

The Cpx Stress Response Confers Resistance to Some, but Not All, Bactericidal Antibiotics

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It has recently been suggested that bactericidal antibiotics, including aminoglycoside antibiotics (AGAs), and toxic small molecules, such as hydroxyurea (HU), kill bacteria the same way, namely, by generating reactive oxygen species (ROS) via a process requiring activation of the Cpx stress response. We suggest an opposite, protective role for Cpx. We have confirmed the initial finding that *cpxA* null mutations confer resistance to HU. However, the two-component sensor CpxA is both a kinase and a phosphatase, and previous work from our lab has shown that removing CpxA can activate the stress response owing to buildup of the phosphorylated response regulator (CpxR~P) that occurs in the absence of the phosphatase activity. We show that a dominant *cpxA** mutation that constitutively activates the Cpx stress response confers a high level of resistance to both HU and AGAs in a CpxR-dependent manner. In contrast, inactivating the CpxR response regulator by mutating the phosphorylation site (D51A) or the putative DNA-binding motif (M199A) does not increase resistance to HU or AGAs. Taken together, these results demonstrate that activation of the Cpx stress response can protect cells from HU and AGAs. However, the Cpx response does not increase resistance to all classes of bactericidal antibiotics, as the *cpxA** mutants are not significantly more resistant to fluoroquinolones or β -lactams than wild-type cells. Thus, it seems unlikely that all bactericidal antibiotics kill by the same mechanism.

The mechanism by which aminoglycoside antibiotics (AGAs) kill bacteria has been a subject of interest for decades, and classic studies link the mechanism with alterations in the cellular membrane (1). It is known that AGAs bind to the A-site of the ribosome, leading to mistranslation (2). Incorporation of these mistranslated proteins into the membrane could alter this essential structure to an extent that renders the cell inviable (2). Consistent with this idea, certain AGAs (such as gentamicin) trigger activation of the Cpx envelope stress response (3). Misfolded proteins, including inner membrane (IM) proteins, are also known inducers of the Cpx response (4). Recent work suggests that induction of the Cpx response contributes to the bactericidal nature of these antibiotics by stimulating the formation of reactive oxygen species (ROS) (5). Antibiotics like fluoroquinolones that inhibit DNA gyrase or small toxic molecules that inhibit DNA replication by targeting ribonucleotide reductases, such as hydroxyurea (HU), are also thought to generate misfolded envelope proteins and lead to killing by a similar mechanism (6, 7). Indeed, many bactericidal antibiotics, including the β -lactams, are thought to ultimately cause cell death by stimulating formation of ROS (5).

The Cpx two-component system is composed of the histidine sensor kinase/phosphatase CpxA and its cognate response regulator CpxR (8–10). Evidence suggests that this system senses extracytoplasmic stresses, such as misfolded envelope proteins, and transcriptionally mediates an adaptive response (10–13). Many of the Cpx-controlled genes upregulated by this adaptive response encode protein folding and degradation factors (14, 15). Signals are transduced from CpxA to CpxR via conserved phosphotransfer reactions that are characteristic of two-component systems (11). Inducing cues cause a conformational change in CpxA, stimulating the autophosphorylation of a conserved histidine residue. Once this residue is phosphorylated, CpxA acts as a kinase and phosphorylates a conserved aspartate residue on CpxR. Phosphorylated CpxR (CpxR~P) acts as a transcriptional activator (8, 16). In the absence of induction, unphosphorylated CpxA acts as a

phosphatase that removes phosphoryl groups from CpxR, thereby maintaining the response regulator in an inactive state even in the presence of heterologous kinases and small-molecule phospho donors such as acetyl-phosphate (8). In mutant strains exhibiting constitutive Cpx activity (*cpxA**), the mutant CpxA* protein still functions as an autokinase and CpxR kinase but lacks phosphatase activity. This leads to high levels of CpxR~P and hyperactivation of the Cpx response (8, 11).

