Induction of Cpx envelope stress pathway contributes to *Escherichia coli* tolerance to antimicrobial peptides

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ABSTRACT

Antimicrobial peptides produced by multicellular organisms as part of their innate system of defense against microorganisms are currently considered as potential alternative to conventional antibiotics in case of infection by multiresistant bacteria. However, while the mode of action of antimicrobial peptides is relatively well described, resistance mechanisms potentially induced or selected by these peptides are still poorly understood. In this work, we studied the mechanisms of action and resistance potentially induced by ApoEdpL-W, a new antimicrobial peptide derived from human apolipoprotein E. Investigation of the genetic response of *Escherichia coli* upon exposure to sublethal concentrations of ApoEdpL-W revealed that this antimicrobial peptide triggers activation of RcsCDB, CpxAR and $\sigma^E$ envelope stress pathways. This genetic response is not restricted to ApoEdpL-W, since several other antimicrobial peptides, including polymyxin B, melittin, LL-37 and modified S4 dermaseptin also activate several *E. coli* envelope stress pathways. Finally, we demonstrate that induction of the CpxAR two-component system directly contributes to *E. coli* tolerance toward ApoEdpL-W, polymyxin B and melittin. These results therefore show that *E. coli* senses and responds to different antimicrobial peptides by activation of the CpxAR pathway. While this study further extends the understanding of the array of peptide-induced stress signaling systems, it also provides insight on the contribution of the Cpx envelope stress to *E. coli* tolerance to antimicrobial peptide.
INTRODUCTION

Administration of antibiotics is the most efficient strategy for combatting pathogenic bacteria. However, decades of extensive use of antibiotics has led to the emergence of bacterial strains with a higher or wider resistance spectrum, causing increasing difficulty worldwide in management of bacterial infections (1). In parallel with research on new antibiotics, antimicrobial peptides (AMPs), mainly produced by epithelial surfaces of multicellular organisms as part of their innate defense system, have emerged as a plausible alternative to conventional antibiotics (2). Although AMPs vary in sequence, length and structural conformation, they are mostly amphipathic compounds with spatially organized clusters of hydrophobic and cationic amino acids (3). The AMP net positive charge enables their binding to the negatively charged microbial surface, while the presence of hydrophobic residues promotes their insertion into membranes (3). Many AMPs form deleterious channels in bacterial membranes (4). Alternatively, AMPs can translocate across membranes into the cytoplasm, where they may inhibit essential processes such as nucleic acid, protein, enzyme and cell wall syntheses (5-9).

In light of the wide distribution of AMPs in multicellular organisms and the long interplay between bacteria and their host during evolution, bacteria have acquired different mechanisms for minimizing the killing impact of AMPs (10). Mechanisms of resistance to AMPs can be classified into three major categories, namely: (i) destruction/modification of AMP by proteolytic cleavage (11-13); (ii) exclusion of AMP from the cell via low-specificity efflux pumps (14-16); and (iii) reduction of bacterial susceptibility to AMPs by altering membrane net charge, thereby impairing physico-chemical interactions between the cationic antimicrobial molecule and the negatively charged bacteria cell surface (17-20). Moreover, exposure to AMPs results in strong alterations in the bacterial gene transcription profile and induction of mostly non-specific poorly understood resistance mechanisms (21-25).

Recently, a new family of antimicrobial peptides derived from human apolipoprotein E (ApoE; sequence: LRKLRKRLRLRKLRLKLL), acting via perturbation of the membrane lipid bilayer has been described (26, 27). The original ApoEdp peptide showed direct, broad anti-infective activity against bacteria, fungi and viruses (27). Substitution of all leucine residues with tryptophan amino acids in ApoEdpL-W (sequence: WRKWRKRWWRKWRKRW) led to production of a variant with increased potency and high antimicrobial activity against viruses,
parasite and *Staphylococcus aureus*, despite a slight decrease of antibacterial activity against *Pseudomonas aeruginosa* (28-30). To identify bacterial resistance potentially induced upon exposure to antimicrobial peptides, we studied *E. coli* genetic response to ApoEdpL-W and demonstrated the contribution of CpxAR envelope stress signaling pathway in *E. coli* resistance to ApoEdpL-W and several other antimicrobial peptides.
MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* Keio collection was derived from wild-type BW25113 (31). pCA24N and derivatives were isolated from *E. coli* K12 carrying the different plasmids (ASKA collection) (32). Deletion mutants were generated by P1 transduction from corresponding Keio mutants or mutants from our laboratory collection into *E. coli* MG1655 or *E. coli* MG1655F'. pBAD18-nlpE was constructed by cloning the *nlpE* gene into the pBAD18 plasmid containing an arabinose inducible promoter. Primers used to clone *nlpE* or verify genetic constructions are listed in Table S1.

