATGTGCATTGCGCGCCCCAC-TAG-3'). The second step used the product of the first PCR as a primer and the forward universal primer 1567 or 1484 to yield the whole promoter region with the desired mutation. The DNase I footprinting assay was carried out as described above.

RESULTS

The Twin Arginine Translocation System Plays an Important Role in Protamine Resistance in Enteric Bacteria *S. typhimurium* and *E. coli*—A collection of over 1,400 *S. typhimurium* mutants (26), each carrying a unique deletion at a chromosomal locus, was used to identify loci responsible for bacterial resistance to the CAMP protamine. Individual strains were grown in LB broth to log phase (4 h), diluted, inoculated ($\sim 10^2$ - 10^3 cells) onto LB agar plates with varied concentrations of protamine, and incubated at 37 °C overnight. The candidate mutants that were selected displayed increased susceptibility to protamine and thus could not grow or formed significantly fewer colonies when compared with the wild-type strain (14028s) in the presence of protamine. As expected, *phoP* and *phoQ* mutants in the collection were blindly characterized as sensitive strains from the screen because none yielded colonies on a plate supplemented with protamine (1.2 mg/ml), whereas the wild-type colonies remained at levels similar to those of the control plate without protamine (Fig. 1A) (data not shown). A strain carrying a deletion at the *tatC* locus, which encodes a subunit of the twin arginine translocation complex (Tat) used for protein secretion (20), exhibited increased susceptibility to protamine, because the *tatC* mutant cells, like the *phoP* mutant cells, were unable to form colonies on the protamine plate (Fig. 1A). The phenotype of the *tatC* mutant was solely the result of a lack of the TatC protein because it was reversed by a plasmid containing an arabinose-inducible wild-type copy of the *tatC* gene (pBAD-*tatC*) (Fig. 1B). Similarly, *E. coli* mutants unable to produce respective essential components of the Tat complex (*i.e.* TatA, TatB, and TatC) (20) were rendered susceptible to protamine (data not shown), demonstrating that the Tat system contributes to resistance of protamine in *Salmonella* and *E. coli*.

Two Tat-dependent N-Acetylmuramoyl-L-alanine Amidases Contribute to Bacterial Resistance to Protamine—We assume that the Tat system could confer protamine resistance by mediating secretion of specific protein substrate(s); therefore, a strain deficient in the substrate production could display in-

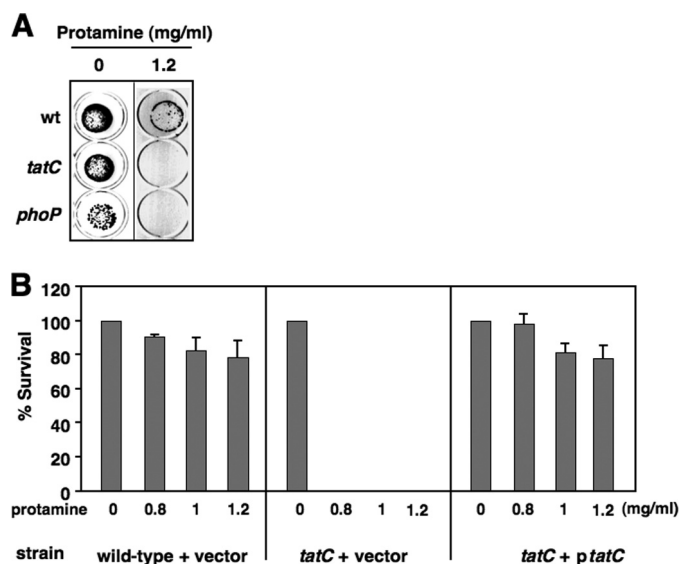


FIGURE 1. The Tat system is required for *Salmonella* resistance to protamine. A, protamine susceptibility screening for wild type (*wt*; 14028s), *tatC* (YS13007), and *phoP* (YS11590) on LB plates containing protamine (0 or 1.2 mg/ml). B, plasmid harboring a wild-type copy of *tatC* restores resistance to protamine. Shown is percentage survival of wild type carrying plasmid pBAD TOPO (vector) and *tatC* mutant carrying plasmid vector or pBAD-*tatC* after incubation with protamine (0, 0.8, 1.0, or 1.2 mg/ml). Data correspond to mean values from at least two independent experiments performed in duplicate. Error bars, S.D.

creased susceptibility to protamine. We used the mutants from the Keio collection, an *E. coli* library with in-frame, single-gene deletion mutants (23), and examined 34 individual mutants, each of which carried a deletion at one Tat-dependent locus, identified or predicted previously (31, 32). We found that the *amiA* and *amiC* mutants exhibited increased susceptibility to protamine (0.8 mg/ml), and the survival rate was reduced to 30 and 46%, respectively, when compared with their wild-type parent (BW25113). Both *amiA* and *amiC* genes encode *N*-acetylmuramoyl-L-alanine amidases, AmiA and AmiC, which cleave the bond between the L-alanine and *N*-acetylmuramoyl residues in the cell wall component peptidoglycan (33, 34). AmiA and AmiC should be translocated in *Salmonella* similarly as in *E. coli* (27, 35) because the amino acid sequence is 100% conserved between *E. coli* and *Salmonella* in the signal peptide, which is recognized by the Tat system. Therefore, we carried out one-step gene disruption procedure to construct *Salmonella* single mutants with deletions at the *amiA* and *amiC* genes. Similar to *E. coli*, both *Salmonella* *amiA* and *amiC* mutants displayed increased susceptibility to protamine when challenged by protamine (1.2 mg/ml), and the survival rate was 27 and 36%, respectively, when compared with wild type (85%) (Fig. 2A), confirming that *Salmonella* and *E. coli* share the mechanism of peptidoglycan amidase-dependent resistance to protamine. Resistance could be restored to wild-type levels in both *amiA* and *amiC* mutants when they harbored plasmids carrying an IPTG-inducible wild-type open reading frame (ORF) of *amiA* (pYS2011) and *amiC* (pYS2012), respectively (Fig. 2A). The *tatC* mutant, like a *phoP* mutant, was hypersensitive to protamine and was more susceptible than *amiA* or *amiC* mutant because it could not grow on the protamine-containing plate (Fig. 2A), sug-