A role for Cpx in the generation of ROS that would contribute to the cell death caused by various antibiotics was unexpected, given the proposed function of the Cpx system in combating envelope stress as described above. Indeed, there are several experimental observations that argue against a role for the Cpx response in promoting stress-induced cell death. First, the *cpxA** mutations were initially identified as suppressors of the toxicity caused by misfolded or mistargeted proteins in the cell envelope (4, 10, 17, 18). Second, the *cpxA** mutation confers resistance to amikacin, an AGA (19). Work presented in this study explains these seemingly paradoxical results and also suggests that all bactericidal drugs do not kill via the same mechanism.

MATERIALS AND METHODS

Strains, media, and culture conditions. All strains used in this study are isogenic derivatives of *Escherichia coli* MC4100 constructed by generalized transduction with bacteriophage P1 and are listed in Table 1 (22). To construct the *cpxA24 cpxR::kan* double mutant, a *cpxR::kan* insertion/deletion construct was introduced into the *cpxA24* strain by homologous

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TABLE 1 *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference(s) and/or source
Strains		
MC4100	F ⁻ <i>araD139</i> Δ(<i>argF-lac</i>)U169 <i>rpsL150 relA1 flbB5301</i> <i>deoC1 ptsF25 thi</i>	3
PTM163	MC4100 <i>cpxA24 zii::Tn10</i>	This study; 8, 16
PTM180	MC4100 <i>cpxA17 metF::kan</i>	This study; 18; lab stock
PTM197	MC4100 <i>cpxR M199A zii::Tn10</i>	This study; 13
PTM198	MC4100 <i>cpxR D51A zii::Tn10</i>	This study; lab stock
PTM202	MC4100 carrying pLD404	This study; 17
PTM208	MC4100(pLD404) <i>cpxR D51A zii::Tn10</i>	This study
PTM329	MC4100 <i>cpxA::kan</i>	This study; 20
PTM436	MC4100 <i>cpxR::spc</i>	This study; 15
PTM441	MC4100 <i>cpxA24 cpxR::kan</i>	This study
PTM467	MC4100 <i>baeS::Tn10-cam</i>	This study; 21
PTM472	MC4100 <i>rseA(Oc) nadB::Tn10</i>	Jaelyn Schwalm, unpublished data
Plasmid		
pLD404	NlpE overexpression plasmid	17

recombination using previously described methods (23). This *kan* insertion does not confer resistance to amikacin or gentamicin.

Media and chemicals. All strains were grown in Luria-Bertani (LB) broth or agar at 37°C unless otherwise indicated. Strains containing *cpxA** alleles were maintained on LB agar containing amikacin (Sigma) at 3 μg/ml. Plasmid-containing strains were grown in LB agar supplemented with ampicillin (Sigma) at 100 μg/ml.

Sensitivity assays. For drug sensitivity assays, strains were serially diluted and plated on LB agar containing increasing amounts of HU, gentamicin, or amikacin. CFU were enumerated after 24 h of growth at 37°C. Between 10 mM and 30 mM HU were required to observe significant cell death in plating assays. Sensitivity of strains was also determined by efficiency of plating (EOP). Strains were serially diluted and replica plated onto LB agar plates supplemented with either 20 mM HU, 3 μg/ml amikacin, or 5 μg/ml gentamicin.

RESULTS

Activation of the Cpx response confers resistance to AGAs and HU. Recent work has shown that null mutations in *cpxA* confer resistance to AGAs and HU (6, 24), and we have confirmed this result: strains carrying *cpxA::kan* are resistant to HU and the AGAs amikacin and gentamicin (Fig. 1). Previous studies suggested that this increased level of resistance occurs because HUs and AGAs induce the Cpx response, and this in turn increases production of ROS that cause cellular damage leading to cell death (5, 7, 24). Like many two-component sensors, CpxA functions not only as an autokinase and a kinase to activate CpxR but also as a phosphatase that maintains CpxR in an unphosphorylated state in the absence of inducing stimuli (8). Importantly, null mutations of *cpxA* disrupt all three activities. The absence of phosphatase activity in particular raises the possibility that the Cpx response will actually be upregulated in *cpxA* null mutants owing to the unrestrained phosphorylation of CpxR by small-molecule phospho donors or heterologous kinases (15, 16). This alternative explanation suggests that *cpxA* null mutations confer resistance not by inactivating but by activating the Cpx stress response. According to this view, the Cpx response is activated in *cpxA* null mutant strains,

and this activation does not contribute to cell death but rather prevents it.

