

The CpxR/CpxA Two-component Regulatory System Up-regulates the Multidrug Resistance Cascade to Facilitate *Escherichia coli* Resistance to a Model Antimicrobial Peptide*

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Background: The CpxR/CpxA system facilitates *E. coli* resistance to antimicrobial peptides. However, many CpxR-dependent mechanisms remain elusive.

Results: The CpxR/CpxA system activates *mar* transcription, subsequently facilitating *E. coli* resistance to protamine via tripartite multidrug transporters.

Conclusion: A CpxR/CpxA-dependent regulatory cascade controls MarRA for CAMP resistance in response to aromatic metabolites.

Significance: This is novel regulatory mechanism controlling bacterial resistance to CAMPs and antibiotics.

A genome-wide susceptibility assay was used to identify specific CpxR-dependent genes that facilitate *Escherichia coli* resistance to a model cationic antimicrobial peptide, protamine. A total of 115 strains from the Keio Collection, each of which contained a deletion at a demonstrated or predicted CpxR/CpxA-dependent locus, were tested for protamine susceptibility. One strain that exhibited high susceptibility carried a deletion of *tolC*, a gene that encodes the outer membrane component of multiple tripartite multidrug transporters. Concomitantly, two of these efflux systems, AcrAB/TolC and EmrAB/TolC, play major roles in protamine resistance. Activation of the CpxR/CpxA system stimulates *mar* transcription, suggesting a new regulatory circuit that enhances the multidrug resistance cascade. Tripartite multidrug efflux systems contribute to bacterial resistance to protamine differently from the Tat system. DNase I footprinting analysis demonstrated that the CpxR protein binds to a sequence located in the –35 and –10 regions of *mar* promoter. This sequence resembles the consensus CpxR binding site, however, on the opposite strand. *aroK*, a CpxR-dependent gene that encodes a shikimate kinase in the tryptophan biosynthesis pathway, was also found to facilitate protamine resistance. Specific aromatic metabolites from this pathway, such as indole, can stimulate expression of well studied CpxR-dependent genes *degP* and *cpxP*, which are not components of the tripartite multidrug transporters. Thus, we propose a novel mechanism for *E. coli* to modulate resistance to protamine and likely other cationic antimicrobial peptides in which the CpxR/CpxA system up-regulates *mar* transcription in response to specific aromatic metabolites, subsequently stimulating the multidrug resistance cascade.

In enteric bacteria, the CpxR/CpxA two-component system regulates a vast number of genetic loci in response to periplasmic stress, including misfolded proteins, inner membrane disruptions, alkaline pH, starvation, and high osmolarity. The *Escherichia coli* CpxR/CpxA system responds to general periplasmic stress and has been used as a model two-component system for transcription regulation in bacteria (for review, see Ref. 1). This system has a global effect in a variety of signal transduction pathways and bacterial resistance as the vastness of its circuitries becomes evident as it continues to be implicated in virulence (1), biofilm formation (2), chemotaxis (3), and recently, resistance to antimicrobials (4–7). To date, there are >150 demonstrated or putative members of the CpxR/CpxA regulon. In a previous study we showed that the CpxR/CpxA system facilitates *Salmonella* and *E. coli* resistance to cationic antimicrobial peptides (*i.e.* CAMPs)³ including a model peptide protamine and α -helical CAMPs by activating transcription of two genetic loci, *i.e.* *amiA* and *amiC*, that encode two peptidoglycan amidases (4). On the other hand, activation of the CpxR/CpxA system in a Δ *tatC* mutant or Δ *amiA* Δ *amiC* double mutant could still facilitate resistance to protamine, suggesting that an additional gene(s) other than *amiA* and *amiC* is also required for the CpxR-dependent resistance.

The multidrug-resistant operon, *marRAB*, encodes a repressor, MarR, an activator, MarA, and an unknown function protein, MarB, which coordinately regulate the *mar* transcription to maintain intrinsic resistance to antimicrobial substances (8, 9). The natural signal for the *marRAB* operon has not been identified as it remains unknown whether plant-derived naphthoquinones are natural inducers (10). It has been shown that the operon is activated by compounds such as salicylate, chloramphenicol, tetracycline, acetaminophen, sodium benzoate, 2,4-dinitrophenol, cinnamate, carbonyl cyanide *m*-chloro-

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³ The abbreviations used are: CAMP, cationic antimicrobial peptide; *mar*, the *marRAB* operon; IPTG, isopropyl 1-thio- β -D-galactopyranoside.

A CpxR/CpxA-up-regulated marRAB Transcription

TABLE 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>E. coli</i>		
Keio collection (parent strain, BW25113)	F ⁻ , DE(<i>araD-araB</i>)567 <i>lacZ</i> 4787(del)::rrnB-3 LAM ⁻ <i>rph</i> -1 DE(<i>rhaD-rhaB</i>)568 <i>hsdR</i> 514	(21)
YS Collection 7a	$\Delta emrB \Delta acrB::Km^R$	This work
YS Collection 7b	$\Delta emrB \Delta acrB \Delta acrE::Km^R$	This work
YS Collection 7c	$\Delta emrB \Delta acrB \Delta emrY::Km^R$	This work
YS Collection 7d	$\Delta emrB \Delta acrB \Delta macB::Km^R$	This work
YS14530	$\Delta marRA::Cm^R$	This work
YS13007	$\Delta tatC$	(4)
YS15910	$\Delta tolC \Delta tatC$	This work
YS15924	$\Delta degP \Delta dsbA$	This work
YS15925	$\Delta tolC::Km^R \Delta degP \Delta dsbA$	This work
YS15926	$\Delta tatC::Km^R \Delta degP \Delta dsbA::Km^R$	This work
YS15537	<i>tolC-lac::Km^R</i>	This work
YS15538	<i>tolC-lac::Km^R cpxR</i>	This work
PND2000	MC4100, <i>kRS88[degP-lacZ]</i>	(42)
SP1	MC4100 <i>ara</i> ⁺ λ p <i>lac</i> Mu53[<i>cpxP-lacZ</i>]	(43)
MG1301	MC4100mgrB:: λ p <i>lac</i> Mu55	(44)
DH5 α	F ⁻ <i>supE</i> 44 Δ <i>lac</i> U169 (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR</i> 17 <i>recA</i> 1 <i>endA</i> 1 <i>gyrA</i> 96 <i>thi</i> -1 <i>relA</i> 1	(New England Biolabs)
BL21-Gold (DE3)	<i>E. coli</i> B F ⁻ <i>ompT</i> <i>hsdS</i> _B (<i>r</i> _B ⁻ <i>m</i> _B ⁻) <i>dcm</i> ⁺ Tet ^r <i>gal</i> λ (DE3) <i>endA</i> Hte	(Agilent Technologies)
Plasmids		
pKD4	rep _{R6K} γ Ap ^R FRT Km ^R FRT	(22)
pKD46	rep _{pSC101} ^{ts} Ap ^R P _{<i>araBAD</i>} γ <i>exo</i>	(22)
pCP20	rep _{pSC101} ^{ts} Ap ^R Cm ^R <i>cI</i> 857 λ P _R	(22)
pKG136	rep _{R6K} γ Km ^R FRT <i>lacZY</i> <i>t</i> _{his}	(23)
pACYC184	rep _{p15A} Cm ^R Tc ^R	(New England Biolabs)
pUHE21-2 <i>lac</i> ⁴	rep _{pMB1} Ap ^R <i>lacI</i> ⁴	(24)
pYS2132	rep _{pMB1} Ap ^R <i>lacI</i> ⁴ <i>nlpE</i>	(4)
pYS1755	rep _{pMB1} Ap ^R <i>lacI</i> ⁴ <i>tolC</i>	This work
pYS1000	rep _{p15A} Cm ^R P _{<i>lac1-6</i>} <i>lacZ</i> <i>t</i> _{his}	(25)
pYS1736	rep _{p15A} Cm ^R P _{<i>lac1-6</i>} P _{<i>mar</i>}	This work
pET28a	rep _{ColE1} Km ^R <i>lacI</i> P _{T7}	EMD Biosciences
pYS2211	rep _{ColE1} Km ^R <i>lacI</i> P _{T7} (Eco) <i>cpxR-His</i> ₆	This work

phenylhydrazine, menadione, and plumbagin (11, 12). In the presence of specific antibiotics (such as tetracycline and chloramphenicol; see Refs. 11 and 13), bile salts, and reactive oxygen species, MarA, in concert with global regulators Rob and SoxS, binds to the *mar* box sequence to activate transcription of the *mar* operon (14–17). In contrast, transcriptional repressor MarR, which binds at the operator region (*marO*), is only released once a compound such as salicylate binds, allowing for transcription (8, 9, 18). Interestingly, the *marRAB* operon can also be activated by sublethal concentrations of CAMPs via Rob (which is required for polymyxin B-induced up-regulation of *micF*) and facilitate resistance at least in part by overexpressing the AcrAB/TolC efflux pump (19, 20).