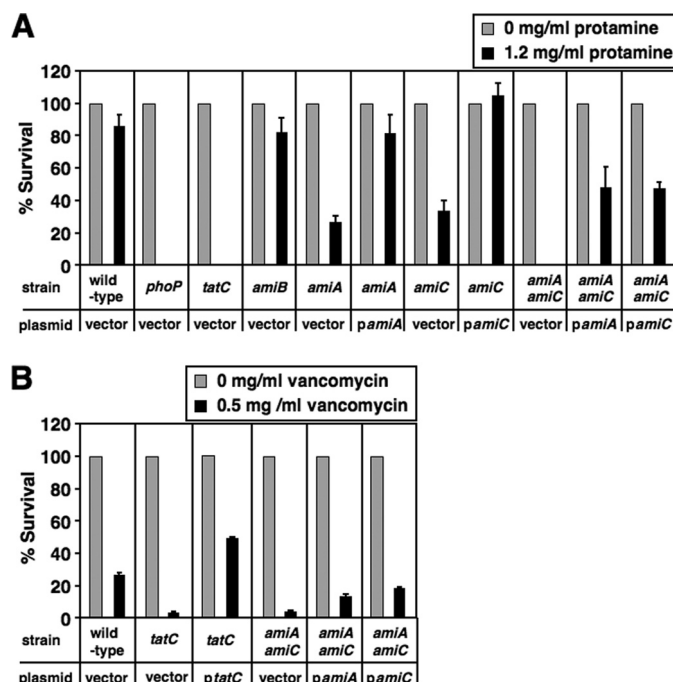


FIGURE 2. Tat-dependent amidases are required for *Salmonella* resistance to protamine and vancomycin. A, percentage survival of wild-type (14028s), *phoP* (YS11590), *tatC* (YS13007), *amiB* (YS13764), *amiA* (YS13629), *amiC* (YS13630), and *amiA* *amiC* (YS13766) strains after incubation with protamine (0 or 1.2 mg/ml). Each strain carries pUHE21 (vector). Additionally, *amiA* and *amiA* *amiC* each carry *pamiA* (pYS2011), whereas *amiC* and *amiA* *amiC* each carry *pamiC* (pYS2012). B, percentage survival of wild-type, *tatC*, and *amiA* *amiC* strains after incubation with vancomycin (0 or 0.5 mg/ml). Each strain carries pUHE21. Additionally, *tatC* mutant carries pBAD-*tatC*, whereas *amiA* *amiC* mutant carries *pamiA* or *pamiC*. Data correspond to mean values from at least two independent experiments performed in duplicate. Error bars, S.D.

gesting that these Tat-dependent amidases have an additive effect on resistance to protamine. Consistent with this notion, an *amiA* *amiC* double mutant exhibited much higher susceptibility to protamine (0% survival) than either the *amiA* or *amiC* single mutant in *Salmonella* (Fig. 2A). The heterologous production of an amidase, either AmiA or AmiC, did not rescue the protamine resistance to a wild-type level because the susceptibility of the double mutant could only be rescued partly because the survival rate increased to 47 or 43% when AmiA or AmiC was expressed from plasmid pYS2011 (*i.e.* *pamiA*) or pYS2012 (*i.e.* *pamiC*), respectively (Fig. 2A). Our results demonstrate that the Tat-dependent peptidoglycan amidases AmiA and AmiC contribute to bacterial resistance to protamine.

Salmonella *tatC* and *amiA* *amiC* Mutants Have a Defective Outer Membrane—*E. coli* *tatC* and *amiA* *amiC* mutants displayed a pleiotropic lesion in the outer membrane and became sensitive to hydrophobic antibiotics and detergents (27, 35). We found that *Salmonella* *tatC* and *amiA* *amiC* mutants exhibited increased susceptibility to the high molecular weight antibiotic vancomycin (Fig. 2B), implying that the outer membrane of these strains, like the *E. coli* mutants, was defective, which allowed this antibiotic to pass through and target the cell wall. The phenotype was fully restored in the *tatC* mutant harboring pBAD-*tatC* but partly complemented

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in *amiA amiC* mutant harboring either *pamiA* or *pamiC* (Fig. 2B).

Both *amiA* and *amiC* Genes Are Required for Resistance to α -Helical Magainin 2 and Melittin but Not β -Sheet Defensin HNP-1 and Lipopeptide Polymyxin B—We examined the susceptibility of the *amiA* and *amiC* mutants to more physiologically relevant peptides belonging to various families. The *amiA* and *amiC* mutants displayed increased susceptibility to magainin 2 and melittin (data not shown), both of which can adopt amphipathic α -helical structures and form pores in bacterial cytoplasmic membrane (8, 36). Consistently, the *tatC* mutant exhibited hypersensitivity to magainin 2, similarly as the *phoP* mutant. Resistance to magainin 2 could be restored in *amiA* and *amiC* mutants by *pamiA* or *pamiC*, respectively (data not shown). The *amiA amiC* mutant also displayed much higher susceptibility to magainin 2 than the single mutants, and the resistance could only be partly rescued by *pamiA* or *pamiC* (data not shown). On the other hand, the *amiA* and *amiC* loci were not required for the resistance to lipopeptide polymyxin B or β -sheet defensin HNP-1 because the *amiA amiC* double mutant strain exhibited a wild-type resistance to these peptides, whereas the *phoP* mutant cells were killed (data not shown).

