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Published in:
Molecular Therapy - Nucleic Acids

DOI:
10.1016/j.omtn.2021.06.009

Publication date:
2021

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Frimodt-Møller, J., Koulouktsis, A., Charbon, G., Otterlei, M., Nielsen, P. E., & Løbner-Olesen, A. (2021). Activating the Cpx response induces tolerance to antisense PNA delivered by an arginine-rich peptide in Escherichia coli. Molecular Therapy - Nucleic Acids, 25, 444-454. https://doi.org/10.1016/j.omtn.2021.06.009
Activating the Cpx response induces tolerance to antisense PNA delivered by an arginine-rich peptide in *Escherichia coli*

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Cell-penetrating peptides (CPPs) are increasingly used for cellular drug delivery in both pro- and eukaryotic cells, and oligoarginines have attracted special attention. How arginine-rich CPPs translocate across the cell envelope, particularly for prokaryotes, is still unknown. Arginine-rich CPPs efficiently deliver antimicrobial peptide nucleic acid (PNA) to its intracellular mRNA target in bacteria. We show that resistance to PNA conjugated to an arginine-rich CPP in *Escherichia coli* requires multiple genetic modifications and is specific for the CPP part and not to the PNA part. An integral part of the resistance was the constitutively activated Cpx-envelope stress response system (cpx*), which decreased the cytoplasmic membrane potential. This indicates an indirect energy-dependent uptake mechanism for antimicrobials conjugated to arginine-rich CPPs. In agreement, cpx* mutants showed low-level resistance to aminoglycosides and an arginine-rich CPP conjugated to a peptide targeting the DNA sliding clamp, i.e., similar uptake in *E. coli* for these antimicrobial compounds.

INTRODUCTION

Antimicrobial resistance is one of the major challenges of the 21st century. Thus, efforts to bring novel antimicrobial compounds, including antimicrobial peptides (AMPs), into clinical use are accelerating. Antisense technology, as a gene-targeted precision drug modality, has recently produced several new drugs in clinical use (e.g., nusinersen, inotersen, valonesorsen, and golodirsen), and agents based on the pseudopeptide DNA mimic peptide nucleic acid (PNA) and phosphorodiimide morpholino oligomer (PMO) have been proposed as future antibiotics. The hydrophobic nature of cellular membranes makes them impermeable for most proteins, peptides, and oligonucleotides, including PNA and PMO. Hence, these require a carrier molecule (e.g., cell-penetrating peptide [CPP]) for efficient translocation into the cytoplasm. CPPs are generally short oligomers rich in basic (lysine and arginine) and hydrophobic amino acids. Polyarginine enters the cell more efficiently than other polycationic homopolymers (including polylysine), and arginine-rich CPPs are also the most extensively employed and studied. Nonetheless, it is poorly understood how these translocate into both eukaryotic and prokaryotic cells. In eukaryotes, evidence supports predominantly an energy-dependent endocytotic pathway, although energy-independent direct translocation may also play a role. Very little is known about how CPPs conjugated to PNA/PMO/AMPs penetrate through the lipid membranes of the envelope in prokaryotes, and for CPPs conjugated to PNA (CPP-PNA) only one paper has addressed this topic. Here, PNA conjugated to the lysine-phenylalanine-rich CPP L((KFF)₉)K enters *Escherichia coli* by the non-essential inner membrane transporter SbmA; consequently, SbmA-deficient *E. coli* are highly resistant to PNA delivered by this peptide. On the other hand, PNA conjugated to an arginine-rich CPP, (R-Ahx-R)₄-Ahx-(βAla) (RXR), enters the cell in an unknown and SbmA-independent manner.