Peptides and growth conditions

ApoEdpL-W and *Fluo*-ApoEdpL-W used in this study were manufactured by Alta Bioscience (Birmingham, United Kingdom) and resuspended in water plus 5% DMSO. Melittin, LL-37 and polymyxin B were purchased from Sigma and diluted in water. K₄K₂₀S₄ dermaseptin was also diluted in water (33).

All experiments were carried out in Mueller Hinton medium (MH) at 37°C except for those using LL-37, which were conducted in 0.4% glucose-M63B1 minimal medium (M63B1-G). When required, antibiotics were added to the medium at the following final concentrations: kanamycin (Km, 50 mg/l), chloramphenicol (Cm, 25 mg/l) and tetracycline (Tet, 7.5 mg/l).

MIC determination

Strains cultured for 6 hours in MH or M63B1-G medium were inoculated in fresh medium at an optical density at 600 nm (OD₆₀₀) of 0.0001. Peptides were diluted in water and 10 µl of peptide solution (concentration ten times higher than the final concentration) were placed in wells of microtiter 96-well polystyrene plates. 90 µl of the diluted bacterial culture (see above) were added to wells. Microtiter plates were incubated overnight at 37°C. The minimum inhibitory concentration (MIC) of the antimicrobial peptide is defined as the lowest concentration that inhibits bacterial growth.
Bacterial killing assay
Overnight cultures were diluted in fresh medium containing IPTG and chloramphenicol when necessary at OD$_{600}$ 0.005 and incubated at 37°C with aeration until reaching OD$_{600}$ 0.1. Then, bacteria were exposed to different concentrations of peptide and incubated at 37°C. At different times, samples were taken, centrifuged and resuspended in equivalent volume of PBS 1X. Then, they were serially diluted and plated on appropriate LB agar plates to enumerate viable colonies (CFU).

RNA isolation
Overnight cultures were diluted in fresh MH medium at OD$_{600}$ 0.005 and incubated at 37°C until reaching OD$_{600}$ 0.1. Bacteria were then exposed or not to 3 µM of ApoEdpL-W for 30 min at 37°C with agitation. OD$_{600}$ of the cultures was adjusted to 3 with corresponding used medium (3 ml final), and 2 volumes of RNA Protect reagent (Qiagen, Valencia, CA) were added (6 ml) in order to maintain the stability and integrity of the RNA. Bacterial cells were centrifuged at 8,000 rpm for 10 min at 4°C and total RNA was then extracted using a modification of the RNeasy kit (Qiagen) protocol described below. Both conditions were compared using three biological replicates. Briefly, bacterial pellets were resuspended in 200 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, DEPC (0.1% (v/v)) containing 1 mg/ml of lysozyme and incubated for 5 min at room temperature. Then, cells were homogenized with 700 µl RLT lysis buffer and the homogenate was vortexed for approximately 1 min. 500 µl ethanol (100%) were then added, and the solution was applied to an RNeasy column. From this point on, instructions from the manufacturer were followed, including on-column DNase treatment. Purified total RNA concentrations were measured using a Nanodrop spectrophotometer at 260 nm.

Microarray and data analysis
Three biological repetitions of RNA extractions from each condition were treated as described in the Affymetrix GeneChip Expression Analysis Technical Manual (P/N 702232 Rev. 2). Quality of the sample hybridizations was checked on Agilent RNA Nano LabChips (Agilent Technologies) before performing data analysis. Data analysis was performed using R software based on the Bioconductor package. For each experimental condition, probe intensities from
three independent biological replicates were analyzed. Pre-processing of the gene expression array was carried out using the model-based Robust Multichip Average algorithm (RMA)(34), enabling global background correction, quantile normalization and summarization of the eleven probe values into a single probe set. The LPE test was used for pairwise expression comparison, (35) followed by the Benjamini and Hochberg $p$-value adjustment method (threshold $p<0.05$). Only fold Changes (FC) superior or inferior to 2 were considered to identify induced or repressed genes, respectively.

**β-galactosidase assays**

β-galactosidase activities were measured as described below. Overnight cultures were diluted in fresh MH or M63B1-G medium containing antibiotics and IPTG when necessary (as indicated in figure legends) at an OD$_{600}$ of 0.005 and incubated at 37°C with agitation until reaching an OD$_{600}$ of 0.1. 1 ml of cultures was transferred to a 12-well plate containing or not an antimicrobial peptide. Microtiter plates were then incubated 30 min with agitation at 37°C. β-galactosidase activity was assayed in duplicate for each strain as previously described (36).

**Fluorescence microscopy**

Exponentially growing bacterial cells (OD$_{600}$ 0.1) were exposed to Fluo-ApoEdpL-W (3 μM) in MH medium for 30 min. 500 μl were centrifuged, washed 2 times with PBS 1X and then resuspended in 100 μl of PBS1X. 30 μl of bacterial cells were loaded on an 8-well black epoxy slide, previously treated with 0.1% poly-L-lysine for 2 min. Bacteria were fixed with 3% paraformaldehyde solution and stained with 25 μl of DAPI at 20 mg/l for 45 min at room temperature. Fluorescence microscopy was performed using a Nikon Eclipse E4000 microscope and images were taken using a 100x lens.