To test the alternative explanation that Cpx activity actually protects cells from AGAs and HU, we took advantage of a constitutively active mutation that abolishes the phosphatase activity, but not the kinase activity, of CpxA (termed *cpxA**). Compared with null mutations in *cpxA*, these *cpxA** mutations confer resistance to HU and AGAs to comparable levels (Fig. 1B). Multiple *cpxA** alleles have been isolated, with all identified mutations clustering in three regions of CpxA: the periplasmic domain, the second transmembrane helix, and the region adjacent to the predicted site of autophosphorylation (8). The *cpxA24* allele, which encodes a variant of CpxA lacking 32 amino acids in a periplasmic loop, exhibits one of the highest levels of induction among all constitutive *cpxA* mutations (8) and is the *cpxA** allele used exclusively hereafter.

To confirm that the resistance to HU and AGAs conferred by a *cpxA* null mutation is due to the unrestrained phosphorylation of CpxR and the accumulation of CpxR~P, we determined drug sensitivity in a mutant strain lacking both CpxA and CpxR. A spectinomycin resistance cassette insertion in *cpxR* has polar effects on *cpxA* and is effectively null for both genes (13). The *cpxR::spc* allele does not ameliorate sensitivity to HU or AGAs (Fig. 1B). This mutant, which is null for both *cpxR* and *cpxA*, suggests that the resistance of the *cpxA* null mutant strain to HU and AGAs is dependent on the presence of functional *cpxR* and is due to the accumulation of CpxR~P. This demonstrates that the activation of the Cpx response increases survival in the presence of HU or AGAs.

To demonstrate that inactivating the Cpx response did not confer resistance to HU or AGAs, we used characterized point mutations in *cpxR* that abolish or reduce function of the response regulator. Mutating the phosphorylation site of CpxR (D51A) did not confer resistance to HU (Fig. 1). Similarly, mutating the putative DNA-binding site of CpxR (M199A) did not confer detectable resistance to HU (Fig. 1) (13). This further demonstrated that it is the activation of the Cpx response that confers resistance to HU and AGAs. Disrupting the response regulator effectively inactivates the Cpx response; survival in the presence of HU and AGAs in this context is similar to that observed for wild-type strains.

Resistance of *cpxA mutants is not due to pleiotropic effects.** The *cpxA** mutation causes many pleiotropic effects, some of which could be the consequence of hyperphosphorylated CpxR and some of which could result from the phosphorylation of heterologous response regulators by CpxA (10). To ensure that the increased resistance of *cpxA** to AGAs and HU was an on-pathway effect, mediated by physiologically normal levels of CpxR~P, we examined the impact of activating the Cpx response by other means.

Overproducing the outer membrane lipoprotein NlpE induces the Cpx response, albeit to a lower level than is observed in the *cpxA** mutant (13, 17). Consistent with our hypothesis, NlpE overproduction caused resistance to HU, but to a lesser degree than the *cpxA** mutation did (Fig. 2). This intermediate level of resistance suggests that the level of Cpx activation determines the degree of resistance to HU.

