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Complex response of the CpxAR two-component system to β -lactams on antibiotic resistance and envelop homeostasis in *Enterobacteriaceae*

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Abstract

The Cpx stress response is widespread among *Enterobacteriaceae*. We have previously reported a mutation in *cpxA* in a multidrug resistant strain of *Klebsiella aerogenes* isolated from a patient treated with imipenem. This mutation yields to a single amino acid substitution (Y144N) located in the periplasmic sensor domain of CpxA. In this work, we sought to characterize this mutation in *Escherichia coli* by using genetic and biochemical approaches. Here, we show that *cpxA*^{Y144N} is an activated allele that confers resistance to β -lactams and aminoglycosides in a CpxR-dependent manner, by regulating the expression of the OmpF porin and the AcrD efflux pump, respectively. We also demonstrate the intimate interconnection between Cpx system and peptidoglycan integrity on the expression of an exogenous AmpC β -lactamase by using imipenem as a cell wall active antibiotic or inactivation of penicillin-binding proteins. Moreover, our data indicate that the Y144N substitution abrogates the interaction between CpxA and CpxP and increase phosphotransfer activity on CpxR. Because the addition of a strong AmpC inducer such as imipenem is known to causes abnormal accumulation of mucopeptides (disaccharide-pentapeptide, *N*-acetylglucosamyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamy-*meso*-diaminopimelic-acid-D-alanyl-D-alanine) in the periplasmic space, we propose these molecules activate the Cpx system by displacing CpxP from the sensor domain of CpxA. Altogether, these data could explain why large perturbations to peptidoglycan caused by imipenem lead to mutational activation of the Cpx system and bacterial adaptation through multidrug resistance. These results also validate the Cpx system, in particular the interaction between CpxA and CpxP, as a promising therapeutic target.

Importance

Cell wall biogenesis is the target of β -lactams, which are potent antibiotics for treating infections caused by Gram-negative bacteria. Resistance to β -lactams is increasing, particularly

39 enteric species such *Klebsiella* and *Enterobacter* spp., both members of the ESKAPE group of
40 problematic clinical pathogen. As a first step to better understand resistance emerged in these
41 organisms, we performed comparative genomics on series of clinical strains of *K. aerogenes*.
42 This allowed in the identification of a number of mutations, all located in genes involved in
43 envelope permeability control. Here, we characterized a gain-of-function mutation in the sensor
44 kinase CpxA. CpxAR is a two-component system that senses and responds to envelope stress.
45 Importantly, characterization of this mutation revealed that the cell wall recycling pathway
46 plays an important role in the resistance of this species when exposed to cell wall-targeting
47 antibiotics.

Introduction

The envelope of Gram-negative bacteria is a complex multilayered structure that is essential for cell viability and provides a protective barrier against environmental stresses. This structure consists of the inner and outer membranes, which are separated by the periplasmic space containing the cell wall or peptidoglycan (PG). PG is a covalently linked scaffold of glycan chains and short peptides that maintains the cell shape and resists against osmotic stress (1). It also provides an oxidizing environment where extracytoplasmic proteins can be stabilized by disulfide bonds (2). PG is synthesized and modified throughout the cell cycle by penicillin binding proteins (PBPs), including high-molecular-weight (HMW) PBPs, which polymerize and crosslink the glycan chains, and low-molecular-weight (LMW) PBPs, often referred to as PG hydrolases, which remodel existing chains (1). In *Escherichia coli* and closely related *Enterobacteriaceae*, the majority of LMW PBPs are D,D-carboxypeptidases — PBPs 5 and 6 and DacD that remove the terminal D-alanine from the pentapeptide chains — and/or endopeptidases — PBPs 4 and 7 and AmpH that cleave the peptide side chains and disconnect the glycan polymers (1). While HWM PBPs are essential (3), LMW PBPs are dispensable in standard laboratory conditions (4).

Bacteria have evolved sophisticated strategies to monitor and maintain the integrity of their envelope. In *E. coli*, several signal transduction pathways sense envelope alterations in the periplasm and control the expression of adaptive genes (5-9). One of these pathways is Cpx, a two-component system that consists of the transmembrane sensor kinase CpxA and the cytoplasmic response regulator CpxR. Although the molecular characteristics of the inducing signal(s) are unknown, activation of CpxA leads to autophosphorylation at a conserved histidine residue and phosphate transfer to a conserved aspartate in CpxR, which then remodels gene expression (10-13). Divergently transcribed from *cpxAR* is *cpxP*, which encodes a periplasmic protein that negatively modulates the activity of the Cpx system by interacting with the

periplasmic sensor domain (SD) of CpxA (14-16). The Cpx system was initially recognized to mediate adaptation to protein misfolding in the periplasm since the first identified genes of the Cpx regulon encoded envelope protein folding and degradation factors such as the protease/chaperone DegP, the peptidyl-prolyl isomerase PpiA and the disulfide oxidase DsbA (12, 17-19). Recent studies have linked the Cpx system to a wider range of other signals and adaptations (20-25), including the detection and repair of perturbations to the PG. First, the Cpx response is activated by the simultaneous deletion of LMW PBPs, resulting in the downregulation of flagellar genes and mobility defects (24). Second, genes of the Cpx regulon are upregulated in the presence of cell wall active antibiotics such as β -lactams (25). Third, Cpx controls the expression of PG-modifying enzymes such as the LdtD (YcbB) transpeptidase (11, 22, 23), which catalyzes unusual 3 \rightarrow 3 crosslinks and mediate resistance to β -lactams (26). We have previously reported a *cpxA* mutation in a clinical strain of *K. aerogenes* isolated from a patient with imipenem, a potent β -lactam antibiotic. This strain presented high-level β -lactam resistance associated with the loss of outer membrane porins and an increased β -lactamase activity (27). This mutation yields to a single amino acid substitution (Y144N) located in CpxA-SD. In this work, we characterized CpxA^{Y144N} as a gain-of-function CpxA* mutant. Biochemical analyses showed that this mutation abrogates the interaction between CpxA-SD and CpxP that normally keeps the system in a resting state in the absence of an inducing stress. Accordingly, it increases phosphotransfer events between CpxA and CpxR. Phenotypic analyses showed it conferred resistance to aminoglycosides and β -lactams by acting on identified Cpx regulon members. We also found that cell wall damage is able induce AmpC in a Cpx-dependent manner, suggesting an intimate interconnection between these two pathways.

Materials and Methods

Strains, media and plasmids

Bacterial strains and plasmids used this study are listed in a Supplementary Table. Unless otherwise specified, all assays were performed in the parental strain *E. coli* MC4100 (28). Single-gene-deleted strains were obtained from the KEIO collection (29), and provided by GE Healthcare Dharmacon. Deletions marked with kanamycin resistance cassettes flanked by FRT sites were introduced into MC4100 by P1 transduction and cured by using the FLP helper plasmid pCP20 for serial deletions (30). Genomic DNA of appropriate bacterial strains was prepared with the Wizard[®] Genomic DNA purification kit (Promega) according to the manufacturer's protocol. Construction of plasmids was performed by using the In-Fusion[®] HD Cloning kit (Clontech) according to the manufacturer's instructions. Point mutations in the *cpxA* gene were generated with the QuickChange II XL site-directed mutagenesis kit (Stratagen) according to the manufacturer's protocol. Oligonucleotides were provided by MWG Eurofins. Cloned DNA inserts were sequenced to confirm the presence of engineered mutations and the absence of other PCR generated mutations (MWG Eurofins). Bacteria were routinely grown at 30 or 37°C in Luria-Bertani (LB) broth or agar (Difco). When needed, ampicillin (100 µg/ml); chloramphenicol (30 µg/ml), kanamycin (50 µg/ml), L-arabinose (0.02-0.2%) or IPTG (1 mM) were added. All chemicals were purchased from Sigma.

β-galactosidase assays

β-galactosidase activity was assayed on late log-phase bacterial cultures as described by Miller (31). Experiments were independently repeated at least three times.

Antibiotic sensitivity assay

Strains susceptibility was determined by efficiency of plating (EOP). Strains containing the empty pBAD33 or *cpxA* derivative plasmids were first induced for 2 h at 37°C with L-arabinose, then serially diluted and plated onto LB agar plates supplemented with antibiotics. Aminoglycosides were amikacin (AMK, 3 µg/ml) and gentamycin (GEN, 5 µg/ml); β-lactams were ceftazidime (CAZ, 0.125 µg/ml) and imipenem (IMP, 0.25 µg/ml); others were fosfomycin (FOF); norfloxacin (NFX, 0.05 µg/ml); erythromycin (ERY, 5 µg/ml). EOP assays were independently repeated at least three times.

Overexpression and purification of CpxR-His

A single colony of BL21(DE3) transformed with pET24a+*-cpxR-His* was grown overnight at 37°C. The next day, the strain was subcultured 1:100 until reaching an OD₆₀₀ of 0.4 and protein expression was induced with 1 mM IPTG for 3 h at 37°C. The cells were harvested by centrifugation and lysed in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl supplemented with 20 mM imidazole by one passage through a cell disruptor (Constant Systems) at 2 kBars. After removal of the cell debris, CpxR-His in the supernatant was subjected to nickel affinity chromatography by using a HiTrap™ Chelating HP column (GE Healthcare Life Sciences) according to the manufacturer's instructions. Purified CpxR-His protein was passed through a HiTrap™ Desalting column (GE Healthcare Life Sciences) to remove imidazole (Figure S1). All protein purification steps were operated by an AKTA protein purification system (Amersham Biosciences).