Transcription of the *amiA* and *amiC* Genes Is Up-regulated through *nlpE* Overexpression in a CpxR-dependent Manner—To explore the possibility that a transcriptional regulator may stimulate resistance to protamine by activating transcription of these amidase loci, we constructed *Salmonella* strains harboring a chromosomal *lac* transcriptional fusion at the *amiA* and *amiC* loci, YS13637 and YS13640, respectively, which could form pale and light blue colonies when grown on a LB agar plate supplemented with 40 μ g/ml X-Gal (data not shown). We prepared a genomic library from the wild-type strain (14028s) in the multicopy number plasmid pUC19, introduced the library into YS13637 and YS13640, and cultured transformants on LB agar plates containing X-Gal. This strategy was based on the premise that overexpression of a positive transcriptional regulator from the P_{lac} promoter in pUC19 may activate transcription of *amiA-lac* in YS13637 or *amiC-lac* in YS13640, resulting in dark blue bacterial colonies. An 1877-bp chromosomal fragment covering a genomic region from 281130 to 283006 of *Salmonella typhimurium* LT2 was characterized in four identical plasmids (pYS0910-R1 and others) recovered from individual dark blue colonies of the *amiA-lac* strain. This region carried two intact open reading frames, *yaeJ* and *nlpE*, under the control of the P_{lac} promoter in the plasmid (Fig. 3A). These two genes, along with *yaeQ*, should form an operon (*i.e.* *yaeQ-yaeJ-nlpE*) in the *S. typhimurium* chromosome (37). To determine which gene was responsible for *amiA* activation, we constructed two plasmids, pYS2131 and pYS2132, which carried a wild-type copy of IPTG-inducible *yaeJ* and *nlpE* genes, respectively, and introduced them into the *amiA-lac* strain. β -Galactosidase activity in this strain harboring *pnlpE* (pYS2132), but not the vector or *pyaeJ* (pYS2131), significantly increased when transformants were grown in LB medium supplemented with IPTG (Fig. 3B). This result indicates that overexpression of *nlpE*, but not *yaeJ*, is able to elevate *amiA* transcription.

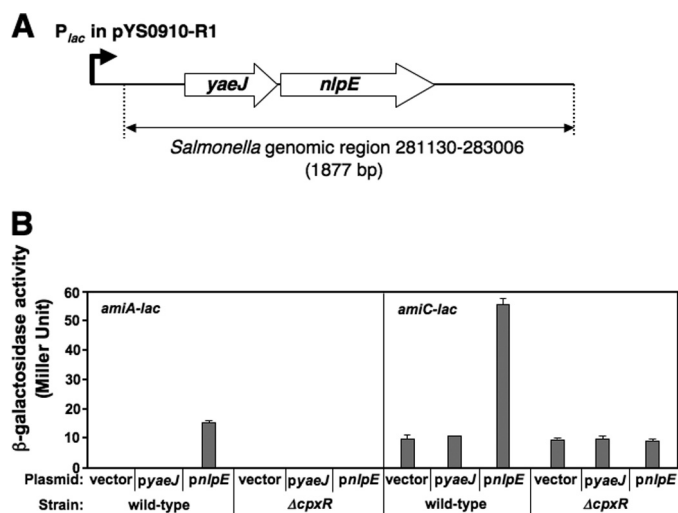


FIGURE 3. Overexpression of *nlpE* up-regulates transcription of *amiA* and *amiC*. A, *Salmonella* genomic region of the insert present in the plasmid, pYS0910-R1, identified as increasing transcription of *amiA* in the *amiA-lac* strain (YS13637) and *amiC* in the *amiC-lac* strain (YS13640). B, expression of *amiA* and *amiC* is up-regulated by the overexpression of *nlpE*, but not *yaeJ*, in a CpxR-dependent manner. Shown is β -galactosidase activity of *amiA-lac* and *amiC-lac* strains, each harboring pUHE21 (vector), *pyaeJ* (pYS2131), or *pnlpE* (pYS2132), grown in LB supplemented with IPTG for 4 h. Assays were conducted in triplicate. Error bars, S.D.

Meanwhile, plasmid pYS0910-R1 was isolated again from darker blue colonies obtained from a screen using the *amiC-lac* strain (YS13640) in a manner analogous to that described above. β -Galactosidase activity from the *amiC-lac* strain harboring *pnlpE* grown in IPTG-containing LB medium was ~ 5.6 -fold higher than that harboring the vector or *pyaeJ* (Fig. 3B). Together, our results show that *nlpE* overexpression can activate transcription of both *amiA* and *amiC*.

The *nlpE* gene encodes an outer membrane lipoprotein that is suggested to be involved in copper homeostasis and adhesion (38, 39); however, its biochemical function remains elusive. The fact that *nlpE* overexpression stimulates the regulatory activity of the CpxR/CpxA system (40) raised the possibility that *amiA* and *amiC* transcription could be activated by this two-component system. Thus, we deleted the *cpxR* gene from the *amiA-lac* and *amiC-lac* strains to construct their isogenic mutants, YS13731 and YS13733. In contrast to the observation from wild type, β -galactosidase activity in YS13731 and YS13733 harboring *pnlpE* remained as low as those harboring the empty vector (Fig. 3B). These observations collectively demonstrate that the CpxR/CpxA system up-regulates transcription of both of the Tat-dependent amidase genes, *amiA* and *amiC*.

The CpxR Protein Binds to the *amiA* and *amiC* Promoters—We carried out primer extension analysis to map the transcription start of the *amiA* and *amiC* genes using total mRNA samples isolated from *Salmonella* wild type and the *cpxR* mutant harboring plasmid vector or *pnlpE*. Two transcription products of *amiA* were observed from wild type harboring the vector, indicating that transcription could start at the nucleotides located 71 and 62 bp upstream of the *amiA* start codon (Fig. 4A; summarized in Fig. 4E). The mRNA level of both transcripts increased when *nlpE* was overexpressed in wild-type but not in the *cpxR* mutant, which further demon-