To show that resistance is conferred by downstream members of the Cpx regulon and is not the consequence of phosphorylation of a noncognate response regulator, we used tests of epistasis. In strains producing CpxR D51A, NlpE overproduction no longer

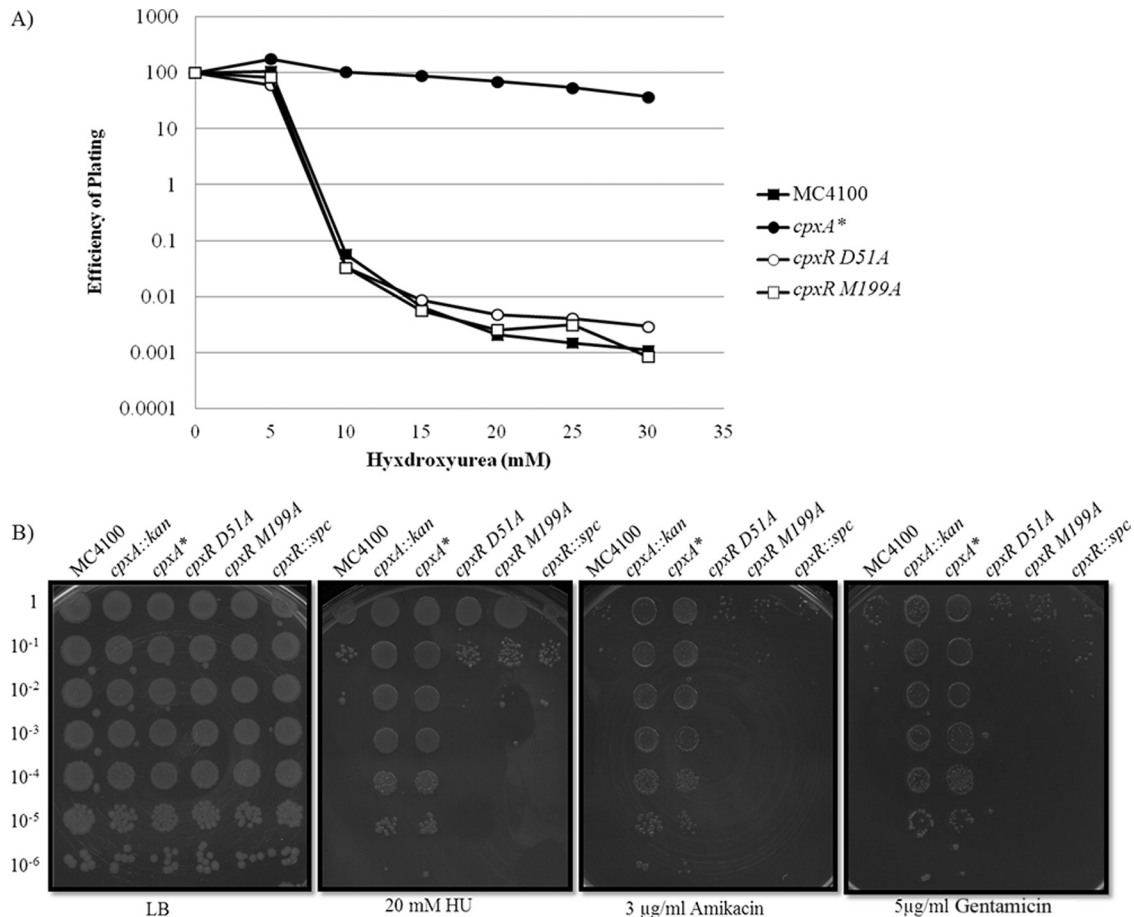


FIG 1 The *cpxA::kan* null and constitutive *cpxA** mutations confer resistance to hydroxyurea (HU) and aminoglycoside antibiotics (AGAs), while null mutations in *cpxR* do not. (A) Survival of strains was measured by enumerating CFU on LB agar plates supplemented with increasing concentrations of HU following overnight growth at 37°C. The strains tested include *E. coli* MC4100, PTM163 (*cpxA**), PTM197 (*cpxR M199A* [*cpxR* gene with an M-to-A change at position 199 that inactivates the putative DNA-binding motif of the CpxR response regulator]), PTM198 (*cpxR D51A* [*cpxR* gene with a D-to-A change at position 51 that mutates the phosphorylation site of the CpxR response regulator]). (B) Efficiency of plating (EOP) assay on LB agar plates supplemented with 20 mM HU, 3 µg/ml amikacin, or 5 µg/ml gentamicin grown at 37°C. Sensitivity was determined for the following strains: MC4100, PTM163 (*cpxA**), PTM197 (*cpxR M199A*), PTM198 (*cpxR D51A*), and PTM436 (*cpxR::spc*).