**Evaluation of ApoEdpL-W antimicrobial activity on biofilm bacteria**

*E. coli* biofilms were formed in MH medium in 96-well polyvinyl chloride (PVC) microtiter plates at 37°C. 24-h biofilms were washed once with PBS 1X using a multichannel pipette to remove unattached cells. 100 μl of ApoEdpL-W diluted in fresh MH medium at different concentrations were added to biofilm and microtiter plates were re-incubated for 24 h. Then, biofilms were washed with PBS 1X once before being resuspended in 100 μl of PBS 1X. Effects
of the peptide were determined by CFU enumeration before adding the peptide (T0) and after 24 h of treatment (T24).

Statistical analysis

Two-tailed unpaired Student’s t-test analyses were performed using Prism 5.0 for Mac OS X (GraphPad Software). Each experiment was performed at least three times. (NS: not significant, *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001).
RESULTS

ApoEdpL-W AMP activity in *E. coli* planktonic and biofilm bacteria

The ApoEdpL-W antimicrobial peptide was previously shown to inhibit growth of both *P. aeruginosa* and *S. aureus* when added at micromolar concentrations on planktonic cultures (26). We tested the activity of ApoEdpL-W on planktonic and biofilm *E. coli* K-12 bacteria using either strain MG1655 or its biofilm-forming isogenic derivative MG1655 F’ (37). Using increasing concentrations of ApoEdpL-W, we determined that the ApoEdpL-W minimal inhibitory concentration (MIC) on exponential phase planktonic *E. coli* was 5 μM. Accordingly, exponential phase cultures displayed a strong drop in viability (1 to 2 log CFU) after 20 min of treatment with 5 μM of ApoEdpL-W (Figure 1A). In order to test ApoEdpL-W activity on biofilm bacteria, which are characterized by high levels of tolerance towards antibiotics, peptide concentrations ranging from 10- to 80-fold the MIC were applied to 24 h *E. coli* K-12 MG1655 F’ biofilms formed in microtiter wells. Under these conditions, a slight growth stimulation was observed using ApoEdpL-W at 10-fold MIC (50 μM), which could be due to the release of nutrients upon low levels of bacterial lysis. However, use of higher ApoEdpL-W concentrations significantly reduced biofilm bacterial viability, with a 4-log CFU reduction after 24 h at 80-fold MIC (400 μM) (Figure 1B).

ApoEdpL-W AMP is localized in the cell envelope

To further explore ApoEdpL-W activity in *E. coli*, we used a fluorescently tagged derivative of ApoEdpL-W (*Fluo*-ApoEdpL-W) and first determined that *Fluo*-ApoEdpL-W and ApoEdpL-W have a similar MIC on *E. coli* K-12 MG1655 (5 μM) (data not shown). To monitor *Fluo*-ApoEdpL-W localization in bacteria exposed to the peptide, the cells were exposed to sublethal concentrations of ApoEdpL-W in order to keep cell lysis to a minimum. *E. coli* MG1655 bacteria in early exponential phase were incubated for 30 min in the presence of the sublethal concentration of 3 μM of *Fluo*-ApoEdpL-W previously shown to have a mild effect on bacterial growth and viability (Figure S1). Epifluorescence microscopy analysis indicated that the *Fluo*-ApoEdpL-W peptide was localized at the periphery of the treated bacteria, but was not detected...
in their cytoplasm, consistent with a potential cell envelope site of action for ApoEdpL-W (Figure 2) (26).