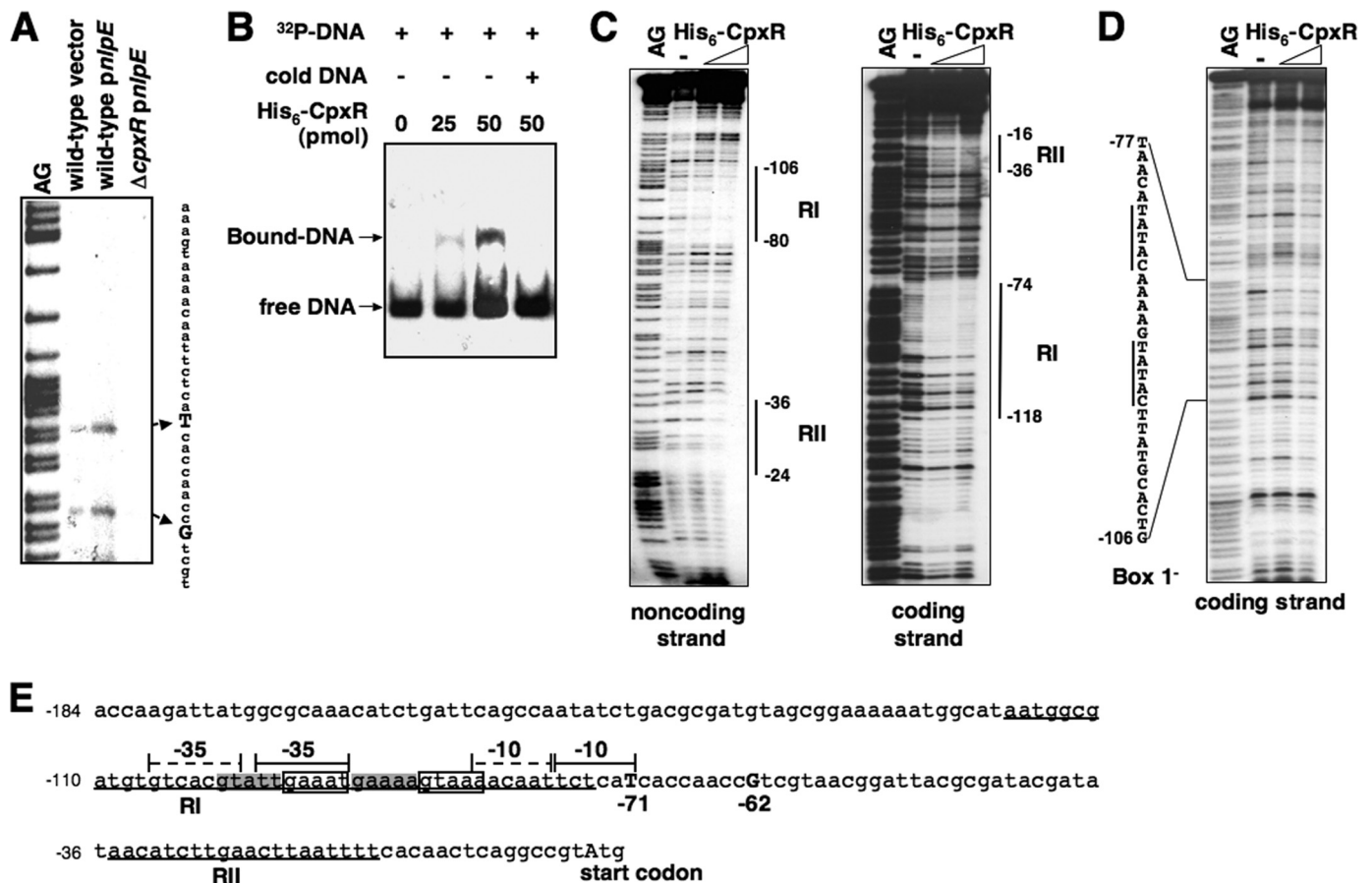


FIGURE 4. The CpxR protein enhances *amiA* transcription by binding to the *amiA* promoter. *A*, primer extension for mapping the transcription start site of *amiA*. The cDNA products were synthesized using ³²P-labeled primer 1472 and total RNA templates isolated from wild-type (14028s) carrying pUHE21 (vector) (lane 2) and *pnlpE* (pYS2132) (lane 3) and *cpxR* mutant carrying *pnlpE* (lane 4). Transcription starts at -71 and -62 are shown in **boldface capital letters**. *B*, EMSA. A ³²P-labeled DNA fragment of wild-type *amiA* promoter was incubated with different amounts of His₆-CpxR, shown in lanes 1–3. Lane 4, same as lane 3 but supplemented with “cold” *amiA* promoter fragment. His₆-CpxR-DNA mixtures were subjected to 4% polyacrylamide electrophoresis. The location of DNA migration was detected by autoradiography. *C*, DNase I footprinting analysis of the wild-type *amiA* promoter with probes for the coding and noncoding strands and increasing amounts of His₆-CpxR protein (50 and 100 pmol). **Solid vertical lines** correspond to the CpxR-binding sites, RI and RII. *D*, DNase I footprinting analysis of the CpxR box (box 1)-substituted *amiA* promoter with probes for the coding strand and increasing amounts of His₆-CpxR protein (50 and 100 pmol). **Solid vertical lines** correspond to the substituted CpxR box. *E*, DNA sequence of the *amiA* promoter region. The **underlines** correspond to the CpxR-binding sites, RI and RII. The **boxes** correspond to sequences (box 1) resembling the consensus CpxR box. The putative -35 and -10 boxes for the transcription started from -62 described in *A* are labeled with **solid braces**. The **highlighted sequences** correspond to an alternative CpxR box (box 2), and the putative -35 and -10 regions for the transcription started from -71 are labeled with **dashed braces**. Numbering in *C–E* is from the predicted start codon of *amiA*.

strated that the CpxR/CpxA system could facilitate *amiA* transcription. Meanwhile, we found that *amiC* transcription was initiated 220 bp upstream of the putative start codon, and the transcript level increased significantly in a CpxR-dependent manner when *nlpE* was overexpressed (Fig. 5A; summarized in Fig. 5E), similarly to *amiA* transcription.