conferred resistance to HU (Fig. 2). Similarly, the ameliorating effect of *cpxA** is also dependent on functional *cpxR*; resistance to both HU and AGAs is abolished when a *cpxR* null mutation is introduced into a *cpxA** background (Fig. 3), a fact noted previously for amikacin resistance (25). Hence, removing the cognate response regulator of the Cpx response abolished the resistance conferred by an activated CpxA kinase. These results demonstrate that resistance to HU and AGAs conferred by the activated Cpx response is due to phosphorylation of CpxR and concomitant changes in Cpx regulon expression.

Multiple Cpx regulon members may be responsible for resistance. The Cpx response is one of the primary envelope stress response systems in *E. coli*, which play overlapping but distinct roles in monitoring the integrity of the cell envelope (11). Some important Cpx regulon members, such as *spy* and *degP*, are coregulated by other envelope stress responses. If any of these coregulated Cpx regulon members were involved in conferring resistance to AGAs or HU, then activation of the other stress response involved in their regulation should also cause resistance. The two envelope stress responses that coregulate Cpx regulon

members are σ^E and BaERS. As with *cpxA**, mutations that cause constitutive activation of the σ^E and BaERS stress responses have been isolated. The activity of σ^E is controlled by the anti- σ factor RseA. Degradation of RseA frees σ^E to become active, so null mutations in *rseA* cause constitutive activity of σ^E (26, 27). The BaERS response can be activated by a gain-of-function mutation *baeS::Tn10-cam* (21). However, upregulation of neither σ^E nor BaERS led to an increase in resistance to HU or AGAs (Fig. 4). This demonstrates that the increased resistance to HU and AGAs conferred by *cpxA** is specific to the Cpx response. It also suggests that the Cpx regulon member responsible for conferring resistance is not coregulated by the σ^E or Bae stress response systems.

Having clarified the relationship between Cpx activation and antibiotic resistance, we sought to identify the Cpx regulon member(s) responsible for resistance to HU and AGAs. Using a candidate approach, we individually constructed null mutations in published Cpx regulon members in both wild-type and *cpxA** strain backgrounds (Table 2). None of the mutations in the regulon members upregulated by the Cpx response decreased the resistance conferred by *cpxA**. Similarly, disrupting genes that are

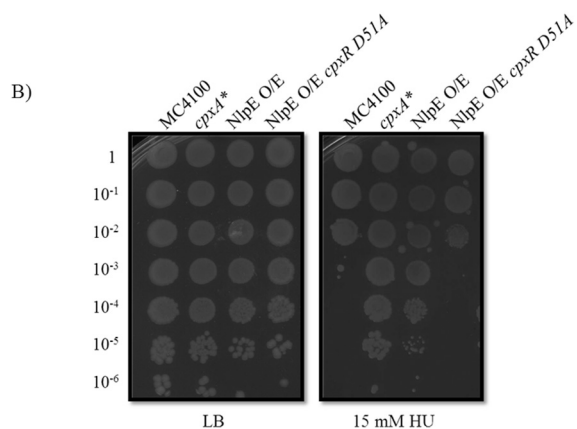
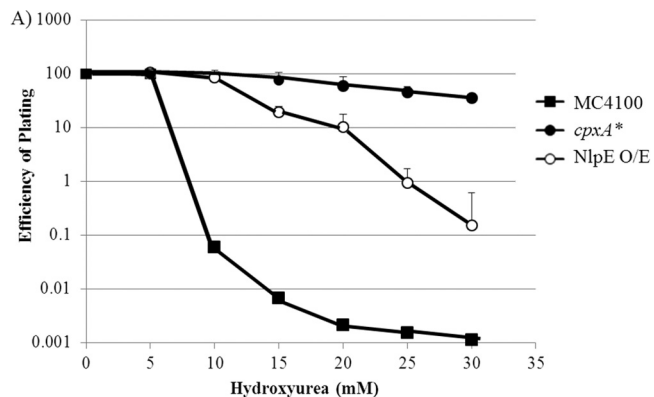