Overexpression of CpxA-His and CpxA^{Y144N}-His and preparation of inner membrane vesicles

Single colonies of C41(DE3) transformed with pET24a+*-cpxA-His* or pET24a+*-cpxA^{Y144N}-His* were grown overnight at 37°C. Strains were subcultured 1:100 at 37°C until reaching an OD₆₀₀

of 0.4 and protein expression was induced with 1 mM IPTG for 3 h at 30°C. Cells were harvested by centrifugation and lysed in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl by one passage through a cell disruptor (Constant Systems) at 2 kBars. This mainly yields to inside-out inner membrane vesicles (IMVs) (32). After removal of cell debris by low-speed centrifugation ($7,000 \times g$, 20 min, 4°C), the supernatant was ultracentrifuged ($100,000 \times g$, 60 min, 4°C) to collect whole-cell envelopes. These were separated into inner and outer membranes by ultracentrifugation through a 30 to 55% (wt/vol) sucrose density gradient as previously described (33). After SDS-PAGE, fractions corresponding inner membranes enriched in CpxA-His or CpxA^{Y144N}-His were pooled, resuspended in phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol (vol/vol), 2 mM dithiothreitol, 50 mM KCl, 5 mM MgCl₂) after removal of sucrose (Figure S2), and used in phosphorylation assays immediately after preparation.

Analyzes of CpxA autokinase, kinase and phosphatase activities *in vitro*

Purified IMVs containing CpxA-His or CpxA^{Y144N}-His were diluted in phosphorylation buffer at a concentration of approximately 20 mg/ml. Autophosphorylation was tested by using the ADP-Glo™ kinase assay (Promega) according to the manufacturer's instructions (see also supplementary Materials and Methods and Figure S3).

For testing kinase and phosphatase activities, CpxA-His and CpxA^{Y144N}-His in IMVs were allowed to autophosphorylate in the presence of ATP, then pelleted by centrifugation and washed twice in phosphorylation buffer to remove residual ATP. For kinase activity assays, CpxR-His was added at a concentration of 1.5 μ M (CpxA:CpxR molar ratio \sim 1:3) and incubated for 30 min at room temperature. Purified CpxR-His was labeled with 50 μ M acetyl phosphate and used as a positive control (see Supplementary Materials and Methods). All reactions were stopped by the addition of 5 \times Laemmli buffer. Phosphoproteins were separated

on a 50 μ M Phos-tagTM (Wako Laboratory Chemicals) 12%-PAGE in the presence of 100 μ M MnCl₂ and detected by staining with Coomassie Blue R250. For phosphatase activity assays, CpxR~P (from acetyl phosphate labeling) was added and mixtures were incubated for 30 minutes at room temperature. Released phosphate levels were measured by using the phosphate colorimetric assay kit (Sigma) according to the manufacturer's instructions (see also Supplementary Materials and Methods and Figure S4).

Phos-tag analysis of the CpxR phosphorylation *in vivo*

The presence of phosphorylated CpxR (CpxR~P) in lysates of BL21(DE3) co-transformed with pET24a+*-cpxR-His* and pBAD33-*cpxA* or pBAD33-*cpxA*^{Y144N} was assessed by coupling Phos-tagTM PAGE to immunoblotting with HRP-conjugated 6-His Epitope Tag monoclonal antibody (1:2,000) (Sigma). For this, single bacterial colonies were grown overnight at 37°C. The next day, the strains were subcultured 1:100 until reaching an OD₆₀₀ of 0.4. CpxA expression was first induced with 0.2% L-arabinose during 2 h at 37°C, then 1 mM IPTG was added to induce CpxR-His expression for another 3 h at 37°C. 2×10⁹ cells were harvested by centrifugation and cell pellets were vigorously resuspended in 1.2 M formic acid. Whole-cell lysates were solubilized by the addition of 5 × Laemmli buffer and neutralized by the addition of 5 M NaOH. Samples (10 μ l equivalent to 0.2 OD units) were loaded and separated on a 25 μ M Phos-tagTM (Wako Laboratory Chemicals) 12%-PAGE in the presence of 50 μ M MnCl₂. Before transfer onto a PVDF membrane, the gel was washed with transfer buffer supplemented with 1 mM EDTA for 10 min to remove excess of MnCl₂.

Bacterial two-hybrid assay

The interactions between CpxA-SD and CpxP were studied by using a bacterial adenylate cyclase two hybrid (BATCH) assay (Euromedex). The target proteins CpxA and CpxP of *K.*

aerogenes were separately fused to the C- termini of the T25 and T18 domains, respectively, as previously described (14). To estimate the interaction between CpxA and CpxP, DHM1 *cya* cells were transformed with the recombinant plasmids pKT25-*cpxA/cpxA*^{Y144N}, and pUT18C-*cpxP*. For detection of lactose metabolizing clones, bacteria were grown overnight at 30°C. The next day, 10 µl of each culture was spotted onto LB agar supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal, 40 µg/ml) and 0.5 mM IPTG, and incubated for 24 h at 30°C. DHM1 cells were transformed with pKT25-*zip* and pUT18C-*zip* as a positive control (Blue, Lac⁺), and with empty pKT25 and pUT18C plasmids as a negative control (White, Lac⁻).

Other protein methods

Whole cell envelopes were prepared as described above and resuspended in 20 mM HEPES-NaOH buffer (pH 7.2). All samples were diluted in Laemmli buffer and heated for 5 min at 100°C before loading. Samples corresponding to 0.2 OD units were separated on 10% SDS-PAGE. To better resolve OmpF and OmpC 4 M urea was added to the running gel. Proteins were either visualized after staining with Coomassie Brilliant Blue R250 or transferred onto PVDF Blotting membranes (Bio Rad) as described (27). Protein quantification was performed by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Quantification of AmpC β-lactamase activity

β-lactamase activity was assessed by using a microtiter plate nitrocefin hydrolysis assay as previously described (34). All chemicals were from Sigma, excepting nitrocefin (Merck) and IMP (Sequoia chemicals Ltd.).

Statistical analyses

220 All assays were repeated at least three times. Results are presented on the basis of the calculated
221 means with standard deviations. When appropriate, P -values were calculated and indicated with
222 asterisks as followed: *** $P \leq 0.01$, ** $0.01 < P \leq 0.05$, * $0.05 < P \leq 0.1$.

Results

cpxA^{Y144N} is an autoactivated *cpxA* allele (*cpxA*^{*}) that confers antibiotic resistance to β -lactams and aminoglycosides

In a previous study, we have reported the evolution of antibiotic resistance in four *K. aerogenes* strains, which were sequentially isolated during the clinical course of a patient under treatment with imipenem. Comparative genomics of these isolates — herein referred as to P1 to P4 — showed that P4 carried mutations in *omp36* (encoding the *E. coli* OmpC ortholog), leading to a premature stop codon, and in *cpxA*, producing a Y144N substitution located in CpxA-SD. Interestingly, we observed that P4 did not produce Omp35 (the *E. coli* OmpF ortholog), although the gene sequence was not altered, and presented increased β -lactamase activity, which could both contribute to high level β -lactam resistance in this strain (27). In order to elucidate the specific effects of the *cpxA*^{Y144N} mutation on antibiotic resistance, wild-type *cpxA* and *cpxA*^{Y144N} were amplified from *K. aerogenes* G7 and P4, respectively, cloned into the pBAD33 plasmid under the control the *P_{ara}* arabinose inducible promoter and expressed in *E. coli* K12 MC4100 Δ *ara174*. Several gain-of-function *cpxA*^{*} mutations have been previously isolated and characterized. Among these, *cpxA104*(R33C) and *cpxA101*(T253P) were generated by site-directed mutagenesis and used as positive controls (13). The effects of the CpxA mutants as well as that of the overexpression of the lipoprotein NlpE, a well-characterized Cpx-inducing cue (35), were tested on a chromosomal *ppiA-lacZ* fusion whose activity directly depends on the CpxAR system (17, 36). Consistent with previous results, *ppiA::lacZ* activity increased 2-3 fold in response to the overexpression of NlpE, CpxA^{R33C} and CpxA^{T253P}. Similar activation was observed when the CpxA^{Y144N} but not wild-type CpxA was overexpressed, suggesting that *cpxA*^{Y144N} is a new constitutively activated *cpxA*^{*} allele (Figure 1).

Antibiotic resistance of *cpxA mutants is due to specific activation of CpxR**

Several recent works suggested that the CpxAR is involved in antibiotic resistance and laboratory and clinical strains of enterobacteria (11, 22, 37-41). Null mutations of *cpxA* — thus lacking both kinase and phosphatase activities — have been shown to activate the Cpx response resulting from the phosphorylation of CpxR by small molecule phosphodonors and to protect cells against external stress caused by hydroxyurea and aminoglycosides (42, 43). Here, we took advantage of the dominant effect of the plasmid-expressed *cpxA** mutations to analyze their effect on antibiotic resistance by using an efficiency of plating assay in a wild-type background. Compared with the empty plasmid or wild-type *cpxA*, *cpxA** mutations conferred some resistance to several antibiotic classes including β -lactams, aminoglycosides and fosfomycin, but not to fluoroquinolones and macrolides (Figures 2A and S5).

*cpxA** mutations could cause pleiotropic effects resulting from the phosphorylation of heterologous response regulators other than CpxR. We eliminated this possibility by showing that antibiotic resistance is abolished when a *cpxR* null mutation is introduced in the presence the of a *cpxA*^{Y144N} allele (Figure 2B). Altogether, these results demonstrate that antibiotic resistance conferred by the Cpx response is due to specific phosphorylation of CpxR and concomitant changes in the expression of genes that belong to the Cpx regulon.