An EMSA was performed using purified His₆-CpxR protein and 206- and 188-bp DNA fragments corresponding to an upstream region of the *amiA* and *amiC* start codons, respectively. We determined that this CpxR protein, whose phosphorylated ratio remained unknown, could shift these two DNA fragments (Figs. 4B and 5B), indicating the presence of CpxR-binding sites in the *amiA* and *amiC* promoter regions. We carried out DNase I footprinting analysis to determine the DNA sequences recognized by CpxR. The His₆-CpxR protein protected the *amiA* promoter at the -118 to -74 and -36 to -16 regions (numbering from the *amiA* start codon) in the coding strand and the -106 to -80 and -36 to -24 regions

in the noncoding strand (Fig. 4C). The region protected by CpxR located upstream of the *amiA* transcription starts (labeled as *RI*) includes an imperfect direct repeat sequence, 5'-GAAATN₅GTAAA-3' (from -96 to -82 boxed sequences, named box 1; Fig. 4E), which is similar to the consensus CpxR box 5'-GTAAAN₅GTAAA-3' described previously (41). It seems that the CpxR box in the *amiA* promoter is located upstream of the putative -10 region and partly overlaps with the putative -35 region for σ^{70} that initiates transcription from -62 (Fig. 4E). Furthermore, a CpxR box-like sequence, 5'-GTATTN₅GAAAA-3', is located 5 bp upstream of, and overlapping with, the CpxR box in the *amiA* promoter (from -101 to -87 , highlighted sequences, named box 2; Fig. 4E), which is located between the alternative putative -10 and -35 regions for σ^{70} that initiates transcription from -71 . Another region (labeled as *RII*), which is weakly protected by CpxR (Fig. 4C), is located downstream of the transcription start and does not contain a sequence homologous to the

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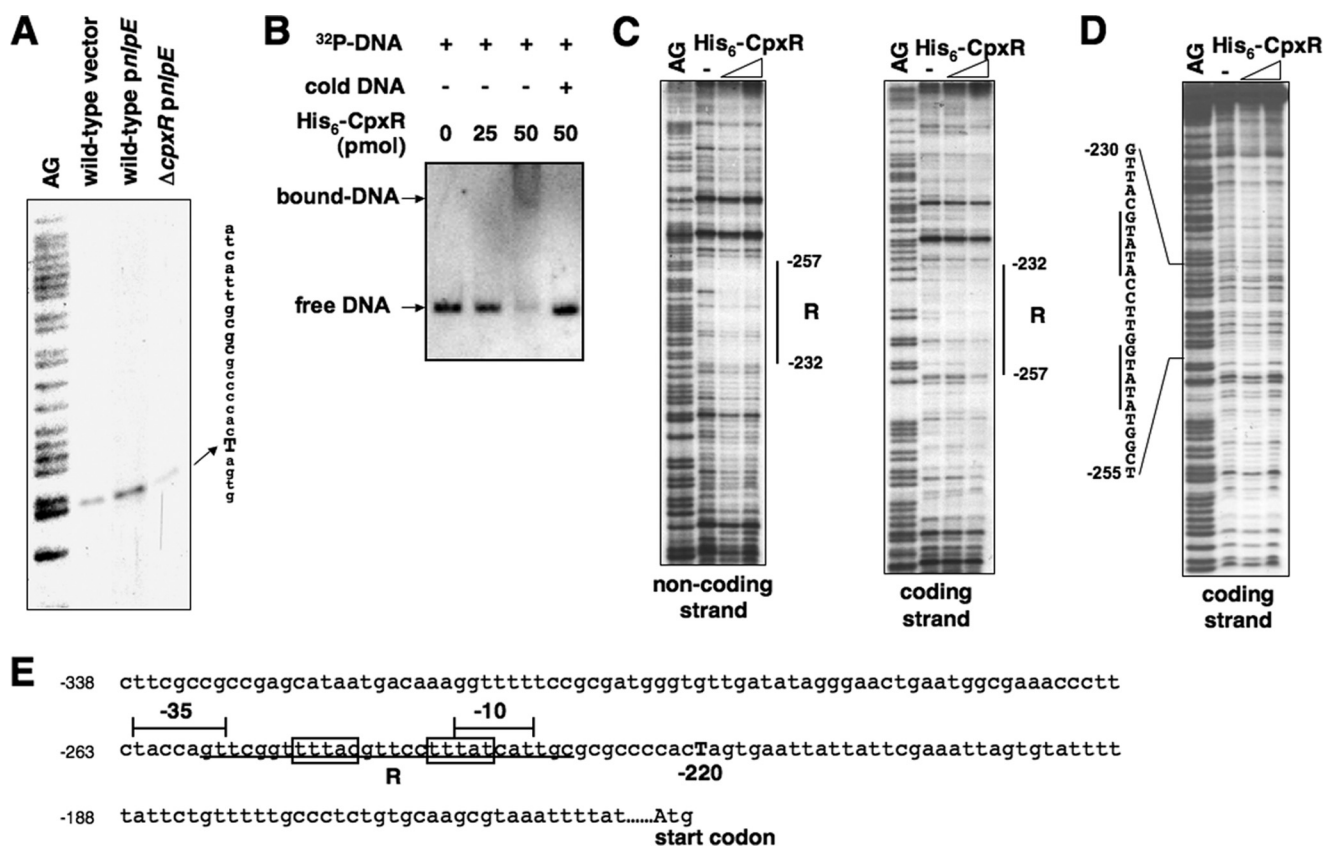