FIG 2 Overexpression of the outer membrane lipoprotein NlpE activates the Cpx response, which is sufficient to confer resistance to hydroxyurea (HU). (A) Survival of strains was measured by enumerating CFU on LB agar plates supplemented with increasing concentrations of HU. (B) EOP assay (as described in the legend to Fig. 1) of *E. coli* strains MC4100, PTM163 (*cpxA**), PTM202 (NlpE overexpressed [NlpE O/E]), and PTM208 (NlpE O/E *cpxR D51A*) on 15 mM HU.

known to be downregulated by the Cpx response in a wild-type background did not increase resistance to HU or AGAs. This seems to suggest that no single known regulon member is necessary to confer resistance to HU and AGAs, although it is possible that one regulon member may be sufficient.

The Cpx response confers resistance to some, but not all, bactericidal antibiotics. It has been suggested that all bactericidal antibiotics kill via the same mechanism (namely, the generation of ROS) to which the activation of the Cpx response contributes. Accordingly, we sought to determine whether activation of Cpx confers resistance to other antimicrobial compounds. Although *cpxA** confers resistance to HU and AGAs, this mutation does not increase resistance to fluoroquinolones, such as norfloxacin, or β -lactams, such as ampicillin (Fig. 5). Thus, the Cpx response confers resistance to a subset of toxic molecules and antibiotics, not to all bactericidal antibiotics. The fact that activation of the Cpx response causes resistance only to a subset of bactericidal antibiotics suggests that this class of antibiotic may not kill bacteria via a common mechanism.

DISCUSSION

We describe a novel role for the Cpx two-component regulatory system in combating the stress caused by AGAs and the small toxic

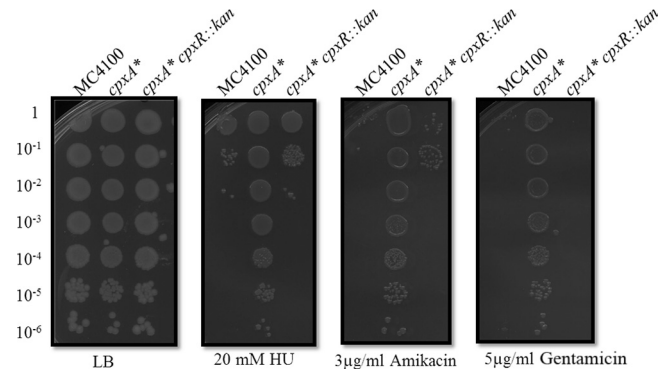


FIG 3 Deletion of *cpxR* in a *cpxA** background abolishes resistance to HU and AGAs. EOPs of strains MC4100, PTM163 (*cpxA**), and PTM441 (*cpxA* cpxR::kan*) on LB agar supplemented with 20 mM HU, 3 μ g/ml amikacin, or 5 μ g/ml gentamicin at 37°C.

molecule HU. Although it has been known for years that *cpxA** mutations confer resistance to amikacin (19), this phenotype was regarded as a consequence of the pleiotropic effects caused by hyperphosphorylation of the response regulator CpxR. Our results suggest that resistance to these toxic small molecules is directly mediated by the Cpx response. Our results also indicate that the combined actions of multiple Cpx regulon members are required to confer resistance to these toxic agents, as no single documented regulon member is epistatic to *cpxA**.