Although deletion of *marA* had no apparent phenotypic effect regarding susceptibility to CAMPs, constitutive expression of *marA* by either a point mutation in *marR* or a plasmid harboring a wild-type copy of *marA* raised bacterial resistance to multiple CAMPs, which represent various classes including α -helical and β -sheet CAMPs (such as LL-37 and HBD-1, respectively) as well as cyclic lipopeptide polymyxin B (19). However, mutation of *acrAB* or *tolC* eliminates this resistance in this constitutive *marA* strain (19). In addition, deletion of *tolC* in a *marA* constitutive expression strain causes a higher susceptibility to CAMPs than deletion of *acrAB* (19), suggesting that an additional TolC-dependent tripartite efflux system(s) other than AcrAB/TolC should contribute to this resistance. AcrAB and other resistance-nodulation-cell division superfamily efflux pumps probably use these export systems to mediate efflux of CAMPs because a loss of proton motive force in the presence of an uncoupler, *i.e.* carbonyl cyanide *m*-chlorophenyl hydrazone, causes an increased level of LL-37 susceptibility (19).

Here we investigated the role of those known CpxR-dependent loci in *E. coli* resistance to protamine. Our study revealed a new regulatory mechanism for bacterial resistance to this model CAMP. The CpxR/CpxA system can activate transcription of *marRAB* operon, thus facilitating multidrug-resistant regulator to enhance expression of TolC-dependent tripartite multidrug transporters. This *E. coli* resistance to protamine via tripartite multidrug efflux systems should represent a novel CpxR-mediated mechanism that is independent from the Tat system. Our results also suggest that the *aroK* gene is involved in the resistance by mediating synthesis of aromatic metabolites, which probably act as signal molecules to stimulate the CpxR/CpxA system and Mar regulators.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—All bacterial strains used in this study are provided in Table 1. *E. coli* strains were obtained or derived from mutant strains in the Keio Collection (21) or derived from the wild-type strain BW25113 using the one-step gene deletion method (22). Bacteria were grown at 37 °C in Luria-Bertani broth (LB). 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 the host for the preparation of plasmid DNA. *E. coli* BL21-Gold (Stratagene, Inc.) was used for protein expression. P1(*vir*) transduction was carried out for construction of *E. coli* multiple mutants from the Keio Collection.

Construction of Strains with Chromosomal Deletions and lac Fusions—Simultaneous deletion of the *marR* and *marA* genes was carried out using primer pairs 1746 (5'-TGC AAC TAA TTA CTT GCC AGG GCA ACT AAT CAT ATG AAT ATC

CTC CTT AG-3') and 1747 (5'-ATT GCC TCA GTG ACG TTG TCA CGT TTT CAA GTG TAG GCT GGA GCT GCT TC-3') to amplify the kanamycin resistance cassette (K_m^R) from plasmid pKD4 and integrate the resulting PCR product into the chromosome of wild-type harboring pKD46. The kanamycin-resistant cassette was removed using plasmid pCP20, and the *lac* transcriptional fusion plasmid pKG136 was integrated into the tyrosine DNA recombinase (FLP) recombination target sequence in the deleted locus (22, 23). P1 transduction was carried out to construct double and triple mutants.

Plasmid Construction—All plasmids used in this study are described in Table 1. pYS1755 (pUHE-*tolC*) was constructed using PCR fragments containing the full-length *tolC* coding region generated with primers 2126 (5'-CGG GAT CCA CAA GGA ATG CAA ATG-3') and 2127 (5'-CCC AAG CTT GTC GTC ATC AGT TAC GG-3') and wild-type BW25113 chromosomal template that were digested with BamHI and HindIII and then ligated between BamHI and HindIII sites of pUHE21-2*lacI*^q (24). pYS1736 was constructed by cloning 120 bp of the *marR* promoter region amplified using primers 1731 (5'-CCG CTC GTT CAT TGA ACA GAT CGC TGG TAC TTT TCA C-3') and 1736 (5'-GCG TCG ACC TTT AGC TAG CCT TGC ATC GCA TTG AAC AA-3') and wild-type BW25113 chromosomal template into the Sall and XhoI sites of pYS1000 with a *lacZ* reporter (25). pYS2211 was constructed to produce the *E. coli* CpxR protein fused to a His₆ tag at its N terminus by PCR amplification with the primers 1512 (5'-GTT TCA TAT GAA TAA AAT CCT G-3') and 1714 (5'-ACG CGT CGA CTC ATG AAG CAG AAA CCA T-3') and wild-type BW25113 chromosomal template. The PCR product was digested with NdeI and Sall and then ligated between the NdeI and Sall sites of pET28a.

Protamine Susceptibility Assay to Test CpxR-dependent Genes—Specific strains from the Keio Collection (21) were picked out to test their susceptibility to protamine (26). Bacterial cells were cultured overnight, re-inoculated (1:100) in LB broth, and grown for 4 h at 37 °C. Cultures were diluted, and $\sim 1 \times 10^3$ cells were dropped onto LB agar plates containing varying concentrations (0.5–1.5 mg/ml) of protamine sulfate (MP Biomedicals) and incubated overnight at 37 °C to determine the colony-forming units (cfu). The percentage survival of each strain was calculated by comparing cfu from plates supplemented with and without protamine.

β -Galactosidase Assay— β -Galactosidase assays (27) were carried out in triplicate, and the activity (Miller Unit) was determined using a VERSAmax plate reader (Molecular Device). Data correspond to three independent assays conducted in duplicate, and all values are the mean \pm S.D.

Purification of CpxR-His₆ Protein—The *E. coli* CpxR-His₆ 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 CpxR-His₆ protein were desalted and concentrated using an Amicon Ultra centrifugal filter (Millipore).

DNase I Footprinting Assay—DNase I footprinting assays were carried out using a DNA fragment whose non-coding strand was radioactively labeled. Primer 2051 (5'-CAG ATC GCT GGT ACT TTT CAC-3') was labeled with [γ -³²P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase

(New England Biolabs). A 332-bp DNA fragment was amplified by PCR using BW25113 chromosomal DNA as template and primers ³²P-labeled 2051 and 2050 (5'-TTG CCT GCC AGG CCA-3'). Approximately 25 pmol of labeled DNA and increasing amounts of CpxR-His₆ protein were mixed in a 100- μ l reaction containing 2 mM HEPES, pH 8.0, 10 mM KCl, 20 μ M EDTA, 0.5 mg/ml BSA, 20 μ g ml⁻¹ poly(dI-dC), 2% glycerol (28). The reaction mixture was incubated at room temperature for 20 min. Then a DNase I solution (10 mM CaCl₂, 10 mM MgCl₂, and 0.01 units of DNase I (Fermentas)) was added, and the mixture was incubated at room temperature for 3 min. The DNase I digestion was stopped by phenol treatment, and the DNA was precipitated. Samples were analyzed by 6% polyacrylamide DNA sequencing electrophoresis supplemented with 8 M urea by comparison with a DNA sequence ladder generated with the same primers using a Maxam and Gilbert A+G reaction. The bands were detected by autoradiography.