Multiple Cpx regulon members may be responsible for antibiotic resistance

Having clarified the relationship between the Cpx response and antibiotic resistance, we sought to identify Cpx regulon members responsible for this phenotype by using a candidate approach. This consists in generating null mutants of genes that are known to be up-regulated by Cpx or overproducing genes that are known to be down-regulated by Cpx, then looking for a decrease in resistance in the presence of *cpxA**. AcrD is an inner membrane efflux pump that functions with outer membrane TolC to expel aminoglycosides (44). EOP assays showed that disruption

of *acrD* or *tolC* decreased resistance to aminoglycosides in a *cpxA** background, suggesting that resistance to this class of antibiotics is somehow attributable to an elevated expression of *acrD* (Figures 3A). Activation of Cpx decreases *ompF* expression. This occurs at directly at the transcriptional level through the binding of CpxR~P to *ompF* promoter and indirectly through the activation of MzrA that connects CpxAR to EnvZ-OmpR (11, 12, 36, 45). We confirmed the effect of Cpx activation on porin expression by Western blot analysis of whole cell lysates after transient overproduction of NlpE or CpxA* (Figure 3B). In addition, EOP assays showed that plasmid-expression of Omp35, the OmpF ortholog of *K. aerogenes*, decreased resistance to β -lactams but not to aminoglycosides in a *cpxA** background (Figure 3C). Interestingly, the replacement of OmpF to OmpC is often associated with an increased resistance to β -lactams in clinical isolates of problematic enteric bacteria such as *K. aerogenes* and *K. pneumoniae* (27, 46, 47). These data suggest that OmpF is the preferred route for the uptake of β -lactams and that the Cpx-dependent inhibition of *ompF* expression is responsible for resistance to this class of antibiotics. Although not tested here, Kurabayashi and colleagues showed that activation of Cpx down-regulated *glpT* and *uhpT*, which encode transporters for fosfomycin together with their native substrates glycerol-3-phosphate and glucose-6-phosphate (39).

***In vitro* and *in vivo* biochemical characterization of CpxA^{Y144N}**

Like many bacterial sensor kinases, CpxA possesses three enzymatic activities that are critical for signal transduction: autokinase, response regulator kinase and phosphorylated response regulator phosphatase (8). To compare the *in vitro* biochemical activities of CpxA to that of CpxA^{Y144N}, we used IMVs enriched in CpxA-His or CpxA^{Y144N}-His. Autokinase activity was quantified using the ADP-GloTM kinase assay. This assay is performed in two steps: after the kinase reaction in the presence of ATP, the ADP-GloTM reagent is added to terminate the kinase reaction and deplete the remaining ATP then the kinase detection reagent is added to convert

ADP into ATP. Newly synthesized ATP is measured using a luciferase/luciferin reaction. The light generated correlates to the amount of ADP generated in the kinase assay, which is indicative of the kinase activity. When the two proteins were incubated with ATP, similar levels of ADP were generated (Figure 4A). This indicated that both proteins catalyze autophosphorylation and that the Y144N substitution does not modify the autokinase activity. Kinase activities were tested by using IMVs and purified CpxR-His. Phosphorylated CpxR was visualized by using the Phos-tag™ PAGE system. CpxR can be phosphorylated *in vitro* by small phosphodonor molecules such as acetyl phosphate (48) and used as a positive control (Figure 4B). However, the Y144N substitution does not increase kinase activity of CpxA on CpxR. Phosphatase activities were tested by using IMVs and phosphorylated CpxR-His. However, similar amounts of phosphate were measured by using a phosphate colorimetric assay. (Figure 4C). Altogether, results from *in vitro* assays suggest CpxA and CpxA^{Y144N} behave similarly.

Overproduction of the small periplasmic CpxP protein inhibits the Cpx response and prevents Cpx activation (49). This effect depends on an intact SD as *cpxA24*, which encodes a variant of CpxA lacking 32 amino acids in its periplasmic loop, is a strong *cpxA** allele (13). It has been shown that CpxP interacts with CpxA-SD in unstressed cells and that CpxP inhibits CpxA autophosphorylation in reconstituted proteoliposomes by direct interaction (14, 16). A peptide array indicated that the C-terminal region of CpxA-SD (E₁₃₈DNYQLYLIRPASSSSQSDEINLLFD₁₆₂) might play an important role for the interaction with CpxP (15). Because this region comprises Y144 (underlined), we investigated the impact of the Y144N substitution on the ability of CpxA-SD to interact with CpxP using a bacterial two-hybrid assay (BACTH). BACTH is based on the functional complementation of the T18 and T25 domains of the adenylate cyclase of *Bordetella pertussis*, resulting in cAMP synthesis and activation of the lactose operon in an appropriate adenylate cyclase deficient reporter strain

(50). A previous study showed efficient interaction when CpxA-SD (P₂₈-P₁₆₄) and signal sequenceless CpxP were fused to the N- or the C-termini of T25 and T18 domains (14). Here, *E. coli* DHM1 was co-transformed with CpxP-T18 and T25-CpxA-SD or T25-CpxA^{Y144N}-SD and transformants were tested for their ability to metabolize Xgal on supplemented LB plates. Co-expression of CpxP-T18 and T25-CpxA-SD but not T25-CpxA^{Y144N}-SD showed blue colonies (Figure 4D). This suggests that the Y144N substitution disrupts the interaction with CpxP and likely explains the constitutive activation of *cpxA*^{Y144N} *in vivo*. In order to confirm this, we analyzed the phosphorylation status of CpxR in intact *E. coli* BL21(DE3) co-transformed with pBAD33-*cpxA* or pBAD33-*cpxA*^{Y144N} and pET24a+-*cpxR*-His by using the Phos-tag PAGE system and immunoblotting with anti-His antibodies. Here, phosphorylated CpxR-His could only be detected in cells expressing the mutant but not the wild-type CpxA (Figure 4E).

Interplay between the Cpx system, PBPs and the cell-wall active antibiotic imipenem for AmpC β -lactamase induction

Enterobacter and *Klebsiella* spp. but not *E. coli* encode an inducible chromosomal AmpC β -lactamase (51-53). Although AmpC is produced at very low levels wild-type strains cultured in standard laboratory conditions, its expression is highly inducible in the presence of β -lactams (AmpC inducers) such as cefoxitin and imipenem. The AmpC induction mechanism is complex and involves at least three proteins that are involved in the PG recycling pathway: AmpR (transcriptional regulator of the LysR family), AmpD (a cytoplasmic amidase) and AmpG (an inner membrane permease) (Figure 6) (54-60). In the current model and under non-inducing conditions, muropeptides from normal PG degradation are removed from the cell wall and transported via AmpG into the cytoplasm where they are cleaved by AmpD to generate free peptides and recycled into the cell wall synthesis. Under inducing conditions, AmpD is unable

348 to process high levels of mucopeptides, which interact with AmpR, creating a conformation that
349 activates the transcription of *ampC*. In clinical strains, high level resistance to β -lactams
350 (especially to third-generation cephalosporins) is due to the derepression of *ampC* mainly
351 resulting from *ampD* mutations (59-62). Results described above showed that expression of
352 *cpxA** in *E. coli* provided some resistance to β -lactams but not to the level to that observed in
353 *K. aerogenes* P4, suggesting the presence an additional mechanism. As compared to strains P1-
354 P3, *K. aerogenes* P4 shows an increased (~8.5 fold) β -lactamase activity in non-inducing
355 conditions (Figure 5A). This activity was attributed to a derepressed AmpC, as it could be only
356 inhibited by boronic acid (inhibitor of class C β -lactamases such as AmpC) but not by a mixture
357 of tazobactam and clavulanic acid (inhibitors of class A β -lactamases such as TEM-3) (Figure
358 5B). Because previous whole genome analysis did not reveal mutations in *ampD* (27), we
359 examined the effect of the *cpxA*^{Y144N} mutation on the expression of AmpC. The *ampR-ampC*
360 operon from *K. aerogenes* ATCC13048 was cloned into the pACYC184 vector and co-
361 expressed with *cpxA* or *cpxA*^{Y144N} in *E. coli* C41(DE3). Production of CpxA or CpxA^{Y144N} was
362 induced with IPTG for 1 h then cells were treated with imipenem or not. AmpC levels were
363 monitored by using a nitrocefin degradation assay. Although the two strains were responsive to
364 imipenem, the expression CpxA^{Y144N} showed a significant (~5 fold) increase in AmpC activity
365 in the absence of imipenem. This suggested that the Cpx system could be an alternative pathway
366 to induce the expression of *ampC*. To test this hypothesis, different genetic backgrounds of *E.*
367 *coli* K12 MC4100 were transformed with pACYC184-*ampRC* and AmpC levels were analyzed
368 as above. As expected, AmpC activity was highly induced in the presence of imipenem in a
369 wild-type background (~12 fold activation) (Figure 5D). In contrast, the absence of AmpG or
370 CpxAR decreased AmpC induction to only 3.5-4.5 fold and the triple mutant showed no
371 induction at all, suggesting that both the AmpG permease and the Cpx system are required for
372 a maximal induction (Figure 5D).

373 The parental strain *E. coli* CS109 and the derivative mutant CS448.3 have been described by
374 K. Young and colleagues in a large study that surveyed the impact of PBPs on bacterial motility
375 (24). Interestingly, the simultaneous removal of PBP4, PBP5, PBP7 and AmpH in CS448.3
376 comprised migration as a result of the activation of the Cpx stress response, which inhibits the
377 production of flagellar proteins. Figure 5E shows AmpC activity is increased CS448.3 as
378 compared to that in CS109. In addition, inactivation of *cpxA* in CS448.3 reduces AmpC activity
379 to the level of the parental strain. While *ampG* is still essential for full AmpC induction, this
380 indicates that activation of Cpx is connecting PBP loss and AmpC activation. This is also
381 consistent with studies that previously linked these two events (63, 64). Altogether, these results
382 demonstrate that the inactivation of PBPs, either by gene deletion or by inhibitory interactions
383 with imipenem, can be sensed by the Cpx system in the periplasmic space and that Cpx
384 activation is providing an adapted response through *ampC* induction.