The Cpx response was first characterized as an envelope stress response triggered by misfolded or mistargeted outer membrane proteins. A processing-defective mutant of the maltoporin, LamBA23D (18) and a toxic “tribrid” fusion of LamB-LacZ-PhoA (18, 28) both cause extracytoplasmic toxicity that is suppressed by *cpxA**. The nature of the toxicity caused by LamB-LacZ-PhoA is unclear. However, LamBA23D is tethered to the inner membrane (IM) by its uncleaved signal sequence, and it is likely that it alters the IM in some deleterious way. Recent work has reinforced the likelihood that the Cpx response both senses and alleviates problems at the IM (4, 29, 30), and we suggest that this may be the primary role of this envelope stress response. In support of this idea, several known Cpx regulon members involved in protein quality control are located at the IM (4, 14, 29, 30).

A role for the Cpx two-component system in AGA resistance is consistent both with a role for this stress response in combating IM toxicity and with the current view that AGAs kill by promoting incorporation of mistranslated proteins into (and the consequent perturbation of) this compartment (2). It seems likely that the Cpx response combats the problem by promoting the degradation of mistranslated IM proteins. However, we do not yet understand how these aberrant proteins are selectively recognized, and we have not identified the protease that degrades them.

A role for Cpx in conferring resistance to HU is much less clear. The target of HU is class I ribonucleotide reductase (31, 32). Inhibition of this enzyme causes a fatal stalling of the replication fork owing to a lack of available deoxyribonucleotide triphosphate substrates (31, 32). It is not immediately obvious how a block to DNA replication could cause fatal problems at the IM. One theory posits that all bactericidal antibiotics, including AGAs, and toxic molecules like HU lead to IM perturbations that result in the generation of reactive oxygen species (ROS), which in turn cause cell

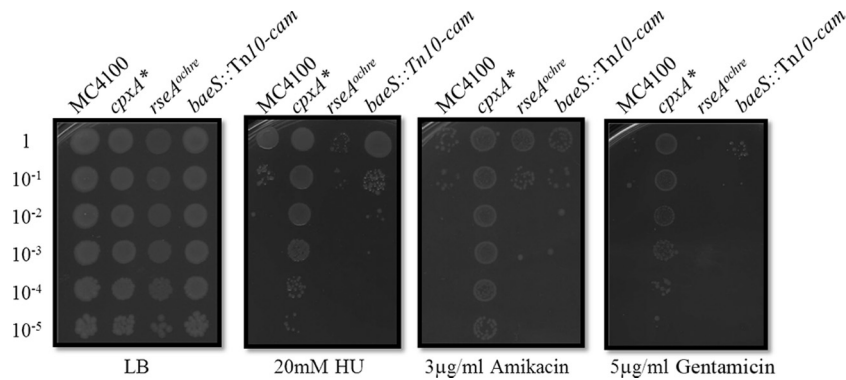


FIG 4 Activation of the σ^E or BaeRS stress responses does not confer resistance to HU or AGAs. EOP assays were performed on strains MC4100, PTM163 (*cpxA**), PTM472 (*rseA* with ochre stop codon [*rseA*(Oc)]), and PTM467 (*baeS*::*Tn10-cam*). *rseA* null mutations such as *rseA*(Oc) activate the σ^E stress response to its highest level. Similarly, a *Tn10-cam* insertion in *baeS* activates the Bae response. In neither case, does activation of these envelope stress response systems increase resistance to HU or AGAs.

death by damaging cellular macromolecules (5, 7). This theory predicts that a stress response that effectively combats these IM perturbations would confer some level of resistance to all bactericidal antibiotics and relevant toxic small molecules. However, we find that this is not the case. The Cpx response specifically confers resistance to AGAs and HU, but not to all bactericidal antibiotics. For example, the *cpxA** mutation does not confer detectable resis-

tance to either fluoroquinolones or β -lactams. This is true even though the fluoroquinolones, like HU, block DNA replication. Accordingly, we think it unlikely that all bactericidal antibiotics cause cell death by a similar, ROS-related mechanism.