RESULTS AND DISCUSSION

CpxR-regulated *tolC* Gene Contributes to *E. coli* Resistance to Protamine—Our previous study has implied that the CpxR/CpxA system may control a Tat-independent mechanism that facilitates bacterial resistance to a model CAMP, protamine (4). To study this new mechanism, we carried out a susceptibility assay to compare protamine resistance of an *E. coli* wild type (BW25113) with particular isogenic strains from the Keio Collection, *i.e.* a mutant library carrying in-frame single-gene deletion (21). Protamine could kill this *E. coli* wild-type strain in a concentration-dependent manner because the percentage survival of BW25113 dropped when protamine concentration was gradually elevated (Fig. 1A). To genome-wide-characterize the CpxR-dependent loci that contribute to *E. coli* resistance to this CAMP, a total of 115 single mutants, each harboring a deletion at one demonstrated or predicted CpxR-regulated locus (29–31), were tested in our susceptibility assays. Bacterial cells were challenged by protamine at 1.0 mg/ml, which allowed $\sim 40\%$ of wild-type cells to survive (Fig. 1A). Thus, a susceptible mutant should exhibit a survival rate significantly lower than the wild-type strain in the presence of this protamine. Individual strains were cultured in LB broth to log phase (4 h) and diluted with fresh medium. Bacterial cells ($\sim 10^3$) were inoculated onto LB agar plates with and without protamine and incubated at 37 °C overnight to develop colonies. Previous studies have suggested that the CpxR/CpxA system enhances the resistance of *E. coli* and *Salmonella* to protamine through activation of the Tat-dependent *amiA* and *amiC* loci as well as the *yqjA* gene, which is also regulated by the PhoP/PhoQ system (4, 32, 33). Consistently, the Δ *amiA*, Δ *amiC*, and Δ *yqjA* single mutants from the Keio Collection displayed increased susceptibility to protamine (Table 2 and data not shown).

A susceptible mutant characterized from our susceptibility assay carried deletion at the *tolC* locus. The *tolC* gene encodes the surface component of tripartite multidrug efflux systems (34). The Δ *tolC* mutant cells exhibited hypersusceptibility to protamine and were killed completely (*i.e.* 0% survival) in the presence of 1 mg/ml protamine. Actually, only 4.4% of these mutant cells survived when challenged by protamine at a low concentration (0.5 mg/ml), which could not kill wild-type cells

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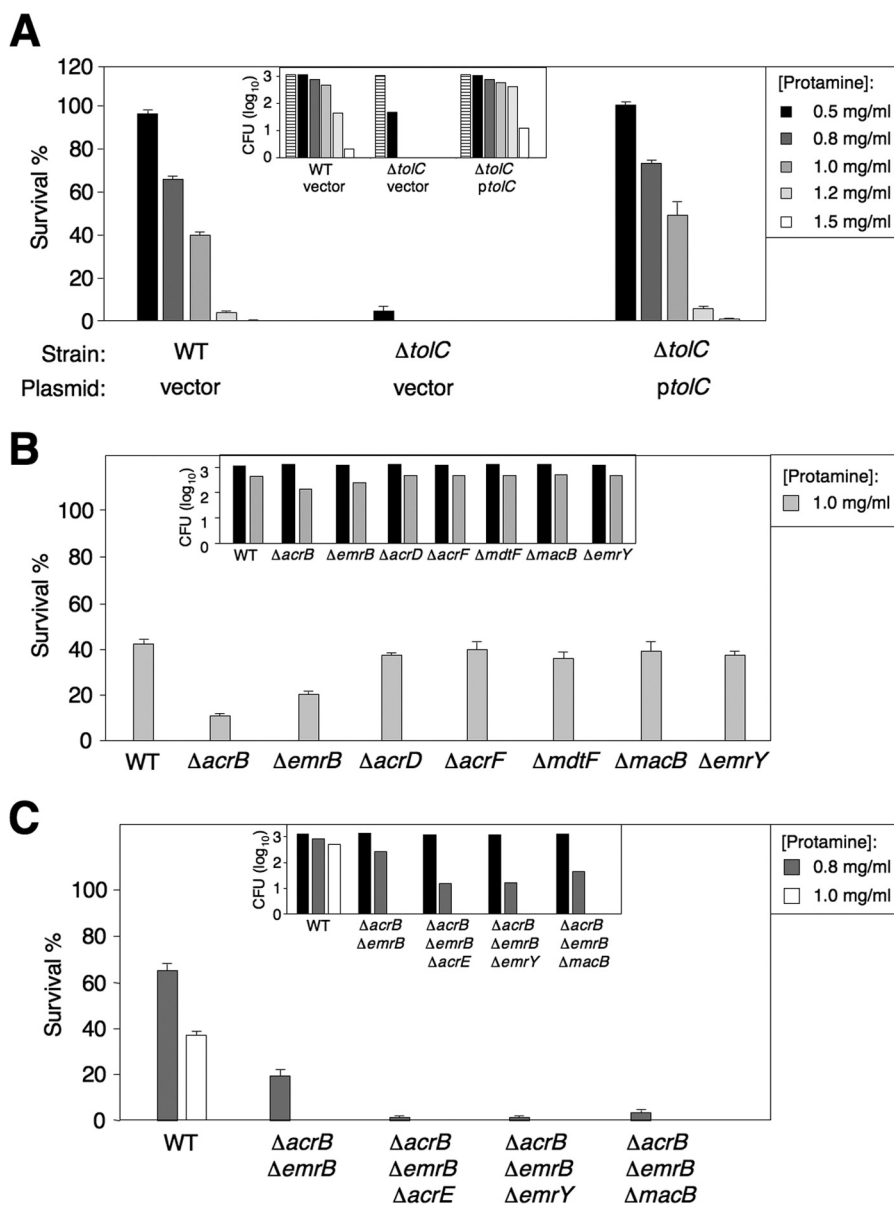


FIGURE 1. TolC-dependent multidrug transporters are required for *E. coli* resistance to protamine. *A*, protamine susceptibility assay for *E. coli* $\Delta tolC$ mutant and complementation. Plasmid harboring a wild-type copy of *tolC* restores resistance to protamine. Shown is the percentage survival and cfu in \log_{10} (inlet) of wild type (BW25113) carrying plasmid pUHE21 (vector) and $\Delta tolC$ mutant (from the Keio Collection) carrying plasmid vector or pYS1755 (pUHE-*tolC* or *ptoIC*) after incubation with protamine (0, 0.5, 0.8, 1.0, 1.2, or 1.5 mg/ml). *B*, protamine susceptibility assay for wild-type (BW25113) and selected single TolC-dependent multidrug transporter mutants (the Keio Collection) on LB plates containing protamine (0 or 1.0 mg/ml). Shown is the percentage survival and cfu in \log_{10} (inlet) of wild type, $\Delta acrB$, $\Delta emrB$, $\Delta acrD$, $\Delta acrF$, $\Delta mdtF$, $\Delta macB$, and $\Delta emrY$ single mutants (the Keio Collection) after incubation with protamine (0 or 1.0 mg/ml). *C*, percentage survival and cfu in \log_{10} (inlet) of wild-type and $\Delta emrB \Delta acrB$ double mutant (YS Collection 7a) as well as $\Delta emrB \Delta acrB \Delta acrE$ (YS Collection 7b), $\Delta emrB \Delta acrB \Delta emrY$ (YS Collection 7c), and $\Delta emrB \Delta acrB \Delta macB$ (YS Collection 7d) triple mutants after incubation with protamine (0, 0.8 or 1.0 mg/ml). Data in these experiments correspond to mean values from at least two independent experiments performed in duplicate. Error bars, S.D. Percentage survival of the strains tested was calculated by comparing cfu from plates with protamine to that from input (*i.e.* the plate without supplementing protamine and shown as the column with light horizontal lines in *A* or column in black in *B* and *C*).