**E. coli** genetic responses upon exposure to ApoEdpL-W antimicrobial peptide

To investigate the mode of action of ApoEdpL-W and potential mechanisms of bacterial resistance to this antimicrobial peptide, we studied the genetic response of **E. coli** upon exposure to subinhibitory concentrations of ApoEdpL-W using **E. coli** DNA microarray. Exponentially growing **E. coli** bacteria were exposed for only 30 min to 3 µM sublethal concentrations of ApoEdpL-W AMP in order to avoid massive cell lysis (see Fig. S1). RNAs corresponding to these experimental conditions were extracted and responses induced by ApoEdpL-W were analyzed using Affymetrix DNA chips. 175 genes were found to be differentially expressed in response to ApoEdpL-W (Table S2 and S3), including 69 downregulated genes involved in general cell metabolism and energetics. Among the 106 upregulated genes, many were related to iron acquisition (**fepA, fes, entABCEF, fhuA, fhuF, sufA** and **exbD**) and surface polysaccharide synthesis (**manA, galU, yjbEGH, rcsA, otsB**). In particular, 17 out of the 19 genes coding for colanic acid synthesis (**wza, wzc, wcaABCDEF, gmd, wcaGHI, manC, wcaJ, wzxC, wcaKL**) were identified (Table S2 and S3). Several genes involved in bacterial stress responses were also upregulated by ApoEdpL-W. They included genes induced by osmotic shock (**osmB, osmC, osmY**), oxidative stress (**katE**) or encoding chaperones from the general stress response (**dnaK, htpG, lolA, clpB, ipbA, ipbB**). Strikingly, 51 out of 106 genes identified as being induced upon exposure to ApoEdpL-W were part of regulons involved in **E. coli** envelope stress, such as the RcsCDB (39 genes), CpxAR (13 genes) two-component systems and the **σ**E pathway (9 genes) (38, 39) (Table S2 and S3). Considering the membrane localization and potential mode of action of ApoEdpL-W, we focused the rest of our analyses on the contribution of envelope stress factors to resistance to ApoEdpL-W.

The ApoEdpL-W peptide induces **E. coli** envelope stress responses

Reporter gene fusions between the **lacZ** and **manC** promoter (to monitor induction of RcsCDB regulon) and between the **lacZ** and **degP** promoter (to monitor induction of CpxAR and **σ**E regulons) were used to confirm DNA array analysis. Comparison of β-galactosidase activities in bacteria exposed or not to 3 µM sublethal concentrations of ApoEdpL-W showed that both
RcsCDB and CpxAR were induced by ApoEdpL-W (Figure 3A). Introduction of a cpxR mutation did not completely prevent degP-lacZ induction, therefore suggesting that ApoEdpL-W could also induce the $\sigma^E$ system (Figure 3A). Since $\sigma^E$ (rpoE) is an essential gene in E. coli, the possible induction of the $\sigma^E$ pathway by ApoEdpL-W was evaluated by overexpressing the anti-$\sigma^E$ factor RseA that is known to sequester $\sigma^E$ (40). The extent of degP-lacZ induction by ApoEdpL-W was reduced by the introduction of pCA24N-rseA into E. coli degP-lacZ and E. coli ΔcpxR degP-lacZ reporter strains, therefore confirming the induction of the $\sigma^E$ pathway by ApoEdpL-W (Figure 3B). Taken together, these results demonstrated that E. coli induces RcsCDB, CpxAR and $\sigma^E$ envelope stress pathways upon exposure to ApoEdpL-W.

The CpxAR pathway, but not the RcsCDB or $\sigma^E$ pathways, is involved in resistance to ApoEdpL-W

Induction of Rcs, Cpx and $\sigma^E$ envelope stress pathways upon E. coli exposure to subinhibitory concentrations of ApoEdpL-W could result from membrane perturbation induced by ApoEdpL-W insertion into E. coli membranes. To test whether these genetic responses could contribute to resistance to the ApoEdpL-W peptide, we inactivated rcsB and observed that absence of a functional Rcs system did not alter E. coli sensitivity to ApoEdpL-W (data not shown). Similarly, introduction of the pCA24N-rseA plasmid and an associated reduction in $\sigma^E$ activity increased sensitivity to ApoEdpL-W, although not to a statistically significant level (Figure S2A). In contrast, inactivation of cpxR lowered E. coli tolerance to ApoEdpL-W, whereas introduction of pCA24N-cpxR restored the wild type phenotype (Figure 4). This result suggested that induction of the Cpx signaling system could reduce E. coli susceptibility to the ApoEdpL-W antimicrobial peptide. To further identify Cpx-regulated genes involved in resistance to ApoEdpL-W, we took advantage of the Keio collection of ordered single gene deletion mutants performed in E. coli K-12 strain BW25113 (31). As ApoEdpL-W MIC was similar in both strains MG1655 and BW25113, the MIC of 10 BW25113 mutants in genes belonging to the Cpx regulon, including 3 upregulated genes in the presence of ApoEdpL-W ($degP$, spy, cpxP) was determined (see Table 2 and S2). Among these 10 mutants, only the $degP$ mutant displayed 2-fold increased susceptibility to ApoEdpL-W (Table 2). Consistently, MG1655Δ$degP$ also displayed similar increased susceptibility to ApoEdpL-W, which could be partially complemented by pCA24N-$degP$ (Figure S3). In contrast, no restoration of the wild type phenotype was observed in a cpxR mutant.
complemented by the pCA24N-degP plasmid (data not shown); moreover, introduction of a pCA24N-rseA plasmid leading to decreased $\sigma^E$ activity in the cpxR mutant did not significantly increase its susceptibility to ApoEdpL-W (Figure S2B). To confirm that the induction of the Cpx pathway contributes to E. coli tolerance to ApoEdpL-W, the nlpE gene encoding the lipoprotein NlpE, which is known to induce the Cpx system (41), was overexpressed. The impact of nlpE overexpression in wild type and cpxR mutant on E. coli survival after exposure to ApoEdpL-W was evaluated and this assay revealed that NlpE-dependent induction of Cpx pathway led to a 7-fold increase of E. coli tolerance to ApoEdpL-W (Fig. S4).