FIGURE 5. The CpxR protein enhances *amiC* transcription by binding to the *amiC* promoter. *A*, primer extension for mapping the transcription start site of *amiC*. The cDNA products were synthesized using 32 P-labeled primer 1482 and total RNA templates isolated from wild-type (14028s) carrying pUHE21 (vector) (lane 2) and *pnlpE* (pYS2132) (lane 3) and *cpxR* mutant carrying *pnlpE* (lane 4). Transcription start at -220 is shown in boldface capital letters. *B*, EMSA. A 32 P-labeled DNA fragment of the wild-type *amiC* promoter was incubated with different amounts (0, 25, and 50 pmol) of His₆-CpxR, shown in lanes 1–3. Lane 4, same as lane 3 but supplemented with “cold” *amiC* promoter fragment. His₆-CpxR-DNA mixtures were subjected to 4% polyacrylamide electrophoresis. The location of DNA migration was detected by autoradiography. *C*, DNase I footprinting analysis of the wild-type *amiC* promoter with probes for the coding and noncoding strands and increasing amounts of His₆-CpxR protein (50 and 100 pmol). Solid vertical lines correspond to the CpxR-binding site. *D*, DNase I footprinting analysis of the CpxR box-substituted *amiC* promoter with probes for the coding strand and increasing amounts of His₆-CpxR protein (50 and 100 pmol). Solid vertical lines correspond to the substituted CpxR box. *E*, DNA sequence of the *amiC* promoter region. The underline corresponds to the CpxR-binding site, R. The boxes correspond to sequences resembling the consensus CpxR box. The putative -35 and -10 boxes for the transcription start at -220 described in *A* are labeled with braces. Numbering in *C–E* is from the predicted start codon of *amiC*.

CpxR box (Fig. 4E). However, this weakly CpxR-protected sequence is unlikely to be involved in *amiA* regulation *in vivo* because a site-directed substitution of this sequence did not affect CpxR-dependent transcription of *amiA* (data not shown).

To investigate the specificity of the identified sequence for CpxR binding, we synthesized the *amiA* promoter fragment with substituted CpxR box sequence and carried out a footprinting assay described above. When the box 1 sequence was substituted, the CpxR protein failed to protect both the R1 and RII regions in the *amiA* promoter region (Fig. 4D). Thus, it is possible that CpxR either first binds to box 1 and then initiates transcription from -62 or moves from box 1 to box 2 and initiates transcription from -71 .

The CpxR protein protected the *amiC* promoter at the -257 to -232 region of the coding strand and the -257 to -232 region of the noncoding strand (Fig. 5C). We identified a sequence, 5'-ATAAAN₅GTA AAA-3', in this CpxR-protected region (labeled R), which resembles the CpxR box but is located on the opposite strand and embedded between the putative -35 and -10 regions (from -250 to -236 , boxed sequences, numbering from the *amiC* start codon; see Fig. 5E).

Also, the CpxR protein did not protect the *amiC* promoter region containing the substituted reverse CpxR box (Fig. 5D). Taken together, these results suggest that the CpxR box identified is essential for CpxR binding in the *amiA* and *amiC* promoters.

The CpxR/CpxA System Facilitates Salmonella Resistance to Protamine—We compared the survival rate of wild type harboring plasmid vector and *pnlpE* (pYS2132) to determine protamine resistance facilitated by *nlpE* overexpression. Bacterial cells with *pnlpE* were rescued by 61% in a plate supplemented with protamine (1.8 mg/ml), whereas those with vector were completely killed (Fig. 6A). However, a *cpxR* mutant harboring either vector or *pnlpE* was killed completely in the same protamine plate (Fig. 6A), demonstrating that the resistance stimulated by *nlpE* overexpression is CpxR-dependent. Interestingly, *nlpE* overexpression could still facilitate resistance of the *tatC* mutant to protamine in a CpxR-dependent manner because the *tatC* mutant harboring *pnlpE* survived by 54% in a plate supplemented with protamine (1.0 mg/ml), whereas the *tatC* mutant harboring vector or the *tatC* *cpxR* double mutant harboring *pnlpE* was completely killed (Fig. 6B). We conclude that the CpxR/CpxA system also confers protamine resistance in a Tat-inde-

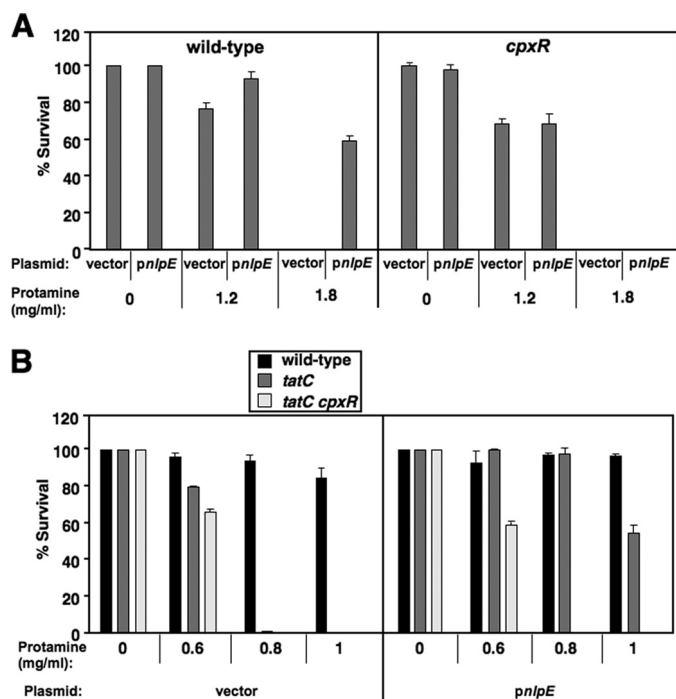


FIGURE 6. Overexpression of *nlpE* enhances *Salmonella* resistance to protamine in a CpxR-dependent manner. A, percentage survival of *Salmonella* wild-type (14028s) and *cpxR* (Y513644), each harboring either the pUHE21 (vector) or *nlpE* (pYS2132) after incubation with protamine (0, 1.2, or 1.8 mg/ml). B, percentage survival of 14028s, *tatC* (Y513007), and *tatC cpxR* (Y513995), each harboring either pUHE21 or *nlpE* after incubation with protamine (0, 0.6, 0.8, or 1.0 mg/ml). Data correspond to mean values from at least two independent experiments performed in duplicate. Error bars, S.D.

pendent manner. We are currently identifying the chromosomal loci contributing to this resistance.