(Fig. 1A). The phenotype of the $\Delta tolC$ mutant was solely the result of a lack of the TolC protein because the survival rate under varied protamine concentrations was reversed to a level similar to wild-type by pYS1755, a plasmid containing an IPTG-inducible wild-type copy of the *tolC* gene (*ptoIC*, Fig. 1A).

Multiple Tripartite Drug Efflux Transporters Play Major Roles in *E. coli* TolC-dependent Resistance to Protamine—TolC makes up tripartite multidrug efflux systems by pairing with AcrAB, AcrAD, AcrEF, MdtEF, MacAB, EmrAB, and EmrKY (35, 36). Susceptibility to protamine caused by *tolC* deletion

suggests a role of drug efflux systems in bacterial resistance to protamine. Thus, we determined the protamine susceptibility of the Keio strains carrying a single deletion in these loci. In the presence of 1.0 mg/ml protamine, the percentage survival of the $\Delta acrB$ and $\Delta emrB$ mutants was 11 and 20%, respectively (Fig. 1B), suggesting that deletion of these genes rendered bacteria more susceptible to protamine when compared with the isogenic wild-type strain (~42%), but not as susceptible as the $\Delta tolC$ mutant cells, which were actually killed completely (Fig. 1B). On the other hand, the survival rate of the $\Delta acrD$, $\Delta acrE$,

TABLE 2

Susceptibility of *E. coli* mutants to protamine

The gene list was summarized from Refs. 8 and 29–31. All strains tested were derived from the Keio Collection. Highlighted mutants are characterized as *E. coli* strains susceptible to protamine.

Mutant	Susceptibility to protamine	Mutant	Susceptibility to protamine	Mutant	Susceptibility to protamine
<i>acpP</i>	no	<i>glk</i>	no	<i>tig</i>	no
<i>acrD</i>	no	<i>hlpA/ompH</i>	no	<i>tolC</i>	yes
<i>adhE</i>	no	<i>hslTS</i>	no	<i>tsr</i>	no
<i>aer</i>	no	<i>htpX</i>	no	<i>udk</i>	no
<i>amiA</i>	yes	<i>intB</i>	no	<i>ung</i>	no
<i>amiC</i>	yes	<i>leuS</i>	no	<i>uxuR</i>	no
<i>argA</i>	no	<i>ligB (yicF)</i>	no	<i>yabC (mraW)</i>	no
<i>aroG</i>	no	<i>mdtABDC</i>	no	<i>ybaQ</i>	no
<i>aroK</i>	yes	<i>mobAB</i>	no	<i>ybaR</i>	no
<i>b2503</i>	no	<i>motAB/cheAW</i>	no	<i>ybaS</i>	no
<i>b2504</i>	no	<i>mviM</i>	no	<i>ybcU (borD)</i>	no
<i>chaA</i>	no	<i>ompF</i>	no	<i>ycbZ</i>	no
<i>chaB</i>	no	<i>pap</i>	no	<i>yccA</i>	no
<i>cobUST</i>	no	<i>pheM</i>	no	<i>ycfS</i>	no
<i>cpxP</i>	no	<i>ppc</i>	no	<i>ycgZ</i>	no
<i>cpxRA</i>	no	<i>ppiA</i>	no	<i>yciF</i>	no
<i>csgBAC</i>	no	<i>ppiD</i>	no	<i>ydeH</i>	no
<i>csgDEFG</i>	no	<i>psd</i>	no	<i>ydjF</i>	no
<i>degP</i>	yes	<i>ptsl</i>	no	<i>yefJ</i>	no
<i>deoC</i>	no	<i>purR</i>	no	<i>yfiD</i>	no
<i>dnaK</i>	yes	<i>puuC (aldH)</i>	no	<i>ygjT</i>	no
<i>dppC</i>	no	<i>qseB</i>	no	<i>yhdG</i>	no
<i>dppD</i>	no	<i>rcaA</i>	no	<i>yhdJ</i>	no
<i>eda</i>	no	<i>rdoA-dsbA</i>	yes	<i>yhfC</i>	no
<i>efeU</i>	no	<i>rpoE rseABC</i>	yes	<i>yhjE</i>	no
<i>endA</i>	no	<i>rpoH</i>	no	<i>yhjV</i>	no
<i>fliY</i>	no	<i>rpsT</i>	no	<i>yjfP</i>	no
<i>flu</i>	no	<i>secA</i>	no	<i>yjiY</i>	no
<i>fryA (ypdD)</i>	no	<i>slt</i>	no	<i>ymgA</i>	no
<i>ftnB</i>	no	<i>smpA</i>	no	<i>yojN</i>	no
<i>ftsJ-hflB</i>	no	<i>spy</i>	no	<i>ypdF</i>	no
<i>fucU</i>	no	<i>sspA</i>	no	<i>yqjA</i>	yes
<i>galP</i>	no	<i>tap</i>	no	<i>yzgL</i>	no
<i>gcvTHP</i>	no				

Δ *acrF*, Δ *mdtE*, Δ *mdtF*, Δ *macA*, Δ *macB*, Δ *emrK*, and Δ *emrY* single mutants was similar to wild-type under the same conditions (Fig. 1B). Therefore, two tripartite drug efflux transporters, AcrAB-TolC and EmrAB-TolC, should play major roles in the protamine resistance. An Δ *acrB* Δ *emrB* double mutant was indeed more susceptible than either Δ *acrB* or Δ *emrB* single mutant and thus was killed completely by 1.0 mg/ml protamine (Fig. 1C). However, this double mutant was still more resistant than the Δ *tolC* mutant because 19.3% of the Δ *acrB* Δ *emrB* mutant cells survived when challenged by a lower concentration (0.8 mg/ml) of protamine (Fig. 1C), whereas the Δ *tolC*

mutant cells were killed completely (Fig. 1A). This observation suggested that an additional TolC-dependent efflux pump(s) contributes to the resistance. We introduced the *acrE*, *emrY*, and *macB* mutations into the Δ *acrB* Δ *emrB* mutant, respectively, and found that all of these triple mutants became more susceptible than the Δ *acrB* Δ *emrB* mutant to protamine (Fig. 1C) regardless of a wild-type level resistance exhibited in their single mutants (Fig. 1B). Thus, multiple tripartite multidrug efflux systems, particularly AcrAB-TolC and EmrAB-TolC, should contribute to TolC-dependent protamine resistance. Taken together, we conclude that a CpxR/CpxA-dependent