Discussion

Cpx, together with Bae, Rcs, σ^E , and Psp are usually considered as the main envelope stress response systems in *E. coli* (9). There is convincing evidence that these systems, especially Cpx, can sense and respond to cell wall damage, while the molecular signals remain mostly unknown. Here, we provide functional characterization of the first gain-of-function mutation of CpxA, which was identified in multidrug resistant clinical strain of *K. aerogenes* (27). We demonstrated this mutation confers resistance to β -lactams and aminoglycosides by regulating the expression of effector genes involved in their uptake (*ompF*) or extrusion (*acrD*). Together with other previous studies, these data enlighten the role of the Cpx system in antibiotic resistance in laboratory and clinical strains of enterobacteria (11, 22, 37-41). This mutation also shed new light on how Cpx might sense and respond to PG damage induced by β -lactam treatment through *ampC* regulation and a putative positive feedback loop involving the lytic murein transglycosylase Slt (Figure 6).

β -lactams are a potent class of antibiotics used for treating infections caused by Gram-negative bacteria. According to the most widely accepted model, cell wall damage that follows β -lactam treatment results from the drug-induced imbalance of the cell wall biosynthesis machinery, between the murein synthetases and hydrolases. β -lactams bind to PBPs with different affinities, thus were considered to have different consequences on cell morphology and viability (65). Noteworthy, carbapenems, including imipenem and meropenem were shown to saturate simultaneously all four essential HMW PBPs (PBP1a, PBP1b, PBP2 and PBP3) as well as LMW PBPs 4, 5 and 6 at low concentrations, leading to rapid cell killing. In an elegant study, Cho et al. showed that β -lactams (mecillinam and cephalexin) induce a toxic malfunctioning of the cell wall biosynthesis machinery, in which Slt acts a quality control enzyme by degrading uncrosslinked glycan chains (66). Interestingly, *slt* belongs to the Cpx regulon (11).

409 Previous transcriptomic studies have revealed that β -lactams lead to the expression of genes
 410 controlled by envelope stress response systems. Indeed, β -lactams specifically targeting the
 411 essential bifunctional PBPs (PBP1a and PBP1b, cefsulodin) and the monofunctional PBP2
 412 (mecillinam), used alone or in combination, increased the expression of genes regulated by the
 413 Rcs, Cpx, σ^E , and Bae systems. Interestingly, Rcs was the only response that was activated in
 414 all tested conditions, suggesting this system preferentially responds to PG damages as compared
 415 to the others (25). A22, a drug that, like mecillinam, targets the cell elongasome, specifically
 416 activates the Rcs response in an RcsF-dependent manner (67). In addition, mecillinam or A22
 417 — targeting PBP2 or MreB, both part of the elongasome — and cephalixin — targeting PBP3
 418 part of the divisome — led to a 2-fold increase in Cpx activation (22). Endogenous signals,
 419 such as genetically blocking a critical step in PG synthesis, can also activate envelope stress
 420 responses. In *E. coli*, the simultaneous deletion of specific PBPs (PBP4, 5, 6 and AmpH) led to
 421 a reduction in motility that was dependent on the activation of both the Cpx and Rcs systems
 422 (24). Interestingly, the activation of the Rcs system was dependent on the activation of the Cpx
 423 system, but not vice versa, suggesting Cpx acts upstream of Rcs.

424 Many Gram-negative bacteria encode an inducible chromosomal AmpC β -lactamase, produced
 425 in response to specific β -lactam treatment such as imipenem or ceftazidime. In enterobacteria,
 426 expression of *ampC* is controlled by the transcriptional regulator AmpR (51-53, 56). Mutants
 427 defective of the cytoplasmic AmpD amidase involved in normal cell wall recycling overexpress
 428 *ampC* even in the absence of inducer (54, 59-62). This and others findings have led to the
 429 conclusion that β -lactam treatment generates major cell wall damage resulting in the
 430 accumulation of anhydro-disaccharide-pentapeptide in the periplasm, which is transported into
 431 the cytoplasm by the AmpG inner membrane permease and converted to anhydro-
 432 monosaccharide-pentapeptide, serving as a signal for *ampC* induction (54-60). Cho *et al.*
 433 showed that Slt is responsible for the degradation of uncrosslinked nascent PG into anhydro-

434 disaccharide-pentapeptide when cells are treated with β -lactams (66, 68). Moreover, previous
435 studies have shown the importance of Slt on *ampC* induction (69, 70) and the hypersensitivity
436 of *slt*-defective mutants to β -lactams (71). Bridging all these data together makes the Cpx
437 system emerge as a central player in bacterial physiology upon β -lactam treatment. Cpx first
438 senses PG end products arising from the concomitant inactivation of PBPs and degradation by
439 Slt; Cpx tunes an adapted response by modulating the expression of a number of cell wall
440 modifying enzymes. Among these are Slt and LdtD (11, 23). LdtD is one of the five L,D-
441 transpeptidases of *E. coli* that catalyze non-canonical 3 \rightarrow 3 peptide crosslinks. The Cpx-
442 dependent or independent expression of *ldtD* was later shown to substantially increase
443 resistance to β -lactams (22, 26). Although the inactivation of *ldtD* in the *cpxA*^{Y144N} background
444 did not significantly affect bacterial resistance to β -lactams using EOP assays, we cannot rule
445 out that *ldtD* expression was altered. In the near future, analyses of the PG composition in
446 untreated and imipenem-treated wild-type and Δ *slt* cells are necessary to investigate whether β -
447 lactams that induce large and little amounts of AmpC β -lactamase produce the same PG
448 turnover. We have observed that imipenem is able to activate the Cpx response by using a
449 specific *ppiA-lacZ* reporter fusion (Figure S6). Experiments are now needed to investigate a
450 feedback mechanism *in vivo* by using *slt*-defective and -overexpressing cells. Additionally, an
451 *in vitro* assay will be valuable to test Cpx activation and identify the nature of (the) signaling
452 muropeptide(s).

453 According to the proposed membrane topology of CpxA, amino acid Y144 is located in its
454 periplasmic SD (13). We have shown that this mutation produced constitutive phosphorylated
455 CpxR. This most likely results from the loss of the periplasmic interaction between CpxA and
456 CpxP, an accessory inhibitory protein, which holds the system in a resting state in the absence
457 of stress (14-16, 49). One can suspect that imipenem therapy can select gain-of-function *cpxA**
458 mutations that mimics the presence of an inducing cue. It is also possible that activation of the

459 Cpx system is maintained in this background *via* the Slt feedback loop. It has been proposed
460 that Slt inhibitors may be used in combination therapies with β -lactams (70). Similarly,
461 inhibiting Cpx function would be an effective way to keep AmpC β -lactamases silent and
462 preserve the activity of potent β -lactams against clinically problematic enterobacteria.
463

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Figure legends

Figure 1. *cpxA*^{Y144N} is a *cpxA** allele. Effect of CpxA, CpxA^{Y144N}, CpxA^{R33C}, CpxA^{T252P} and NlpE on *ppiA* expression by assaying β-galactosidase activities of a chromosomal *lacZ* fusions.

Figure 2. *cpxA** mutations confer resistance to β-lactams, aminoglycosides and fosfomycin. (A) Efficiency of plating (EOP) assay on LB agar plates supplemented with 3 μg/ml amikacin (AMK), 2.5 μg/ml gentamicin (GEN), 0.5 μg/ml imipenem (IMP), 0.125 μg/ml ceftazidime (CAZ) or 4 μg/ml fosfomycin (FOF) and grown at 37°C. Susceptibility was determined for the following strains: RAM1292 transformed with pBAD33, pBAD33-*cpxA*, pBAD33-*cpxA*^{Y144N}, pBAD33-*cpxA*^{R33C}, or pBAD33-*cpxA*^{T252P}. (B) Deleting *cpxR* in RAM1292 pBAD33-*cpxA*^{Y144N} abolished antibiotic resistance.

Figure 3. The Cpx response regulates genes that impact antibiotic resistance. (A), (C): EOP assays. (A) Deleting the *acrD* or *tolC* efflux pump components decreased resistance to aminoglycosides in RAM1292 pBAD33-*cpxA*^{Y144N}. (B) OMP expression level is altered by *cpxA*^{Y144N}. RAM1292 transformed with pBAD33, pBAD33-*cpxA*, pBAD33-*cpxA*^{Y144N}, pBAD33-*cpxA*^{R33C} and pBAD33-*cpxA*^{T252P} were cultured and induced for 2 h with 0.2% L-arabinose. OMPs were detected from whole cell lysates using polyclonal antibodies directed against OmpF/C/A as described in the Materials and Methods section. Equivalent of 0.2 OD₆₀₀ units were loaded per well. OmpF/C levels were normalized using the OmpA intensity signal. (C) Plasmid expression of the OmpF ortholog of *E. aerogenes* Omp35 restores the susceptibility of RAM1292 pBAD33-*cpxA*^{Y144N} to β-lactams.