Finally, comparison of sensitivity to ApoEdpL-W of 24 h biofilms formed by E. coli MG1655 F’ wild type and cpxR and degP mutants showed that both mutants exhibited increased susceptibility to ApoEdpL-W (Figure 5).

Taken together, these results show that ApoEdpL-W-dependent induction of the CpxAR pathway and degP expression contributed to E. coli tolerance to this antimicrobial peptide.

The CpxAR system is induced by different AMPs and contributes to E. coli tolerance to polymyxin B and melittin.

To investigate whether AMPs unrelated to ApoEdpL-W targeting cell membranes could also induce envelope stress response pathways, four different AMPs known or predicted to disrupt or permeabilize bacterial membranes were selected for analysis: (i) polymyxin B derived from Bacillus polymyxa and is a cationic cyclic peptide that binds to lipid A of the LPS, destabilizing and disrupting outer and inner membranes (42, 43); (ii) melittin extracted from bee venom (44, 45); (iii) the human cathelicidin-derived antimicrobial peptide LL-37 (46, 47); Melittin and LL-37 are both $\alpha$-helical peptides and are proposed to form similar types of trans-membrane pores in a lipid bilayer by a toroidal pore mechanism where peptides and lipids together form well-defined pores (45, 47); and (iv) KdK20-S4 dermaseptin derived from dermaseptin S4 isolated from frog skin and proposed to act on bacterial membranes (48, 49). After MIC determination of these different AMPs, E. coli bacteria were exposed to sublethal and non-lytic concentrations of each compound (ranging from 0.5xMIC to 0.8xMIC) to monitor genetic responses induced by the chosen AMP. As done previously, manc-lacZ and degP-lacZ reporter gene fusions were used to monitor expression of Rcs, Cpx and $\sigma^E$ pathways in the presence of polymyxin B 0.1 mg/l, melittin 5 mg/l, LL-37 4 mg/l and K4K20-S4 dermaseptin 0.1 mg/l. While both Rcs and Cpx
systems were induced upon exposure to polymyxin B, K₄K₂₀⁻S₄ dermaseptin and LL-37, melittin only slightly induced the Cpx system (Figure 6). Moreover, induction of degP expression upon exposure to polymyxin B, LL-37 and melittin was likely mediated by both Cpx and σE pathways, whereas K₄K₂₀⁻S₄ dermaseptin activated only the Cpx system, since a cpxR mutation completely abolished degP expression upon exposure to this peptide. We then tested the impact of Rcs and Cpx pathways on E. coli tolerance to polymyxin B, which induces both pathways, and to melittin, which only activates the Cpx pathway. While susceptibility of rcsB mutant to polymyxin B and melittin was unchanged, a cpxR mutant displayed a significantly reduced tolerance regarding both peptides compared to wild-type strain, suggesting CpxAR system involvement in AMP tolerance (Figure 7).
DISCUSSION

In the context of the emergence of antibiotic resistance, antimicrobial peptides stand as a plausible alternative against bacterial infections in some clinical situations (2). In this study, we investigated resistance mechanisms potentially induced by a new antimicrobial peptide derived from human apolipoprotein E, ApoEdpL-W. ApoEdpL-W is an aromatic substituted peptide previously reported to be active against *P. aeruginosa* and *S. aureus* pathogens, and to possibly interact with membranes. (26) We showed that *E. coli* exposure to ApoEdpL-W induces Rcs, Cpx and σ^E^ pathways, three regulatory pathways known to sense envelope stress. Interestingly, transcriptome analyses revealed strong similarities between responses to ApoEdpL-W and to peptidoglycan-disrupting β-lactams (cefusulodin and amdinocillin). Indeed, expression of Rcs-regulated genes was increased in response to cefsulodin and amdinocillin, while induction of Cpx and σ^E^ upon exposure to amdinocillin and amdinocillin plus cefsulodin (50). While antimicrobial peptides are not targeting peptidoglycan, the similarities observed between *E. coli* responses to β-lactams and to ApoEdpL-W, both inducing regulatory systems involved in sensing envelope perturbations, suggest that ApoEdpL-W targets the cell envelope, which is consistent with the demonstrated localization of this peptide in the bacterial cell envelope.