A CpxR/CpxA-up-regulated *marRAB* Transcription

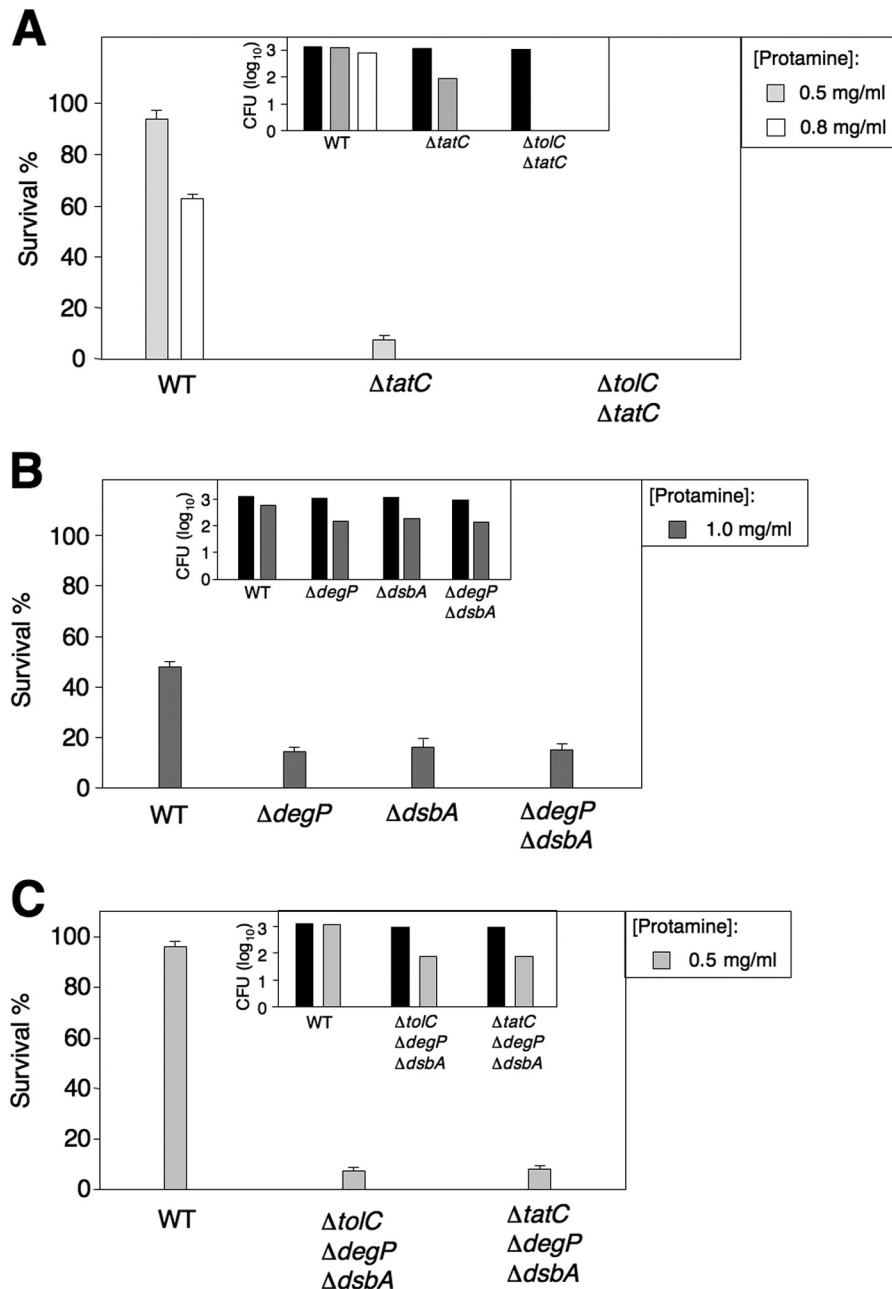


FIGURE 2. Protamine resistance controlled by the CpxR/CpxA system in TolC-dependent and Tat-dependent manners. A, protamine susceptibility assay for wild type, Δ *tatC* single mutant (YS13007), and Δ *tolC* Δ *tatC* double mutant (YS15910) on LB plates containing protamine (0, 0.5, or 0.8 mg/ml). Shown is the percentage survival and cfu in log₁₀ (inlet). B, percentage survival and cfu in log₁₀ (inlet) of wild type, Δ *degP*, and Δ *dsbA* single mutants (the Keio Collection) and Δ *degP* Δ *dsbA* double mutant (YS15924) after incubation with protamine (0 or 1.0 mg/ml). C, percentage survival and cfu in log₁₀ (inlet) of wild type, Δ *tolC* Δ *degP* Δ *dsbA* (YS15925), and Δ *tatC* Δ *degP* Δ *dsbA* (YS15926) triple mutants after incubation with protamine (0 or 0.5 mg/ml). The column in black is input cell number counted from the culture without protamine. Data in these experiments correspond to mean values from at least two independent experiments performed in duplicate. Error bars, S.D. Percentage survival of the strains tested was calculated by comparing cfu from plates with protamine to that from input (shown as the columns in black in A, B, and C).

mechanism that controls *E. coli* resistance to protamine is to facilitate production, assembly, and action of multiple TolC-dependent tripartite drug efflux transporters.

Multiple Mechanisms Facilitate CpxR-dependent Resistance to Protamine in *E. coli*—We constructed a Δ *tolC* Δ *tatC* double mutant to investigate whether the tripartite multidrug efflux systems were components of the Tat-mediated mechanism or functioned independently to confer resistance to protamine. In the presence of a low concentration of protamine (0.5 mg/ml),

4.4% and 7.5% of Δ *tolC* and Δ *tatC* single mutants survived, respectively, whereas all of the Δ *tolC* Δ *tatC* mutant cells were killed (Fig. 1A and Fig. 2A). Because this double mutant displayed an additive effect, we postulate that the tripartite multidrug efflux complexes function independently from the Tat system.

Two more CpxR-regulated genes required for protamine resistance were *degP* and *dsbA* (Table 2). When challenged by 1.0 mg/ml protamine, the percentage survival of the Δ *degP* and