Figure 4. Enzymatic activities of CpxA^{Y144N}. (A) CpxA-His and CpxA^{Y144N}-His exhibit similar autophosphorylation activities. Autophosphorylation was tested by using the ADP-Glo™ kinase assay on inner membrane fractions as described in the Materials and Methods section. Reactions were performed with 5 μl IMVs in the presence of 100 μM or 1 mM ATP for 30 min at room temperature. The two step assay includes the removal of the remaining ATP then the

687 simultaneous conversion the ADP produced by the kinase reaction to ATP and into light by a
688 luciferin/luciferase reaction. Bioluminescent signals are proportional to the ADP produced and
689 the activity of the kinase. The amount of ADP produced from each reaction was calculated by
690 using ATP to ADP standard curves. **(B)** CpxA-His and CpxA^{Y144N}-His exhibit similar *in vitro*
691 kinase activity on CpxR. CpxA-His or CpxA^{Y144N}-His in enriched IMVs (5 μ l) was
692 phosphorylated under standard conditions then incubated in the presence of 1.5 μ M purified
693 CpxR-His for 30 min. Samples were analyzed by using Phos-tagTM PAGE fractionation. **(C)**
694 CpxA-His and CpxA^{Y144N}-His exhibit similar *in vitro* phosphatase activity on CpxR. CpxR-His
695 was phosphorylated with acetyl-phosphate as described in Materials and Methods.
696 Phosphorylated CpxR-His was incubated in the presence of CpxA-His or CpxA^{Y144N}-His
697 enriched IMVs (5 μ l). Phosphate standards and samples (duplicates) were brought to 200 μ l with
698 water in a microplate. 30 μ l of phosphate reagent were added to each well and mixtures were
699 incubated at room temperature in the dark. After 30 min, absorbance at 650 nm was measured.
700 **(D)** Substitution Y144N in the periplasmic sensor domain of CpxA affects binding to CpxP.
701 Protein-protein interaction analysis was performed by using BATCH. CpxP and the sensor
702 domain of the wild-type CpxA or mutant CpxA^{Y144N} (CpxA-SD and CpxA^{Y144N}-SD) were fused
703 to the C- and the N-terminal ends of the T18 and T25 fragments of *B. pertussis* adenylate
704 cyclase, respectively. *E. coli* cells DHM1 co-transformed with plasmids encoding the hybrid
705 proteins were grown overnight, spotted on an Xgal plate and incubated at 30°C for 24 h. **(E)** *In*
706 *vivo* detection of phosphorylated and non-phosphorylated CpxR. *E. coli* BL21(DE3) was co-
707 transformed with pET24+-*cpxR-His* and pBAD33-*cpxA* or pBAD33-*cpxA*^{Y144N}. Cells were
708 grown at 37°C to mid-log phase and protein expression was induced with 0.2% L-arabinose for
709 2 h then 1 mM IPTG for 3 h. Bacteria were pelleted by centrifugation, lysed with formic acid
710 and samples were rapidly fractionated by Phos-tagTM PAGE and blotted with anti-His
711 antibodies.

Figure 5. Interplay between the Cpx system, PBPs and the cell-wall active antibiotic imipenem for β -lactamase induction. **(A)-(D):** Nitrocefin degradation assays were performed as described in the Materials and Methods section. **(A)** *E. aerogenes* clinical strain P4 exhibits high β -lactamase activity. Total β -lactamase activity was assessed in a series of clinical strains of *E. aerogenes* (P1 to P4) sequentially isolated from a patient under treatment with IMP. **(B)** β -lactamase activity in P4 is mainly due to AmpC. Inhibition of the β -lactamase activity in *E. aerogenes* P4 and control strains of *E. coli* expressing constitutive TEM-3 or AmpC from a plasmid. Tazobactam/clavulanic acid and boronic acid were used as specific inhibitors of class A (e.g. TEM-3) and class C (e.g. AmpC) β -lactamases, respectively. **(C)** AmpC activity is increased upon the expression of CpxA^{Y144N} in the absence of IMP. *E. coli* C41(DE3) was co-transformed with pACYC184-*ampRC* and pET24a+*-cpxA-His* or pET24a+*-cpxA^{Y144N}-His*. Expression of the CpxA proteins was induced with 1 mM IPTG for 1 h at 30°C, then cells were exposed or not to IMP (0.32 μ g/ml) for 1 h. **(D)** Functional AmpG and CpxAR are both required for inducing AmpC activity in response to IMP exposure. **(E)** Loss of specific PBPs induces AmpC activity in a Cpx-dependent manner. In **(D)** and **(E)** *E. coli* MC4100, CS109 and derivative mutants were transformed with pACYC184-*ampRC*, grown and induced as described Materials and Methods. Asterisks indicate that the data determined for the mutant samples are significantly different from those of the wild-type control samples; nd, not determined.

Figure 6. Schematic representation of the interplay between the Cpx system and the chromosomal AmpC β -lactamase regulatory pathway in *Enterobacteriaceae*. In a wild-type background and under non-inducing conditions, the PG is recycled. PG degradation products (disaccharide-peptides, aD-peptides) are generated in the periplasm by the activity of PBPs. aD-peptides (aD-tripeptides, aD-tetrapeptides and aD-pentapeptides) are transported into the cytoplasm by the inner membrane permease AmpG and processed by the amidase AmpD into their corresponding monosaccharide-peptides (aM-peptides). aM-peptides are then transformed

737 into UDP-MurNAc-pentapeptides and incorporated to the PG biosynthesis pathway to complete
738 the recycling process. In the cytoplasm, these UDP-MurNAc-pentapeptides interact with
739 AmpR, creating a conformation that represses the transcription of *ampC*. In addition, normal
740 interactions between CpxP and the periplasmic sensor domain of CpxA keeps in the Cpx system
741 « Off ». Strong AmpC inducers such as imipenem simultaneously bind to several PBPs; lead to
742 an increase of aD-pentapeptides in the periplasm (AmpG saturation) and of aM-tripeptides
743 (AmpD saturation) in the cytoplasm. In the cytoplasm, the accumulated aM-tripeptides displace
744 UDP-MurNAc-pentapeptides from AmpR, creating a conformation that activates the
745 transcription of *ampC*. In addition, our data showed that the increased level of aD-pentapeptides
746 in the periplasm is sensed by the Cpx system. The release of CpxP from CpxA-SD by competing
747 interactions with aD-pentapeptides probably switches the Cpx system « On ». High level of
748 CpxR~P leads to AmpC overexpression thus to β -lactam resistance. The proposed feedback
749 loop involving the lytic glycosylase appears in red. It also unknown which of CpxA or CpxP
750 interacts with the putative signaling mucopeptide in the periplasm. The *cpxA*^{Y144N} mutation
751 alters the interaction between the CpxA-SD and CpxP. This activates the Cpx response and
752 leads to *ampC* overexpression, even in the absence of AmpC inducers. This mutation also acts
753 on other genes of the Cpx regulon that impact antibiotic resistance.

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The authors declare that they have no competing interests.

MM and JMP design the research. MM and EP performed the experiments. MM and JMP wrote the paper.