Bacteria two-component systems sense and respond to different stimuli, including membrane stresses caused by antimicrobial peptides. In some cases, they were shown to play a role in tolerance to the recognized AMP. For instance, in *Salmonella enterica* sv Typhimurium, AMP binding to the PhoQ sensor directly activates the PhoP/PhoQ two-component system, which then contributes to tolerance toward different AMPs, notably by regulating genes involved in LPS modifications (18, 24, 51). Recently, the RcsCDB pathway was shown to perceive the action of polymyxin B in *S. enterica* and to contribute to the intrinsic tolerance of bacteria to this antibiotic (25, 52, 53). In this work, whereas exposure to ApoEdpL-W induces all Rcs, Cpx and σ^E^ *E. coli* envelope stress responses, only the Cpx two-component system contributes to *E. coli* tolerance to this peptide. Indeed, the Rcs system is induced upon exposure to ApoEdpL-W, polymyxin B or melittin, it is not required for *E. coli* tolerance to these peptides. This indicates that, in *E. coli*, while a general cell envelope perturbation is sensed upon presence of ApoEdpL-W and is leading to induction of multiple cell-envelope stress pathways, not all upregulated genes are involved in *E. coli* tolerance to ApoEdpL-W. The Cpx system senses envelope perturbations...
such as protein misfolding and accumulation and bacterial contact with surfaces (39, 54). It responds to different physico-chemical stimuli by activating expression of genes encoding periplasmic proteases and chaperone proteins (39). In contrast, its role in antimicrobial resistance is less well understood. The CpxAR system was shown to be involved in *Salmonella* resistance to protamine and several α-helical AMPs (55). More recently, the CpxAR system was shown to be induced upon exposure to an antimicrobial cationic polyethyleneimine in *E. coli* (56), and to confer resistance to several β-lactams and chloramphenicol in *Klebsiella pneumoniae* (57). Here we show that inactivation of *cpxR* increased the sensitivity of *E. coli* to ApoEdpL-W. In addition, induction of the Cpx pathway upon NlpE overexpression increased *E. coli* tolerance to ApoEdpL-W in a CpxR dependent manner. Interestingly, the overexpression of NlpE in a *cpxR* mutant also led to an increased susceptibility to ApoEdpL-W; in these conditions, the *cpxR* mutant could be unable to control the important envelope stress induced by perturbations generated by both ApoEdpL-W and the overexpression of NlpE, explaining this increased susceptibility to ApoEdpL-W treatment. Furthermore, we determined that the *cpx*-regulated gene *degP* is involved in *E. coli* tolerance to ApoEdpL-W. *degP* encodes a periplasmic endopeptidase of the ATP-independent serine protease family and presents both chaperone and proteolytic activities (temperature-dependent switch from chaperone to protease activity) (58). Extracytoplasmic proteases were previously associated with antimicrobial tolerance. For instance, the outer membrane OmpT protease is involved in *E. coli* tolerance to protamine by degrading this membrane-permeabilizing peptide (12), while PgtE, a *Salmonella* OmpT protease homolog (46% identity and 65% similarity), contributes to resistance to several α-helical antimicrobial peptides (11). DegP has also been shown to be involved in *E. coli* tolerance to lactoferricin B, probably by proteolytic degradation of this antimicrobial peptide (59). DegP preferentially cleaves after valine and isoleucine residues, even when additional determinants (sequence, structure) are involved in the cleavage (60). As ApoEdpL-W does not contain either of these two amino acids, this suggests that the peptide might not be a substrate for DegP. Consistently, no effect of DegP on the fluorescent signal was observed in bacteria exposed to the *Fluo*-ApoEdpL-W peptide (data not shown). Hence, while the precise role of DegP in *E. coli* tolerance to ApoEdpL-W remains to be further investigated, we speculate that induction of DegP expression could reduce damages induced by ApoEdpL-W in the periplasm.

We also showed that decreased σ^E activity upon overexpression of RseA led to reduced
levels of degP expression upon exposure to ApoEdpL-W, as in the cpxR mutant. However, only the cpxR mutation, and not depletion of σE activity, increased E. coli sensitivity to ApoEdpL-W. These results indicate that, although degP is required for E. coli tolerance to ApoEdpL-W, increased susceptibility of the cpxR mutant is DegP-independent and involves others cpx-regulated factors, which is consistent with the absence of complementation of the cpxR mutant by overexpressing degP.

Mechanisms of resistance to AMPs are mostly non-specific and tolerance to antimicrobial peptides often relies on ill-understood bacterial adaptation to each peptides rather than on specific resistance mechanisms (10). Here we showed that different tested peptides induce a specific set of envelope stress pathways, depending on their global charge, structure and/or mechanism of action: polymyxin B, ApoEdpL-W and, to a lesser extent, LL-37, all predicted to target membranes, activate Rcs, Cpx and σE envelope stress pathways, whereas K4K20-S4 dermaseptin activated both Rcs and Cpx systems, but not the σE pathway. Finally, melittin led to only weak activation of Cpx and σE pathways. These results are consistent with a recent study showing that in S. enterica sv Typhimurium, the non-canonical Rcs pathway is activated by polymyxin B and several other AMPs, but not by envelope-permeabilizing agents (SDS, EDTA, Triton X-100) or polyamines, suggesting that Rcs activation requires detection of specific outer membrane alterations induced by peptides rather than mere global membrane permeabilization (53).