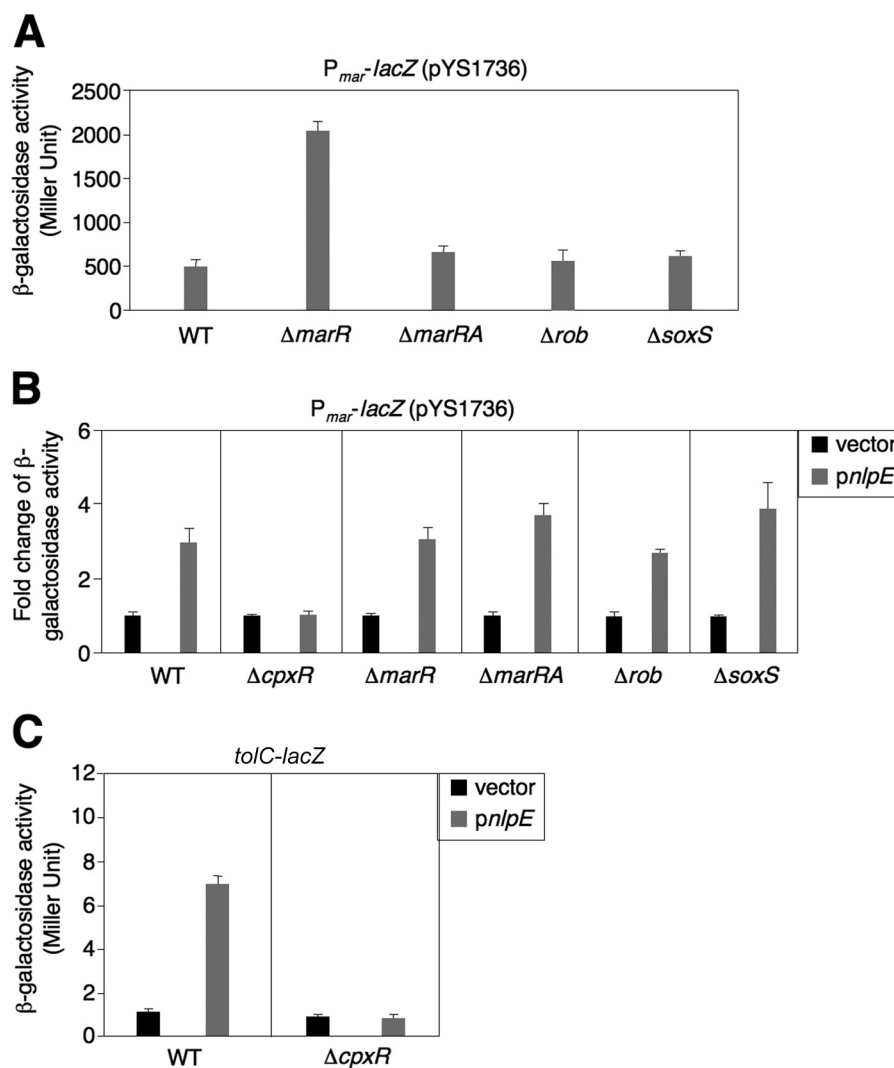


FIGURE 3. **Activation of the CpxR/CpxA system by *nlpE* overexpression up-regulates *mar* transcription.** *A*, a cloned *mar* promoter region including 112 bp of DNA upstream of the transcription start site is sufficient for the MarR and MarA-dependent regulation. Shown is β -galactosidase activity of wild type and $\Delta marR$ (Keio) and $\Delta marRA$ (YS14530) mutants harboring pYS1736 (P_{mar} -*lacZ*) grown in LB for 4 h. *B*, the CpxR/CpxA system activates *mar* transcription in different genetic backgrounds. -Fold change in β -galactosidase activity of wild type and $\Delta cpxR$ (the Keio Collection), $\Delta marR$ (the Keio Collection), and $\Delta marRA$ (YS14530) mutants harboring pYS1736 and either pUHE21 (vector) or pYS2132 (*pnlpE*) grown in LB supplemented with IPTG (0.1 mM) for 4 h. -Fold change was calculated: β -galactosidase activity of a strain grown with IPTG divided by that without. *C*, the CpxR/CpxA system activates *tolC* transcription. Shown is β -galactosidase activity of wild-type and *cpxR* harboring chromosomal *tolC*-*lac* fusion (YS15537 and YS15538, respectively) and either pUHE21 or pYS2132, grown in LB supplemented with IPTG (0.1 mM) for 4 h. All assays were conducted in triplicate. Error bars, S.D.

$\Delta dsbA$ single mutants was 14% and 16%, respectively (Fig. 2*B*). The *degP* and *dsbA* genes encode a heat shock response chaperone/protease and thiol disulfide oxidoreductase, respectively. They play important roles in the release of envelope stress and the maintenance of the outer membrane integrity by assisting in protein folding and assembly (37). Thus, the outer membrane of the $\Delta degP$ and $\Delta dsbA$ mutants can be vulnerable and more susceptible to the membrane-acting antimicrobial peptides. DsbA is required for formation of one disulfide bond in the DegP protein, whose nascent form is unstable and thus becomes absent in a *dsbA* null mutant (38). Thus, the susceptibility of the $\Delta dsbA$ mutant to protamine could result from a lack of functional DegP proteins. Indeed, a $\Delta degP \Delta dsbA$ double mutant was as susceptible as individual single mutants (percentage survival, 14.8%, Fig. 2*B*). We constructed the $\Delta tolC \Delta degP \Delta dsbA$ and $\Delta tatC \Delta degP \Delta dsbA$ triple mutants and found that their susceptibility was similar to the $\Delta tolC$ and

$\Delta tatC$ single mutants, respectively (Fig. 2*C*). Thus, we conclude that the Tat system and tripartite multidrug efflux complexes may require contain components related to DegP and DsbA for *E. coli* resistance to protamine.

The CpxR/CpxA System Up-regulates Transcription of the mar Operon—We postulated that the CpxR/CpxA system indirectly regulated the *tolC* gene because the consensus CpxR binding site (*i.e.* CpxR box, GTAAA-N₅-GTAAA; see Ref. 3) was not present in its promoter region. It is known that the *marA* gene encodes MarA, a regulator that activates transcription of both *tolC* as well as the pump component genes, including *acrAB* (19). Therefore, we investigated whether the CpxR/CpxA system could activate transcription of the *marRAB* operon (marked as *mar*, hereinafter). We constructed a reporter plasmid, pYS1736, in which *lacZ* was fused with a DNA fragment covering 112 bp upstream of the *mar* transcription start site including both the marbox and MarR binding site

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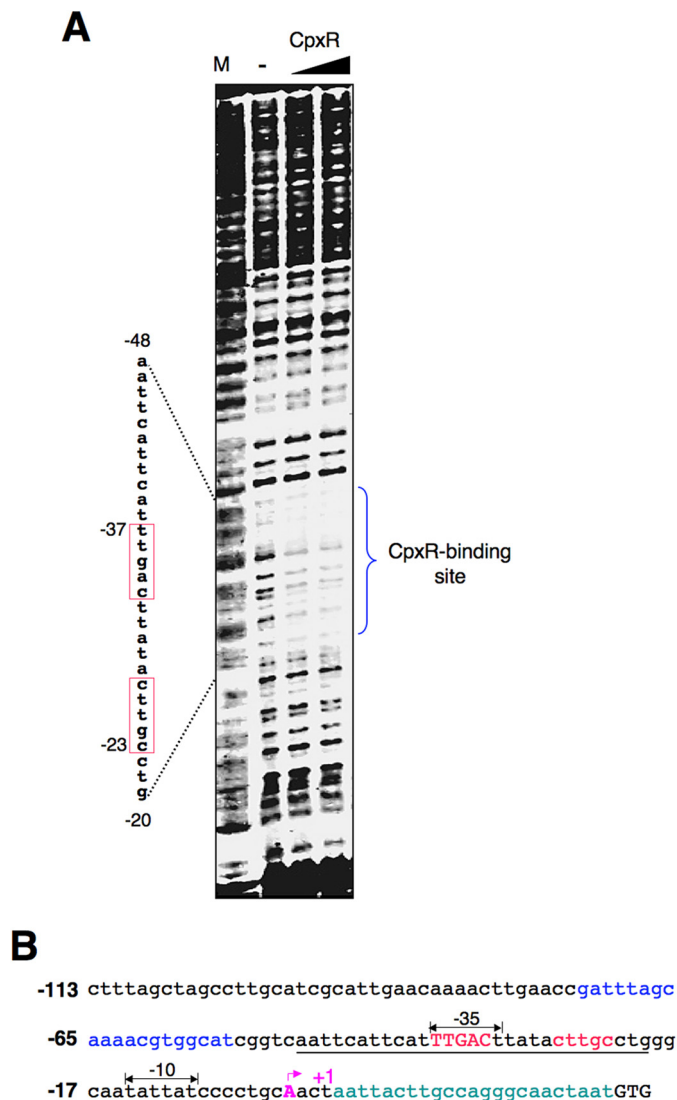


FIGURE 4. The CpxR protein binds to the *mar* promoter. A, DNase I footprinting analysis of the wild-type *mar* promoter with ^{32}P -labeled probe for the noncoding strand and increasing amounts of CpxR-His₆ protein (100 and 200 pmol). Products were subjected to polyacrylamide DNA sequencing electrophoresis, and the bands were detected by autoradiography. The right bracket corresponds to the CpxR binding site, and the sequence represents the DNA region protected by CpxR-His₆ protein. Red boxes are the CpxR box sequence. B, DNA sequence of the *mar* promoter region. The underlines correspond to the CpxR-protected region. Sequences in red resemble a reverse consensus CpxR box. Double arrows indicate the putative -35 and -10 sequences, respectively. The nucleotide A in purple with arrow is the transcription start site (+1). Sequences in blue and green are the marbox and MarR binding site, respectively. Numbering in A and B is from the +1.

characterized previously (18) and introduced it into *E. coli* strains. β -Galactosidase activity from a mutant harboring a deletion of the repressor gene *marR* was 4.1-fold higher than that from wild-type (Fig. 3A). This transcription activation caused by the *marR* mutation was eliminated when the activator gene *marA* was also deleted, and β -galactosidase activity in a $\Delta\textit{marR} \Delta\textit{marA}$ double mutant was reversed to a level comparable to a basal level in wild-type (Fig. 3A). We postulate that MarA functions as an activator required for *mar* transcription but not as an antirepressor to antagonize MarR. These results demonstrated that the *mar* promoter region present in pYS1736 is sufficient for the MarR and MarA-dependent tran-

scription regulation. Without supplementing inducer molecules, the *mar* operon remained at a basal level of expression in the wild-type strain when grown in LB. Furthermore, the level of *mar* transcription in a $\Delta\textit{rob}$ mutant or $\Delta\textit{soxS}$ mutant grown in LB was similar to that in a wild-type strain (Fig. 3A), implying that its expression is also activated by a factor(s) other than these known regulators.