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Fig. 1

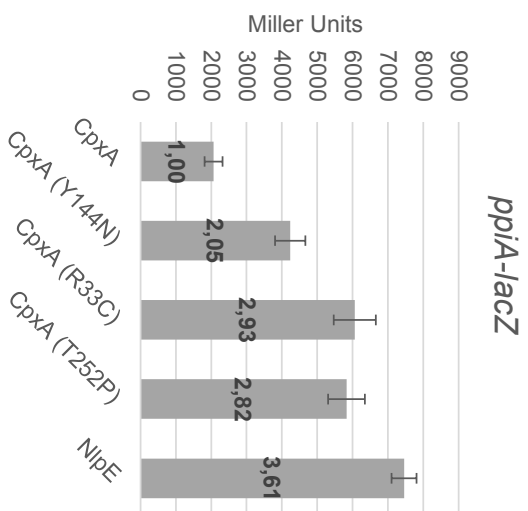


Fig. 2

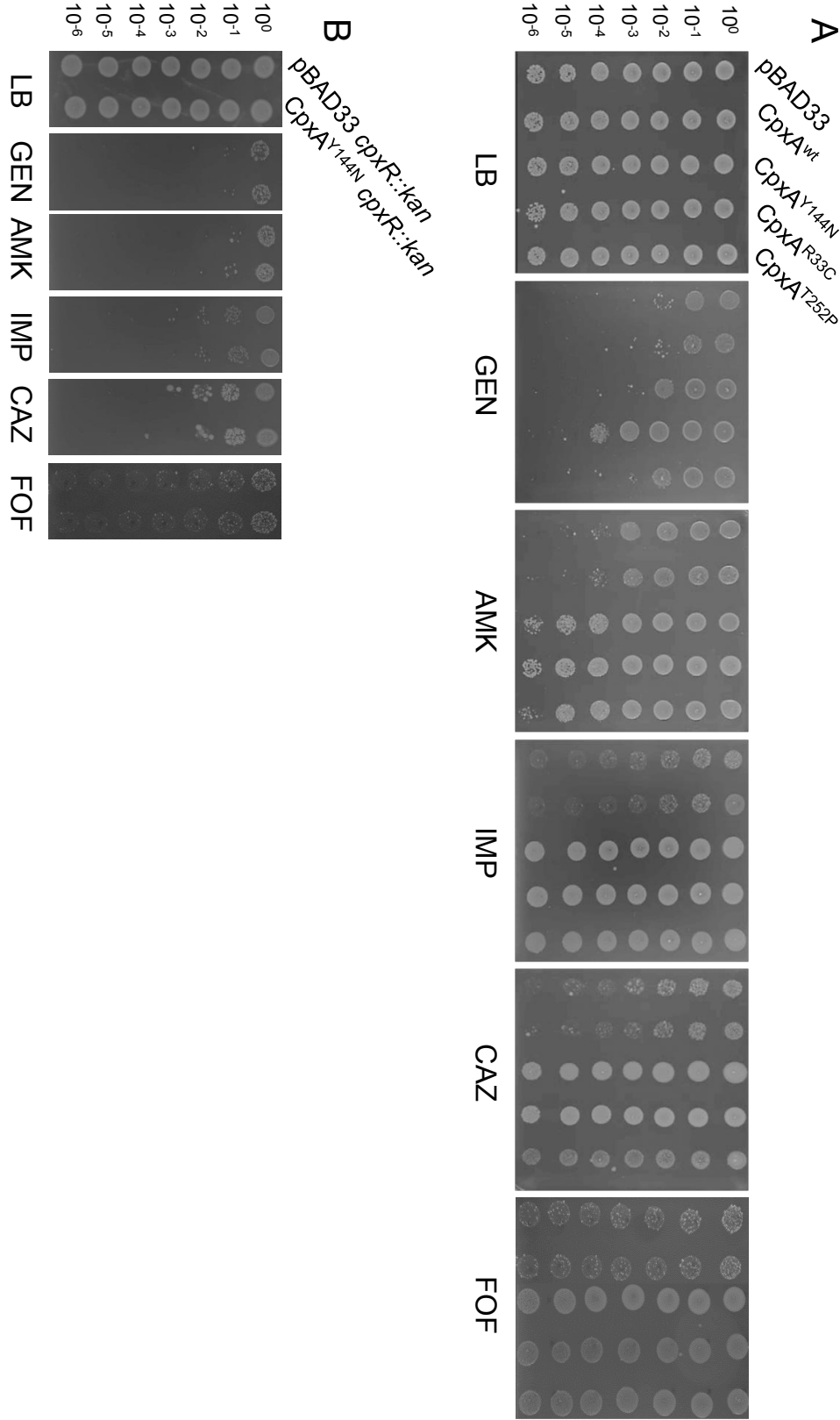


Fig. 3

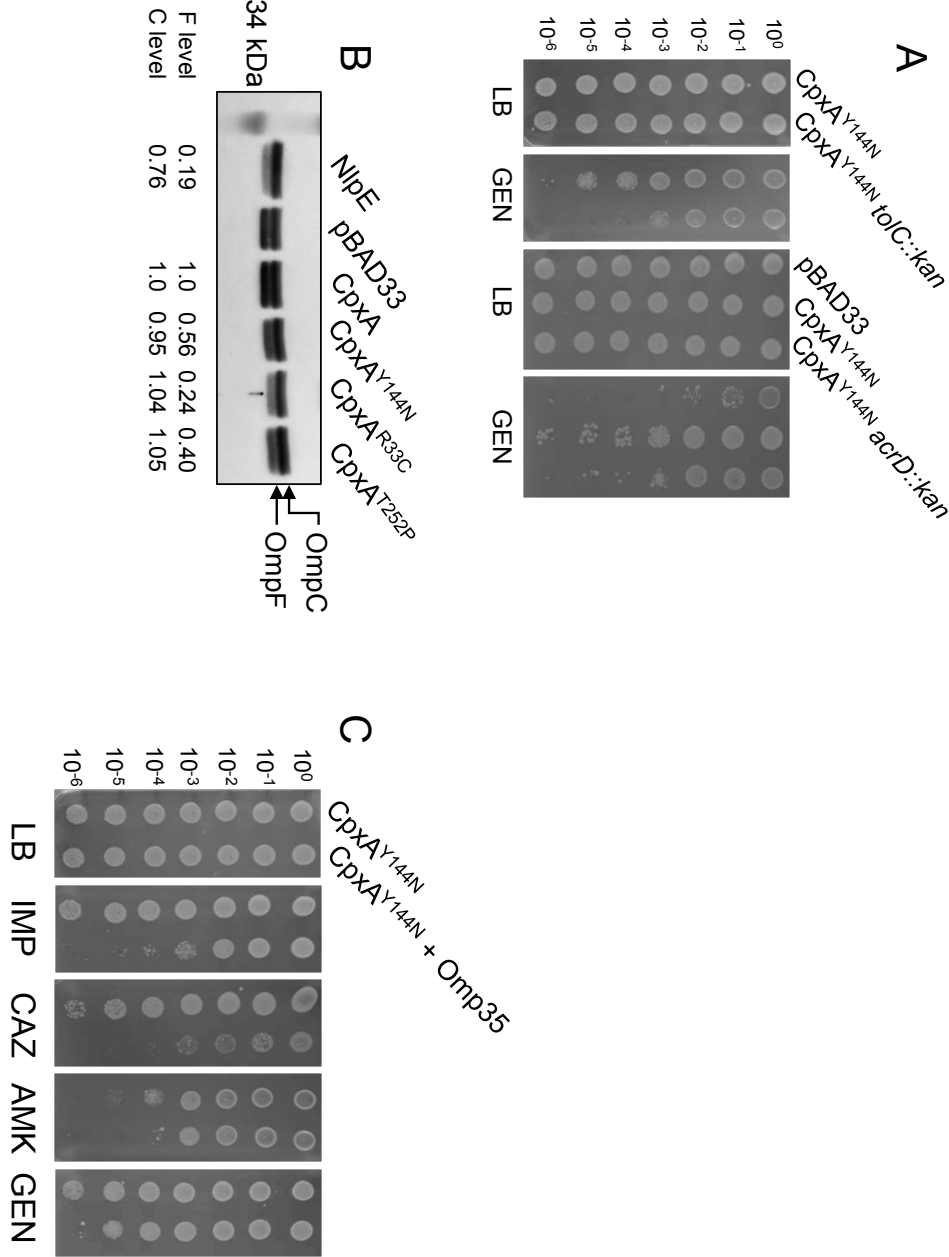


Fig. 4

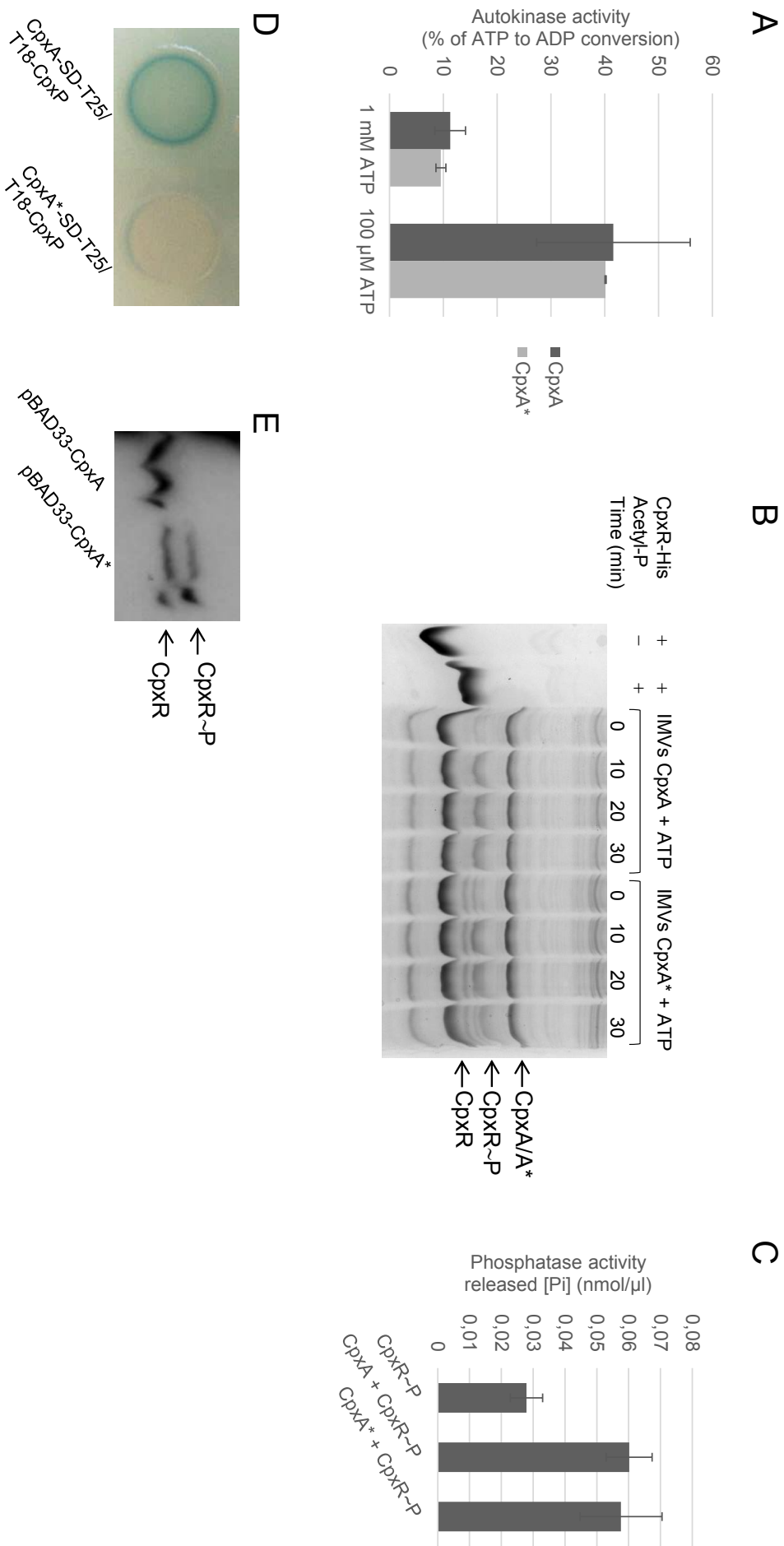
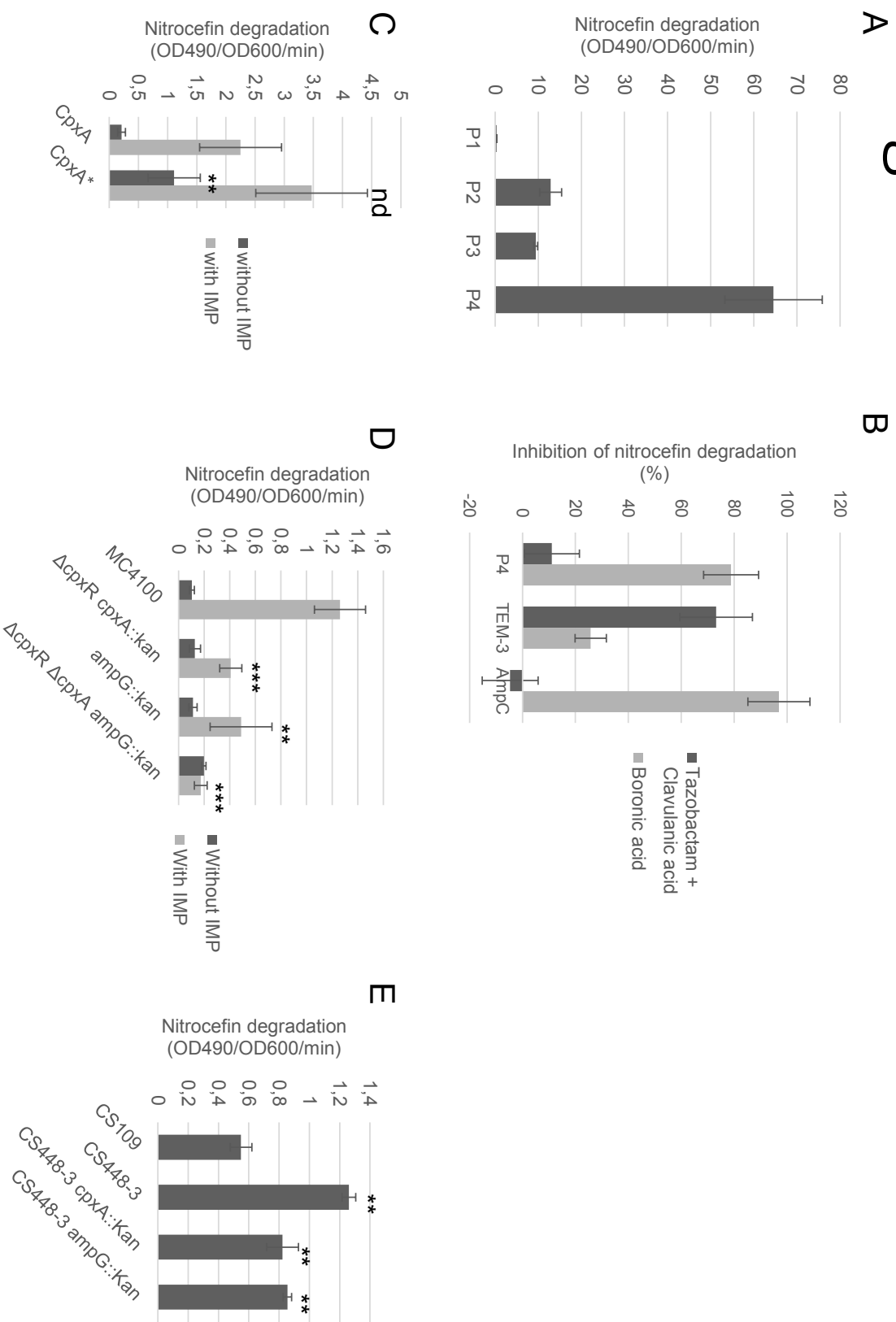