Antimicrobial peptides represent a promising source of novel anti-infection molecules (2). Using several AMPs, we demonstrated that the Cpx pathway is involved in resistance to ApoEdpL-W, polymyxin B or melittin and potentially other predicted to membrane-acting antimicrobial peptides. These results therefore show that the study of peptide-specific genetic responses induced by different antimicrobial peptides improves our fundamental understanding of peptide mode of action, while providing key insights on potential bacterial resistance mechanisms to antimicrobial peptides.
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REFERENCES


### Table 1. Strains and plasmids used in this study.

<table>
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<th>Name</th>
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<td>P1 transduction from JW2205 (Keio collection) into MG1655</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>MGΔdegP::Kmfrt</td>
<td>P1 transduction from JW0157 (Keio collection) into MG1655</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655 F'</td>
<td>Biofilm-forming strain</td>
<td>Tet&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(37)</td>
</tr>
<tr>
<td>MG1655F'let ΔcpxR::Kmfrt</td>
<td>P1 transduction from MGΔcpxR::Kmfrt into MG1655 F'</td>
<td>Tet&lt;sup&gt;a&lt;/sup&gt;, Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655F'let ΔdegP::Kmfrt</td>
<td>P1 transduction from JW0157 (Keio collection) into MG1655 F'</td>
<td>Tet&lt;sup&gt;a&lt;/sup&gt;, Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>SK1938 (manC-lacZ)</td>
<td>MG1655 ΔlacBΔ(cpxB::lacZ)</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(63)</td>
</tr>
<tr>
<td>SK1941 (manC-lacZ ΔrcsB)</td>
<td>MG1655 ΔlacBΔ(cpxB::lacZ) ΔrcsB::cat</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;, Cm&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(64)</td>
</tr>
<tr>
<td>degP-lacZ ΔpsR</td>
<td>P1 transduction from MGΔcpxR::Kmfrt into degP-lacZ</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>BW25113</td>
<td>Parental strain of the Keio collection mutants</td>
<td>-</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔcpxA</td>
<td>BW25113 ΔcpxA::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔdegP</td>
<td>BW25113 ΔdegP::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔmdtA</td>
<td>BW25113 ΔmdtA::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>Δydi</td>
<td>BW25113 Δydi::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔompF</td>
<td>BW25113 ΔompF::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔnanC</td>
<td>BW25113 ΔnanC::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔompC</td>
<td>BW25113 ΔompC::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔcpxP</td>
<td>BW25113 ΔcpxP::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔacrD</td>
<td>BW25113 ΔacrD::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔybaJ</td>
<td>BW25113 ΔybaJ::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>ΔydeE</td>
<td>BW25113 ΔydeE::Kmfrt</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(31)</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pBAD18</td>
<td>Cloning vector, arabinose inducible promoter</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(66)</td>
</tr>
<tr>
<td>pBAD-oriE</td>
<td>nlpE gene cloned in pBAD18</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCA24N</td>
<td>High copy number plasmid with an IPTG-inducible promoter</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(67)</td>
</tr>
<tr>
<td>pCA24N-cpxR</td>
<td>E. coli cpxR cloned in pCA24N</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(67)</td>
</tr>
<tr>
<td>pCA24N-degP</td>
<td>E. coli degP cloned in pCA24N</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(67)</td>
</tr>
<tr>
<td>pCA24N-rcsA</td>
<td>E. coli rcsA cloned in pCA24N</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(67)</td>
</tr>
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</table>
Table 2. Impact of ApoEdpL-W on growth of *E. coli* mutants impaired in the Cpx pathway response.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Inactivated function</th>
<th>MIC (μM)</th>
</tr>
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<tbody>
<tr>
<td>BW25113</td>
<td>Wild type (Keio)</td>
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<tr>
<td>ΔacrD</td>
<td>Component of the AcrAD-TolC multidrug efflux transport system</td>
<td>5</td>
</tr>
<tr>
<td>ΔcpxP</td>
<td>Negative regulator of Cpx response</td>
<td>5</td>
</tr>
<tr>
<td>ΔdegP</td>
<td>Periplasmic serine endoprotease</td>
<td>2.5</td>
</tr>
<tr>
<td>ΔmdtA</td>
<td>Component of the MdtABC multidrug efflux transport system</td>
<td>5</td>
</tr>
<tr>
<td>ΔnanC</td>
<td>N-acetylneuraminic acid outer membrane channel</td>
<td>5</td>
</tr>
<tr>
<td>ΔompC</td>
<td>Outer membrane porin C</td>
<td>5</td>
</tr>
<tr>
<td>ΔompF</td>
<td>Outer membrane porin F</td>
<td>5</td>
</tr>
<tr>
<td>Δspy</td>
<td>Periplasmic protein related to spheroblast formation</td>
<td>5</td>
</tr>
<tr>
<td>ΔtomB (∆ybaJ)</td>
<td>Hha toxin overexpression modulator</td>
<td>5</td>
</tr>
<tr>
<td>∆ydeH</td>
<td>Diguanylate cyclase</td>
<td>5</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Impact of ApoEdpL-W on planktonic and biofilm E. coli bacteria.
(A) E. coli growing cells were exposed to 0 and 5 μM of ApoEdpL-W for 80 min during which samples were taken every 20 min, serially diluted and plated on LB plates. Percent of survival was calculated by CFU counting and compared to numbers obtained at t=0 min. (B) 24 h MG1655 F’ biofilm was treated with increasing concentrations of ApoEdpL-W for 24 h. Viable cells of treated biofilm population were quantified by CFU enumeration and were compared to numbers obtained prior to ApoEdpL-W treatment. Percent survival values represented at least 3 replicates. Statistical analysis: *p<0.05 and **p<0.01 by two-tailed unpaired Student’s t-test.