DISCUSSION

According to the results shown in this study, we present a transcriptional regulation pathway that contributes to resistance of Gram-negative bacteria *Salmonella* and *E. coli* to specific CAMPs. We demonstrate that (i) the Tat system is required for the resistance to protamine and α -helical CAMPs magainin 2 and melittin; (ii) the Tat-dependent resistance is in part mediated by two of its amidase substrates, *AmiA* and *AmiC*; (iii) the CpxR/CpxA two-component system up-regulates *amiA* and *amiC* transcription; (iv) the CpxR protein binds to divergent CpxR boxes in the *amiA* and *amiC* promoters; and (v) the CpxR/CpxA system facilitates resistance to protamine.

The Tat-dependent Cell Wall Amidases Contribute to Resistance to Protamine and α -Helical Antimicrobial Peptides in Enteric Bacteria—Enzymes that can cleave peptidoglycan and damage the integrity of bacterial cell wall play a role in cell division, growth, and cell wall remodeling. The Tat-dependent *N*-acetylmuramoyl-L-alanine amidases, *AmiA* and *AmiC*, catalyze cleavage of the cross-link formed by the peptide strand and polysaccharide strand of peptidoglycan (21, 33, 34). We have shown that *tatC*, *amiA*, and *amiC* mutants exhibit increased susceptibility to protamine and α -helical CAMPs magainin 2 and melittin (Fig. 2A) (data not shown). This phenotype is probably caused by an altered outer mem-

brane that allows penetration of more peptides to the sites of action (e.g. cytoplasmic membrane) that are located within this barrier. This hypothesis is based on the observation that interference of the *AmiA* and *AmiC* function by either disruption of the Tat activity or mutation of the *amiA* and *amiC* genes renders the integrity of the *E. coli* cell envelope highly defective, resulting in increased permeability of the outer membrane to hydrophobic antibiotics and detergents (27). Consistent with this notion, both *tatC* and *amiA/amiC* mutants of *Salmonella* displayed increased susceptibility to the antibiotic vancomycin (Fig. 2B), which targets the peptidoglycan of Gram-positive bacteria. However, this antibiotic is prohibited, probably due to its high molecular weight, by the outer membrane of Gram-negative bacteria. Furthermore, Gram-negative bacteria could be sensitized to many antibiotics when pretreated with polymyxin B nonapeptide, a polymyxin B analog that could enhance the permeability of bacterial outer membrane but not kill bacteria by itself (42).

Notably, antimicrobial peptide nisin exhibits bactericidal activity against Gram-positive bacteria by specifically binding to lipid II, a membrane-bound component involved in peptidoglycan synthesis (43). In Gram-negative bacteria, the pentapeptide moiety of the lipid II intermediates, L-alanyl- γ -D-glutamyl-diaminopimelyl-D-alanyl-D-alanine, should carry negative charges in the physiological condition due to the carboxyl group of the glutamate residue. We hypothesize that the defective outer membrane in the *tatC* and amidase mutants may allow more CAMP molecules to pass through and interact with the pentapeptide domain via electrostatic force. Thus, an alternative mechanism for protamine and α -helical CAMPs to implement their bactericidal activities might be to bind to the glycan chain during peptidoglycan biosynthesis in the periplasm. It is possible that a pentapeptide in the lipid II intermediate, once bound to protamine, cannot integrate into the peptidoglycan meshwork via the transpeptidation step. On the other hand, the periplasmic amidases, *AmiA* and *AmiC*, could probably cleave the pentapeptides to prevent the protamine-glycan complex accumulation, thus conferring resistance to this CAMP. It remains to be investigated why *AmiA* and *AmiC* are not required for *Salmonella* resistance to defensins and polymyxin B although they are also CAMPs.

Interestingly, a *Salmonella* mutant carrying deletion at the *amiB* locus displayed wild-type resistance to protamine (Fig. 2A). It is possible that *AmiB*, a Tat-independent periplasmic amidase (44), has a different specificity to the target bond formed between the L-alanine and *N*-acetylmuramoyl residues, which is located in a particular location of the peptidoglycan meshwork. Although *AmiA* and *AmiC* both contribute to resistance to CAMPs, the *AmiC* amidase appears to act on the peptidoglycan at the septum and is located exclusively at the septal ring during cell division, whereas the *AmiA* amidase can be detected throughout the periplasm (44). *N*-acetylmuramoyl-L-alanine amidases are widely distributed among bacterial species; therefore, the amidase-dependent mechanism characterized in this study may represent a generalized mechanism for bacterial re-

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sistance to α -helical CAMPs and probably other peptides with similar features.

The CpxR/CpxA Two-component System Contributes to Resistance to Protamine and α -Helical Antimicrobial Peptides in Enteric Bacteria—The CpxR/CpxA two-component system senses perturbations in the cell envelope and up-regulates the expression of genes encoding factors that combat the damage (for a review, see Ref. 45). In agreement with its function, we demonstrate that the CpxR/CpxA system contributes to *Salmonella* resistance to protamine and α -helical CAMPs in this study. Our results further show that the *amiA* and *amiC* genes are required for resistance to these peptides (Fig. 2A) (data not shown) and are up-regulated by the CpxR/CpxA system (Fig. 3B). Although the direct action of these Tat-dependent amidases is to modulate cell wall remodeling, they should play a role in the release of bacterial envelope stress because mutations of the *amiA* and *amiC* loci or the *tatC* locus caused a loss of integrity of the bacterial cell envelope (27). Thus, it is plausible that the CpxR/CpxA system confers the resistance to CAMPs by combating damage of the cell envelope caused by these peptides or by modifying the outer membrane to prevent CAMPs from binding.

Activation of the CpxR/CpxA system by overexpressing *nlpE* is still able to elevate resistance of the *tatC* mutant to protamine, indicating that some other CpxR-dependent loci contribute to the peptide resistance in a Tat-independent manner. One of these loci is the *yqjA* gene, whose transcription is up-regulated by both the PhoP/PhoQ and CpxR/CpxA systems (13, 14). The *yqjA* gene encodes a protein with unknown function but unlikely to be a Tat substrate and is required for magainin 2 and protamine resistance (13). In addition to the CpxR/CpxA system, other regulatory mechanism(s) should also be responsible for activation of the *amiA* and *amiC* genes. Both *amiA* and *amiC* are transcribed to similar levels in wild type and the *cpxR* mutant when grown in LB medium without *nlpE* overexpression (Fig. 3B), and the *cpxR* mutant does not display increased susceptibility to protamine when compared with the wild-type parent (Fig. 6A).