Fig. 5



AmpC inducer (imipenem)

periplasm

PG

OM

IM

Porin

PBPs

-aD-peptides

AmpC

amC-peptides/AmpR

ATP

ADP + P

CpxA

CpxP

CpxR

CpxR (P)

Cpx regulon members

- ☒ *ompF* (resistance to β -lactams)
- ☒ *acrD* (resistance to aminoglycosides)
- ☒ *glpT*, *uhpT* (fosfomycin resistance)
- ☒ *sit* (resistance to β -lactams)

☒ *ampC* (resistance to β -lactams)

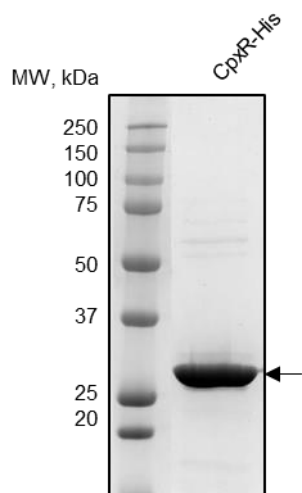
Cpx regulon members

- ☒ *ompF* (resistance to β -lactams)
- ☒ *acrD* (resistance to aminoglycosides)
- ☒ *gfpT*, *uhpT* (tostomycin resistance)
- ☒ *sit* (resistance to β -lactams)

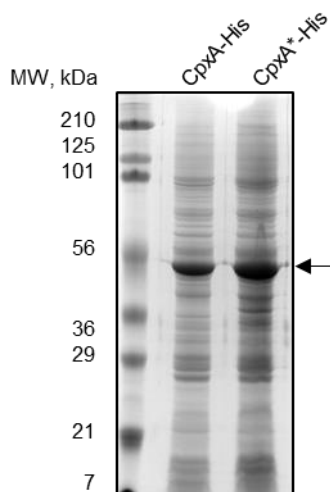
- 1 **Supplementary data**
- 2 **Supplementary Materials and Methods.**
- 3 **Supplementary Table. Bacterial strains and plasmids.**

Strain or plasmid	Relevant characteristics	Reference or source
<i>K. aerogenes</i> strains		
G7	Clinical isolate, source for amplification of <i>cpxA</i> and <i>nlpE</i>	1
P4	Clinical isolate, source of amplification of <i>cpxA</i> ^{Y144N}	1
ATCC13048	Source for amplification of <i>omp35</i> and <i>ampR-ampC</i>	Lab collection
<i>E. coli</i> strains		
MC4100	[<i>araD139</i>] _{B/r} , $\Delta(\arg F-lac)169$, λ^- , <i>e14-</i> , <i>flhD5301</i> , $\Delta(fruK-yeiR)725(fruA25)$, <i>relA1</i> , <i>rpsL150</i> (strR), <i>rbsR22</i> , $\Delta(fimB-fimE)632(::IS1)$, <i>deoC1</i>	2
RAM1292	MC4100 $\Delta ara174$	3
RAM1330	RAM1292 $\Delta tolC::kan$	4
RAM1551	RAM1292 $\Delta ppiA-lacZ$	5
Stellar	Host for cloning, F ⁻ , <i>endA1</i> , <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> , <i>relA1</i> , <i>gyrA96</i> , <i>phoA</i> , $\Phi 80d lacZ\Delta M15$, $\Delta(lacZYA-argF)$ U169, $\Delta(mrr-hsdRMS-mcrBC)$, $\Delta mcrA$, λ^-	CloneTech
BL21(DE3)	Host for protein overexpression, <i>fhuA2</i> [<i>lon</i>] <i>ompT gal</i> (λ DE3) [<i>dcm</i>] $\Delta hsdS$, λ DE3 = λ <i>sBamHI</i> o $\Delta EcoRI$ -B <i>int::(lacI::PlacUV5::T7 gene1)</i> <i>i21</i> $\Delta nin5$	Lab collection
C41(DE3)	Host for toxic membrane protein overexpression, derivative of BL21(DE3)	Lab collection
DHM1	Host adenylate cyclase complementation, F ⁻ , <i>cya-854</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> (Nal ^r), <i>thi1</i> , <i>hsdR17</i> , <i>spoT1</i> , <i>rfbD1</i> , <i>glnV44</i> (AS)	Euromedex
BW25113	Parental strain of single-gene-deletion, $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i>	KEIO collection
BW25113 <i>cpxR::kan</i>	Source for P1 transduction	KEIO collection
BW25113 <i>cpxA::kan</i>	Source for P1 transduction	KEIO collection
BW25113 <i>acrD::kan</i>	Source for P1 transduction	KEIO collection
BW25113 <i>ampG::kan</i>	Source for P1 transduction	KEIO collection
BW25113 <i>ldtD::kan</i>	Source for P1 transduction	KEIO collection
CS109	W1485 <i>rph-1 katF</i> (<i>rpoS</i>)	6
CS448-3	CS109 <i>dacB::res dacA::res pbpG::res ampH::res</i>	7
Plasmids		
pCP20	<i>cI857 repA</i> (Ts) P _R ; <i>flp</i> Amp ^r Cam ^r	8
pAD7	Constitutive Amp ^C	Gift from MGP. Page
pTEM-3	TEM-3	Gift from MGP. Page
pBAD24	Amp ^r ; Expression vector; P _{ara} arabinose inducible	9
pBAD24- <i>nlpE</i>	NlpE of <i>K. aerogenes</i> G7 cloned into pBAD24	This study
pBAD24- <i>omp35</i>	Omp35 of <i>K. aerogenes</i> ATCC13048 cloned into pBAD24	This study

pBAD33	Cm ^r ; Expression vector; <i>P_{ara}</i> arabinose inducible	9
pBAD33- <i>cpxA</i>	CpxA of <i>K. aerogenes</i> G7 cloned into pBAD33	This study
pBAD33- <i>cpxA</i> ^{Y144N}	CpxA ^{Y144N} of <i>K. aerogenes</i> P4 cloned into pBAD33	This study
pBAD33- <i>cpxA</i> ^{R33C}	R33C site-directed mutagenesis from pBAD33- <i>cpxA</i>	This study
pBAD33- <i>cpxA</i> ^{T252P}	T252P site-directed mutagenesis from pBAD33- <i>cpxA</i>	This study
pACYC184	Tet ^r , Cm ^r ; Cloning vector	New England Biolabs
pACYC184- <i>ampR</i> C	AmpR and AmpC of <i>K. aerogenes</i> ATCC13048 cloned into pACYC184	This study
pET24a+	Kan ^r ; Expression vector, <i>P_{T7}</i> IPTG inducible	Novagen
pET24a+- <i>cpxA</i> His	CpxA of <i>K. aerogenes</i> G7 cloned into pET24a+ for overexpression in C41(DE3)	This study
pET24a+- <i>cpxA</i> *His	CpxA ^{Y144N} of <i>K. aerogenes</i> P4 cloned into pET24a+ for overexpression in C41(DE3)	This study
pET24a+- <i>cpxR</i> His	CpxR of <i>K. aerogenes</i> G7 cloned into pET24a+ for overexpression in BL21(DE3)	This study
pKNT25	Kan ^r ; Vector generating N-terminal fusion to T25	Euromedex
pUT18C	Amp ^r ; Vector generating C-terminal fusion to T18	Euromedex
pKT25-zip	T25-zip expression plasmid	Euromedex
pUT18-zip	T18-zip expression plasmid	Euromedex
pCpxA-SD-T25	CpxA-SD cloned into pKNT25	This study
pCpxA-SD*-T25	CpxA ^{Y144N} -SD cloned into pKNT25	This study
pUT18C-CpxP	CpxP cloned into pUT18C without its signal sequence	This study



Supplementary Figure 1. Purified CpxR-His after nickel affinity chromatography and buffer exchange.