To study transcriptional regulation of *mar* controlled by the CpxR/CpxA system, we constructed a set of strains carrying pYS1736 and a compatible plasmid, pYS2132, that carried a wild-type copy of a IPTG-inducible *nlpE* gene (4). Overexpression of *nlpE* induced by IPTG will stimulate the regulatory activity of the CpxR/CpxA system (47). β -Galactosidase activity in wild-type strain was 3.1-fold higher when IPTG (0.1 mM) was supplemented (Fig. 3B). On the contrary, β -galactosidase activity from a *cpxR* deletion mutant remained at similar levels when grown in the medium with or without IPTG as that in the wild-type strain without IPTG induction (Fig. 3B). These results demonstrate that the CpxR/CpxA system is a regulator that activates *mar* transcription through the *mar* promoter fragment in pYS1736. To investigate whether the CpxR/CpxA system could regulate the *mar* transcription indirectly through a known *mar* regulator (14–17), we determined pYS1736-directed *lacZ* expression in the strains harboring a deletion at one of these regulator genes. Overexpression of *nlpE* could still activate *mar* transcription in the absence of these regulators because in the presence of IPTG, β -galactosidase activity in $\Delta\textit{marR}$, $\Delta\textit{marRA}$, $\Delta\textit{rob}$, and $\Delta\textit{soxS}$ mutants was raised by 3.1-, 3.7-, 2.8-, and 3.9-fold, respectively (Fig. 3B). We conclude that the CpxR/CpxA system can activate *mar* expression by bypassing or overcoming these regulators. Concomitantly, percent survival of these mutants was similar to the wild-type strain when challenged by 1.0 mg/ml protamine (data not shown).

We also examined the effect of CpxR/CpxA system on the transcription of *tolC* gene. Supplementing IPTG raised β -galactosidase activity by \sim 6.8-fold in a strain with a chromosomal *tolC-lac* fusion carrying pYS2132 (Fig. 3C). On the contrary, when the *cpxR* gene was deleted, β -galactosidase activity became unchanged regardless of IPTG (Fig. 3C). Taken together, we propose a transcriptional regulatory cascade that controls *E. coli* TolC-dependent resistance to protamine in which the CpxR/CpxA system up-regulates transcription of *mar* operon to facilitate expression of MarA-dependent loci such as *tolC*.

The CpxR Protein Binds to the *mar* Promoter—An electrophoretic mobility shift assay suggests that the CpxR binding site is present in the *mar* promoter because a CpxR-His₆ fusion protein binds to the *mar* promoter (data not shown). To reveal the nucleotide sequence recognized by CpxR, we carried out a DNase I footprinting assay using a ^{32}P -labeled 332-bp DNA fragment containing the *mar* promoter region present in pYS1736. We found that CpxR was able to protect DNA from DNase I cleavage in a region covering the nucleotides from -48 to -20 upstream of the +1 site (*i.e.* the transcription start site) (Fig. 4A and illustrated in Fig. 4B). We observed that this CpxR-protected region possessed a sequence, 5'-TTGACTtataCTTGC-3', which is located in -37 and -23 upstream of +1, whose sequence on the opposite strand, 5'-GCAAGtataaGTCAA-3', resembled a

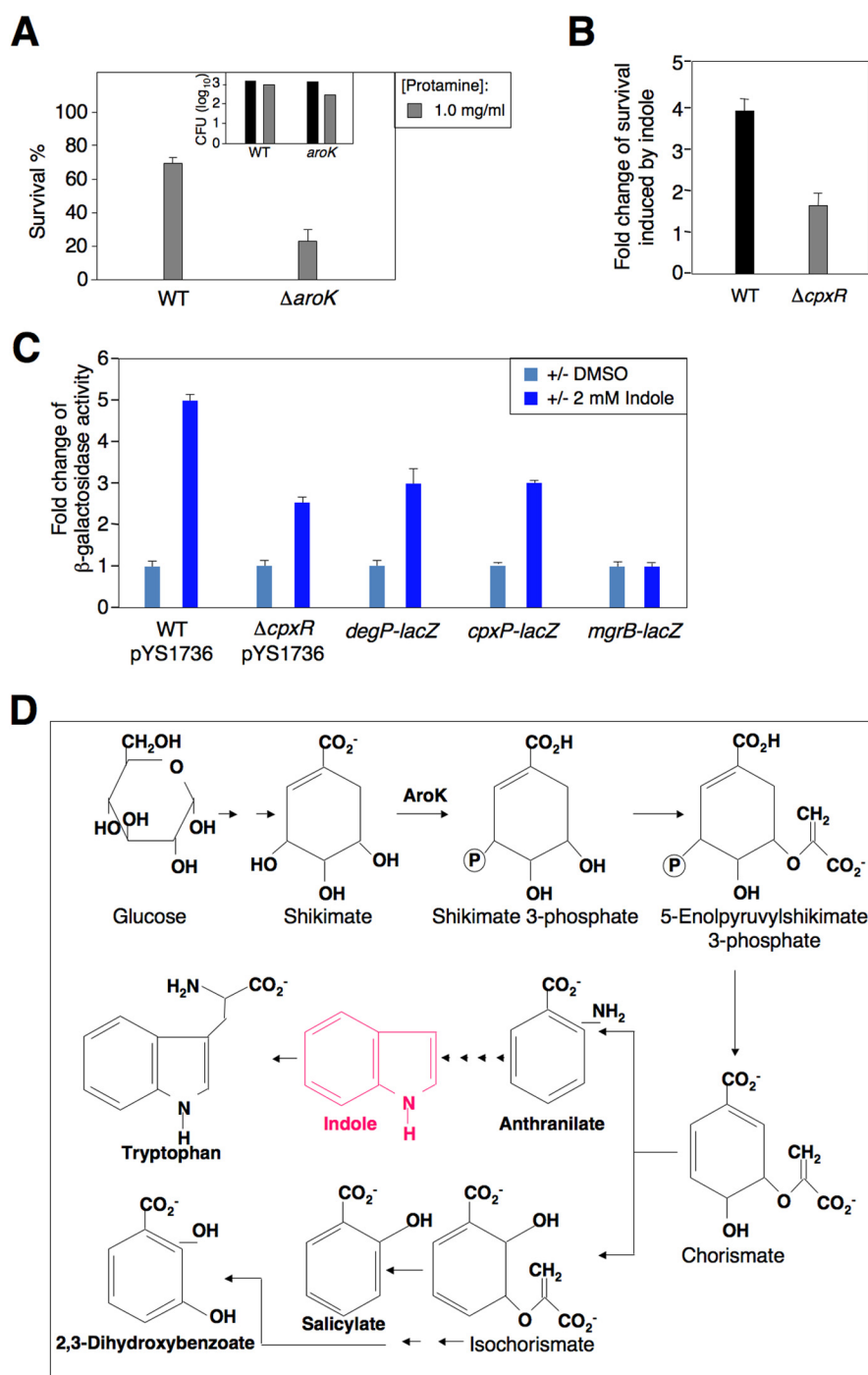


FIGURE 5. **A possible signal transduction required for resistance to protamine.** *A*, percentage survival and cfu in log₁₀ (inlet) of wild-type and Δ *aroK* mutant (Keio) after incubation with protamine (0 or 1.0 mg/ml). Percentage survival was calculated by comparing cfu from plates with protamine to that from input (the column in black). *B*, -fold change of percentage survival of wild-type and Δ *cpxR* mutant (Keio) after incubation in the presence of protamine (1.0 mg/ml) supplemented with indole (2 mM) or without. -Fold change was calculated as follows: percentage survival of the strains with indole divided by that without. *C*, -fold change in β -galactosidase activity of wild-type harboring pYS1736 and strains carrying chromosomal *lacZ* fusions at *degP* (PND2000), *cpxP* (SP1), and *mgrB* (MG1301) grown in LB for 4 h supplemented with or without 2 mM indole or solvent dimethyl sulfoxide (DMSO). -Fold change was calculated as follows: β -galactosidase activity of strains with indole or DMSO divided by that without. *D*, AroK is a kinase that mediates biosynthesis of chorismate, a precursor for the synthesis of aromatic metabolites including anthranilate, salicylate, 2,3-dihydroxybenzoate as well as indole (the structure in red).