It is shown that CAMPs are able to induce regulatory activity of some two-component systems. A number of antimicrobial peptides, including polymyxin B and LL-37 (46), activate the PmrA/PmrB system, thus elevating resistance to these peptides in *Pseudomonas aeruginosa* (47). In addition, LL-37, as well as other CAMPs, was shown to induce the PhoP/PhoQ system, resulting in up-regulation of the PhoP-activated gene, *phoN*, in *Salmonella* (48). However, CAMPs are unlikely to activate the CpxR/CpxA system because β -galactosidase activity remained similar to that of the *amiA-lac* and *amiC-lac* strains grown in LB medium supplemented with or without sublethal concentrations of protamine and magainin 2 (data not shown).

*The CpxR Regulator Protein Binds to the *amiA* and *amiC* Promoters via the CpxR Box*—The CpxR-dependent regulation of the *amiA* and *amiC* genes is validated by identifying the CpxR-binding sites in their promoters. Although the CpxR protein used was not further phosphorylated *in vitro* using acetyl phosphate, the identified CpxR-binding sequences in these genes share similarity to the CpxR box, pre-

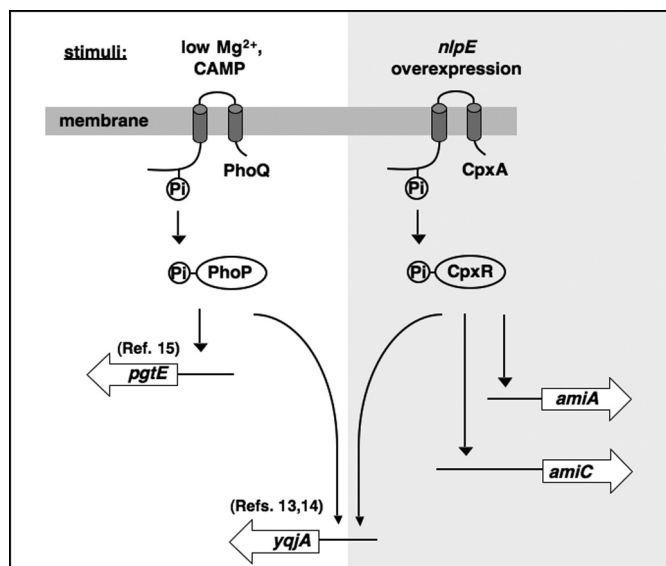


FIGURE 7. Model illustrating the CpxR-dependent resistance to protamine and α -helical CAMPs. In *Salmonella*, induction of the regulatory activity of the CpxR/CpxA system facilitates transcription of the *amiA*, *amiC*, and *yqjA* genes, which confers resistance to protamine and α -helical CAMPs (highlighted pathways). The *yqjA* gene is also activated by the PhoP/PhoQ system, which is a master signaling system controlling bacterial resistance to multiple CAMPs.

viously characterized as the CpxR-binding sequence from demonstrated or predicted CpxR-regulated genes (41). The CpxR box in both *amiA* and *amiC* promoters is localized within the -35 and -10 regions. A preliminary result showed that substitutions of the CpxR box abolished CpxR-dependent activation of *amiA* transcription (data not shown). However, we could not rule out the possibility that the *amiA* promoter was damaged due to these substitutions being located near the -35 and -10 regions. A previous study showed that the PhoP box, the consensus DNA sequence for the PhoP binding (29), was characterized from a region that overlapped with the -35 region in many PhoP-activated genes (29). Thus, it is plausible that binding of these two-component regulators to the regions located near -35 and -10 could facilitate, but not inhibit, interaction of the RNA polymerase to some specific promoter regions they regulate. On the other hand, the CpxR box, similarly to the *amiA* promoter, is also located between the -35 and -10 regions of the *aer* gene in *E. coli*, which mediates down-regulation of its transcription (41). Further study is ongoing to investigate how CpxR can coordinate RNA polymerase in transcription of *amiA* and *aer*.

Although the CpxR box is located in the opposite strand at the *amiC* promoter (Fig. 5E), its transcription is up-regulated in a CpxR-dependent manner (Fig. 3B). A comparable example was found in the P2 promoter of the *rpoE* operon, in which a CpxR box in the reverse direction was overlapped with the -35 for σ^F ; however, it was responsible for negative regulation of *rpoE* transcription (41). According to these observations, it is likely that CpxR interacts with different σ factors, thus facilitating σ^{70} binding or inhibiting σ^F binding when it is located on the opposite strand of a promoter region. Furthermore, it has been shown that the CpxR box is located far upstream of the -35 region in three CpxR-acti-

vated loci, *degP*, *yihE*, and *ppiA* (50). Cumulatively, these results indicate divergent models for the CpxR regulatory function, which provide possibilities for further investigation of this stress response regulator.

It is very interesting that bacteria such as *Salmonella* use multiple signaling systems to confer resistance to antimicrobial peptides. A possibility that might explain this phenomenon is that these systems can modify different cellular components in separate parts of bacterial cells under various stresses, thus protecting different targets from attack by CAMPs. In summary, we demonstrate that the CpxR/CpxA system confers *Salmonella* resistance to protamine and α -helical CAMPs partially dependent on the Tat pathway via amidases, AmiA and AmiC. As illustrated in Fig. 7, our findings provide new insights into the regulatory networks that contribute to bacterial resistance to antimicrobial peptides and greatly enrich our understanding of the new mechanism for envelope stress response governed by the CpxR/CpxA system.

Acknowledgments—We thank Josephine Clark-Curtiss, Rajeev Misra, and Cheryl Nickerson for thoughtful discussion as well as the anonymous reviewers for helpful comments.

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