While activation of the Cpx response confers resistance to AGAs and HU, wild-type cells with an intact Cpx response remain sensitive to both. Why are wild-type cells not protected by this stress response? In particular, AGAs like gentamicin induce the Cpx response (3), yet this induction does not render wild-type cells resistant. We suggest that this disparity reflects a race between Cpx induction and cell killing, both of which are caused by these

TABLE 2 Cpx regulon members tested for a role in resistance to AGAs and HU^a

Cpx regulon members tested

Upregulated genes

cpxP
rdoA-dsbA
psd
ydeH
ppiD
ycfS
yccA
spy
ybaJ
ung
yebE
yqjA
ppiA
ydeH
htpX
ftnB
degP
ompC
acrD
secA

Downregulated genes

efeU
mdtABCD
ompF
tsr
aroK
aer
csgDEFG
glpABC

^a The candidate list was derived from a previously published report (14).

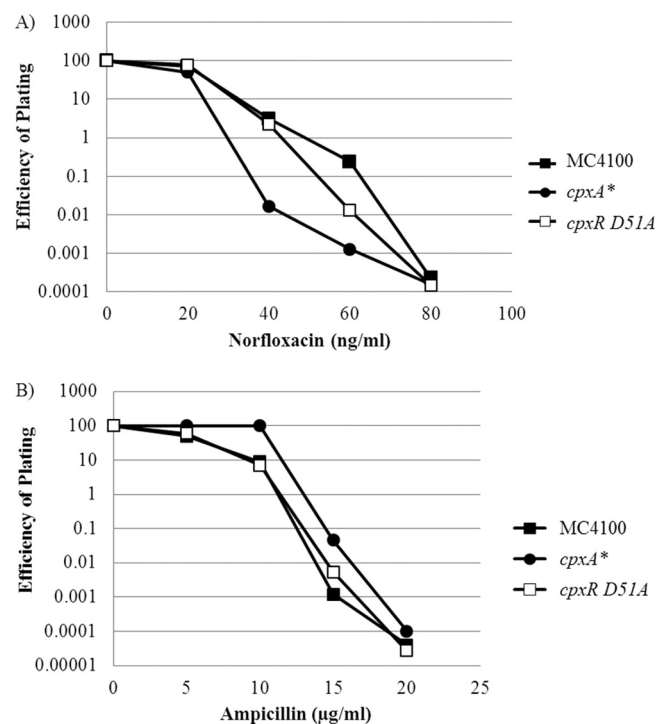


FIG 5 *cpxA** causes resistance to only certain classes of bactericidal antibiotics. (A and B) Sensitivity of strains MC4100, PTM163 (*cpxA**), and PTM198 (*cpxR D51A*) to fluoroquinolones such as norfloxacin (A) or β -lactams such as ampicillin (B) was performed as described in the legend to Fig. 1.

lethal agents. The harmful effects of these compounds reach lethal levels before Cpx induction reaches a sufficient level to confer resistance. In *cpxA** mutants, the continuous presence of high levels of the stress combative proteins prevent the damage caused by these toxic compounds from reaching lethal levels.

Both null mutations in *cpxA* and constitutively active mutations of *cpxA* are sufficient to confer resistance to AGAs and HU. The phenotypes of the *cpxA* null mutant, the *cpxA** constitutively active mutant, and the *cpxR* null mutants underscore the delicacy required when analyzing two-component signaling systems. Atypical two-component signal transduction systems are made up of a sensor histidine kinase and a cognate response regulator. Most of these histidine kinases are multifunctional, acting not only as kinases and autokinases but also as phosphatases. In the absence of an activating signal that stimulates autophosphorylation, sensor kinases act as phosphatases to maintain the proper balance of phosphorylated and unphosphorylated response regulator species (33, 34). Given the dual function of many sensor kinases as phosphatases, analyzing only the phenotypes of null mutations in the gene for the histidine kinase may not provide an accurate picture of the impact of inactivating a given two-component system. In order to understand the full effect of disrupting a two-component signal transduction system, null mutations of both the histidine kinase and response regulator should be analyzed in concert.

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