Figure 2. Localization of fluorescent ApoEdpL-W in the envelope of E. coli cells.
E. coli growing cells were exposed to 3 μM of Fluo-ApoEdpL-W for 30 min and observed by epifluorescence microscopy (Eclipse E400, Nikon). (A) DAPI staining. (B) Fluo-ApoEdpL-W. (C) Merged images.

Figure 3. Induction of ResCDB, CpxAR and σE pathways in response to ApoEdpL-W.
(A) β-galactosidase activity measurements of lacZ transcriptional fusion in genes belonging to each regulon with and without the regulator deletion. (B) β-galactosidase activity measurements of degP-lacZ fusion with and without cpxR deletion and/or decrease of σE activity by overexpressing the repressor RseA. In the latter case, experiments were carried out in MH medium plus chloramphenicol and IPTG at 0.01mM. E. coli growing cells carrying the different reporter fusions were exposed to 0 and 3 μM of ApoEdpL-W for 30 min and β-galactosidase activities were measured as described in Materials and methods. Statistical analysis: *p<0.05, **p<0.01 and ***p<0.001 by two-tailed unpaired Student’s t-test.

Figure 4. Role of the CpxAR system in planktonic bacteria tolerance to ApoEdpL-W.
Wild-type strain, its corresponding cpxR mutant and the complemented strain were grown in MH medium plus chloramphenicol and IPTG (0.01 mM) until reaching OD600 0.1. They were exposed to 0 and 5 μM of ApoEdpL-W for 80 min, during which samples were taken every 20 min.
Survival of each strain was estimated by CFU counting and compared to numbers obtained prior to ApoEdpL-W treatment. Statistical analysis: asterisks indicate values significantly different from wild type strain by the two-tailed unpaired Student’s t-test: *p<0.05.

Figure 5. Impact of the CpxAR system on E. coli biofilm tolerance to ApoEdpL-W.

24 h biofilms formed by wild-type strain and corresponding cpxR and degP mutants were treated with 0 and 400 μM of ApoEdpL-W (80 x MIC). Bacterial survival was estimated after 24 h of treatment by viable cell counts. Percent survival represents viable cells after 24 h of treatment compared to untreated biofilm prior to addition of ApoEdpL-W. Statistical analysis: *p<0.05 and **p<0.01 by two-tailed unpaired Student’s t-test.

Figure 6. Induction of RcsCDB and/or CpxAR pathways in response to different cationic antimicrobial peptides.

β-galactosidase activity measurements of lacZ transcriptional fusion in genes belonging to each regulon with and without the regulator deletion after exposure for 30 min to 0.1 mg/l polymyxin B (0.8 x MIC) (A), 5 mg/l melittin (0.8 x MIC) (B), 4 mg/l LL-37 (0.65 x MIC) (C) and 0.1 mg/l K4K20-S4 dermaseptin (0.5 x MIC) (D). Statistical analysis: *p<0.05, **p<0.01 and ***p<0.001 by two-tailed unpaired Student’s t-test.

Figure 7. Impact of RcsCDB and/or CpxAR systems on E. coli planktonic bacteria tolerance to different cationic antimicrobial peptides.

Wild-type strain and corresponding cpxR and rcsB mutants were grown in MH medium until reaching OD<sub>600</sub> 0.1. They were then exposed for 60 min to 0 and 4 x MIC of polymyxin B (A) or 0 and 2 x MIC of melittin (B). Survival of each strain was estimated by CFU counting and compared to values obtained prior to AMP treatment. Statistical analysis: *p<0.05 and ***p<0.001 by two-tailed unpaired Student’s t-test.