consensus CpxR binding site (the CpxR box, 5'-GTAAA-N₅-ATAAA-3'; see Ref. 29). Similar to a reverse CpxR box characterized from the *amiC* promoter (4), the CpxR binding site is located between the -35 and -10 regions (illustrated in Fig. 4B), which bind σ^{70} to initiate *mar* transcription. On the other hand, the marbox is located far upstream,

approximately two helical turns apart from this CpxR box, providing a region for MarA/Rob/SoxS to interact with α subunits of RNA polymerase. Because of this, CpxR can act on the *mar* promoter in a manner independent from other regulators. Thus, we conclude that CpxR regulates *mar* transcription by directly binding to its promoter.

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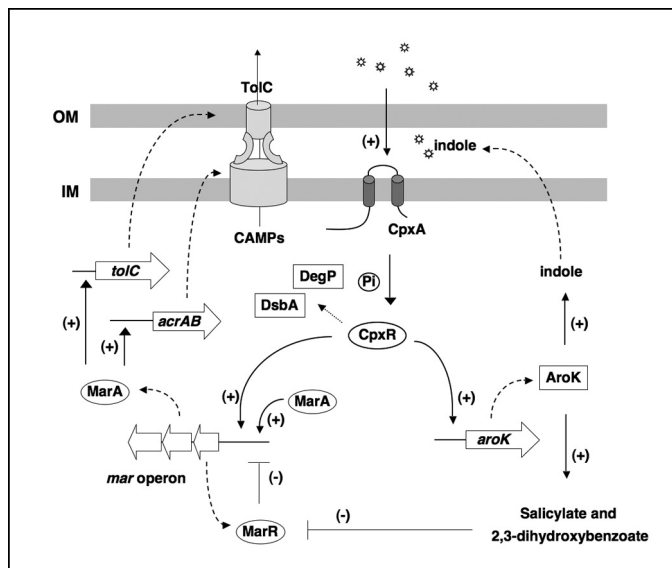


FIGURE 6. A proposed role for CpxR-dependent regulation in *E. coli* resistance to CAMP. The CpxR/CpxA response induces the expression of a variety of genes encoding MarA, MarR, AroK, and other envelope folding factors DsbA, DegP, etc. CpxR-induced MarA activates the genes encoding TolC and other components of tripartite multidrug transporters. DegP and DsbA affect assembly of these transporters, which mediate efflux of the CAMPs into the cytoplasm. AroK mediates production of indole, which is exported and stimulates the CpxR/CpxA system, resulting in activation of *mar* transcription. In the meantime, AroK also mediates production of salicylate and 2,3-dihydroxybenzoate, which bind to MarR to derepress *mar* transcription. OM and IM are bacterial outer and inner membranes, respectively.

Activation of the CpxR/CpxA System through Aromatic Metabolites—We wanted to know how the CpxR/CpxA system could activate *mar* transcription in wild type (Fig. 3B) when MarR was present and presumably bound to the promoter. We postulate that the CpxR/CpxA system actually facilitates production of a signal molecule(s) to destabilize MarR binding to the *mar* promoter, thus allowing an RNA polymerase to pass the MarR binding site. In fact, salicylate and 2,3-dihydroxybenzoate activate the *mar* promoter by directly binding to MarR with a similar affinity (40). We screened the *aroK* locus as a protamine-resistant gene because the Δ *aroK* mutant displayed increased susceptibility when challenged by 1.0 mg/ml protamine (23% survived; Fig. 5A). The *aroK* gene has been demonstrated as a CpxR-dependent gene (29) which encodes a kinase that generates shikimate 3-phosphate in chorismate biosynthesis in *E. coli*. As illustrated in Fig. 5D, chorismate is a precursor for biosynthesis of aromatic amino acids including phenylalanine, tyrosine, and tryptophan and also for the synthesis of specific aromatic metabolites, indole and salicylate, as well as salicylate-like compounds including 2,3-dihydroxybenzoate and anthranilate. Therefore, CpxR-activated *aroK* expression should enhance production of aromatic metabolites to derepress transcription of *mar*, resulting in activation of the *tolC* gene.

Investigation of Indole as a Signal Molecule to Activate the CpxR-dependent Genes—Indole is able to induce the BaeR/BaeS two-component system to activate multidrug exporters including *acrD* and *mdtABC* (41). We found that indole (2 mM) induced a higher level of resistance to protamine in wild type (~3.8-fold) than that in Δ *cpxR* mutant (~1.6-fold) (Fig. 5B). Meanwhile, this indole could also facilitate *mar* transcription in wild type harboring pYS1736 to a higher level (~4.8-fold) than the Δ *cpxR* mutant

(~2.5-fold) (Fig. 5C). To determine if indole could directly act on the CpxR/CpxA system, we constructed strains harboring a chromosomal *lacZ* fusion at *degP* and *cpxP* genes that have been demonstrated previously as CpxR-activated loci (4, 42, 43). Indole was shown to activate transcription of these Mar-independent genes because β -galactosidase activity in *degP-lacZ* and *cpxP-lacZ* strains was ~3- and 2.9-fold higher, respectively, when 2 mM indole was supplemented to the culture. As a negative control, transcription of a PhoP-dependent gene, *mgrB* (44), could not be activated by indole (Fig. 5C). Taken together, we propose that indole may be one of the physiological signals for the CpxR/CpxA system, thus facilitating resistance to protamine.

Conclusion—The results presented in this study extend our understandings of molecular mechanisms for the CpxR-dependent resistance to CAMPs. Many CAMPs are not only membrane-active, thus causing damages of bacterial membranes, but are also membrane-permeable, thus entering the cytoplasm to target-specific cytoplasmic components (for reviews, see Refs. 45 and 46). A damaged bacterial envelope would be changed in membrane permeability or become leaky, resulting in uncontrollable transmembrane exchange of varied harmful substances. On the other hand, bacteria can probably release this stress by removing malicious substances from the cytoplasm through varied tripartite multidrug transporters. CAMPs that entered into the cytoplasm might be recognized as such noxious substances, subsequently exported by these transporters. This is probably a reason why loss of the TolC-dependent efflux can activate the BaeRS and CpxRA systems (39). Some cytoplasmic components, such as indole, could be facilitated to release out of the cytoplasm from a damaged bacterial envelope. We postulate that this extracytoplasmic indole can interact with the periplasmic domain of the CpxA sensor and subsequently activate this two-component system. Therefore, our findings provide new insights into a mechanism of action of the CpxR/CpxA system.

Antimicrobial peptides, rather than conventional antibiotics, are a natural component in the host environments. Thus, it is more likely that expression of the tripartite multidrug efflux systems actually is to facilitate bacterial resistance to antimicrobial peptides when bacteria are located in certain host niches. As illustrated in Fig. 6, we propose a model that outlines the new mechanism we uncovered in this study. The CpxR/CpxA system activates transcription of *mar* operon, thus facilitating generation of tripartite multidrug efflux transporters; meanwhile, it also activates transcription of the *aroK* gene, thus enhancing production of aromatic metabolites including indole, salicylate, and 2,3-dihydroxybenzoate, etc. Salicylate and 2,3-dihydroxybenzoate can act directly on MarR to release it from the *marO* site. Indole released into the extracytoplasm stimulates the CpxA sensor to phosphorylate CpxR, which subsequently activates the *mar* transcription.

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