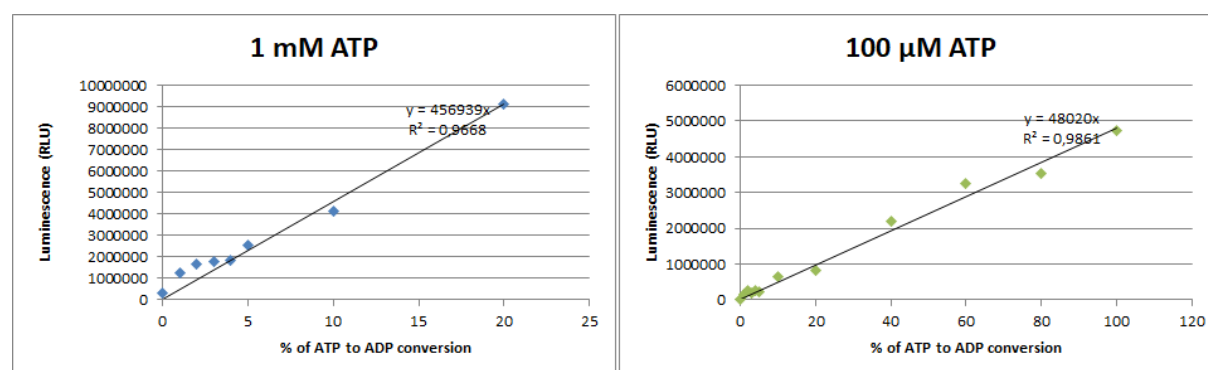


Supplementary Figure 2. Purification of inner membrane vesicles containing CpxA-His and CpxA*-His.

ADP-Glo™ kinase assay

The reactions were performed in a volume of 5 μ l, in the presence of 100 μ M or 1 mM ATP and incubated for 30 min at room temperature in a solid white 384-wells assay plate. Then, the assay is performed in two steps after completion of the kinase reaction: first, the ADP-Glo reagent is added that simultaneously terminates the enzyme reaction and removes remaining ATP; second, after 40 min incubation, the kinase detection reagent is added that converts ADP to ATP and simultaneously converts the generated ATP into light using luciferin/luciferase reaction. The amount of light generated is proportional to the ADP produced and the activity of the kinase. ATP to ADP standard curves were prepared in the phosphorylation buffer to assess the linearity of the assay order to calculate the amount of ADP produced from each reaction. The 1 mM ATP and ADP concentration range was created by mixing proportionally an amount of 1 mM ADP and 1 mM ATP in a microtiter plate to always reach a concentration of 1 mM total nucleotides. This 1 mM series (12 concentration points from 0 to 100% ATP or 100 to 0% ADP) was then diluted 1:10 to make the 100 μ M series. A volume of 5 μ l for each of the 12 points was transferred to the assay plate and ADP-Glo reagents were added as follows: 5 μ l

ADP-Glo reagent was added then the plate was mixed for 30 s and incubated at room temperature for 40 min. Then, 10 μ l of kinase detection reagent was added and the plate was mixed again before incubating it for a minimum of 60 min to allow the highest concentrations of ADP to fully convert to ATP and to be used by luciferase/luciferin reaction (**Figure S3**). For both standard curves and assays, luminescence was recorded by using a TECAN $\text{\textcircled{R}}$ Infinite Pro M200 instrument set to 0.5 sec integration time.



Supplementary Figure 3. Linearity of the ADP-GloTM kinase assay for the 100 μ M and 1 mM ATP series. Luminescence values represent the mean of 2 replicates. Abbreviation: RLU, relative light unit.

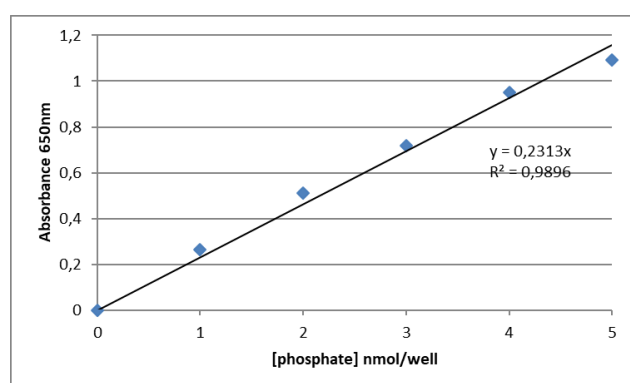
Phosphorylation of CpxR with acetyl phosphate

Acetyl phosphate was synthesized by mixing 2 units of acetate kinase (Sigma) with 500 μ M of ATP diluted in 0.1 M triethanolamine buffer (pH 7.8) containing 10 mM K acetate, 1 mM MgCl_2 , and 10% glycerol at room temperature for 2 h. The solution was then ultrafiltrated through a Microcon Ultracel YM-10 filter (Millipore) at $14,000 \times g$ at 4°C for 30 min. Purified CpxR-His (25 μ M) diluted in phosphorylation buffer was incubated with the ultrafiltrate (200 μ l), at room temperature for 30 min. The reaction mixture was transferred to a concentrator (Vivaspin 500, 10,000 MWC0 PES, Sartorius) and centrifuged at $13,000 \times g$ for 5 min. After

discarding the filtrate, the concentrate was diluted in phosphorylation buffer and centrifuged again. This procedure was repeated 3 times to remove free ATP and acetyl phosphate.

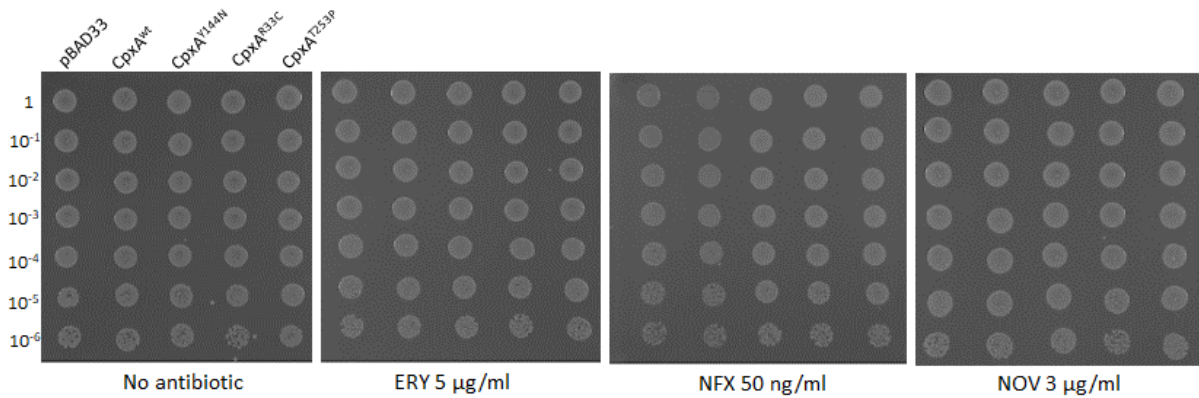
Phosphate colorimetric assay

Phosphate reacts (in standards and samples) with a chromogenic complex, which results in a colorimetric product proportional to the amount of phosphate present (**Figure S4**). The assay was performed in microplates and absorbance was measured at 650 nm using a TECAN ® Infinite Pro M200 instrument.

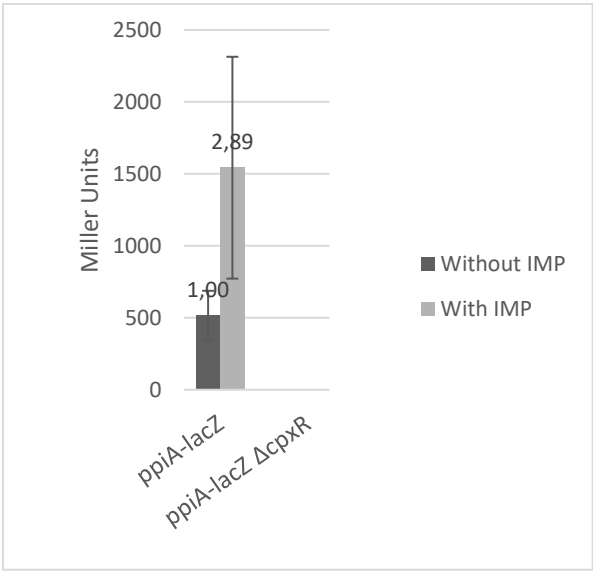


Supplementary Figure 4. Standards for colorimetric phosphate assay. Absorbance values represent the mean of a duplicate. 10 µl of the 10 mM phosphate standard was with 990 µl of water to prepare a 0.1 mM phosphate standard solution. 0, 10, 20, 30, 40, and 50 µl of the 0.1 mM phosphate standard solution were added into a 96 well plate, generating 0 (blank), 1, 2, 3, 4, and 5 nmole/well standards. Water was added to each well to bring the volume to 200 µl. 30 µl of phosphate reagent was added to each well. Mixtures were incubated for 30 min at room temperature before readings.

Supplementary results



Supplementary Figure 5. *cpx*^{*} alleles do not increase resistance macrolides, fluoroquinolones or novobiocin. EOP assays were performed as described in Materials and Methods. ERY, erythromycin; NFX, norfloxacin; NOV, novobiocin.



Supplementary Figure 6. The Cpx system is activated by imipenem. A *ppiA-lacZ* transcriptional fusion was used as a Cpx reporter activity. The fusion was significantly activated (~2-3 fold) in the presence imipenem (0.32 µg/ml for 1 h @ 37°C) to level similar to that *cpxA*^{*} alleles, but completely abolished in a *ΔcpxR* background.

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