The envelope of Gram-negative bacteria consists of three structurally and chemically diverse layers: (1) the inner/cytoplasmic membrane (consisting of phospholipids); (2) the periplasm containing a thin peptidoglycan layer; and (3) the outer membrane that contains phospholipids in the inner leaflet and phospholipids and lipopolysaccharide (LPS) in the outer leaflet. The role of the Gram-negative envelope is multifaceted, i.e., it is a barrier that prevents toxic molecules, such as antimicrobials, from entering, meanwhile facilitating the entry of molecules vital for growth (by either dedicated transport or diffusion). When faced by antimicrobial agents, bacteria are able to adapt and survive this selective pressure in numerous ways, including (over)
expression of efflux pumps, drug target modification, modification of the cell envelope, inactivation, or modification of the drug.\

E. coli can actively transport compounds across the cytoplasmic membrane using either ATP hydrolysis or the chemical proton gradient (part of the proton motive force [PMF]), which is generated by translocating protons from the cytoplasm into the periplasmic space, via the electron transport chain. The energetics of the cell varies considerably between growth with or without oxygen, with oxidative growth conditions providing the highest energy turnover. For instance, PMF is decreased during anoxic growth conditions. Under aerobic conditions the electron transport chain predominantly contains NADH:ubiquinone oxidoreductase complex I (encoded by the nuoABCDEGHJKLMN-operon; NDH-I) and cytochrome bo₃ ubiquinol oxidase (encoded by the cyoABCDE operon; cytochrome bo₃ oxidase). The PMF generated serves as energy storage, which is used to drive, e.g., F₁Fₒ-ATPase, efflux pumps, and transport of metabolites. The PMF across the cytoplasmic membrane is a combination of the electric potential (ΔΨ) and the transmembrane proton gradient (ΔpH: internal pH – external pH). ΔΨ is always negative inside growing neutrophils with a cytosolic pH environment between 6.5 and 7.5 such as E. coli, and ΔΨ decreases as ΔpH increases, i.e., bacteria may regulate ΔΨ and/or ΔpH in order to control PMF.

The Cpx, Bae, Psp, Rcs, and σ⁵² signaling systems detect alterations to the bacterial envelope. These pathways are involved in the biogenesis, maintenance, and repair of the bacterial envelope and thus contribute to cell surface integrity. One of the most well-studied extracytoplasmic stress response systems is the CpxA/R two-component signal transduction system, which when activated controls a number of genes including respiratory enzymes that are downregulated. Like many other histidine kinases, CpxA is localized to the cytoplasmic membrane through two transmembrane helices and contains both periplasmic and cytoplasmic domains. The cytoplasmic response regulator, cpxR, is predicted to encode an OmpR-like transcriptional activator. CpxR consists of an N-terminal receiver domain (phospho-acceptor domain) and a C-terminal DNA-binding domain. Under non-stress conditions the auxiliary regulator CpxP inhibits the Cpx response by a predicted direct interaction with the periplasmic domain of CpxA. Under Cpx-inducing conditions, CpxP is degraded by the periplasmic protease and chaperone DegP. The CpxP relieved CpxA autophosphorylates on a conserved histidine residue (His-248), using ATP as its phosphoryl donor. Subsequently, phosphorylated CpxA donates its phosphoryl group to the conserved aspartate residue (Asp-51) in the N-terminal receiver domain of CpxR. Phosphorylated CpxR binds to DNA and acts as a transcriptional regulator. In addition, CpxA dephosphorylates CpxR-P, which ensures that CpxR remains inactive in the absence of an activating signal. The outer membrane lipoprotein NlpE that senses surface adhesion acts as a “sentry protein” against envelope stress and activates the Cpx-response system through an undefined interaction with CpxA. cpxP is the most highly inducible gene of the Cpx regulon, and expression of this is used as a representative of an activated Cpx response.

Here we show that a constitutive Cpx response, resulting in a decreased ΔΨ across the cytoplasmic membrane, reduces drug delivery by arginine-rich CPPs to their intracellular targets. The decreased ΔΨ also confers cross-resistance to aminoglycosides, indicating a similar uptake mechanism for the two classes of compounds.

RESULTS

To understand bacterial uptake and resistance to PNA conjugated to an arginine-rich CPP, we studied the widely used RXR⁷ (Table 1). Unless stated otherwise, the PNA part always targets translation of the acpP mRNA, encoding the acyl carrier protein, which is essential for fatty acid synthesis. RXR-PNA had a minimum inhibitory concentration (MIC) of 0.5 μM toward E. coli. Because PNA containing two mismatches (PNA_mm) showed no inhibition (up to 16 μM) of bacterial growth, the antibacterial activity of RXR-PNA is specific to the PNA part, whereas the delivery peptide part alone did not confer any antimicrobial effect but only promotes cellular uptake of the conjugated PNA (Table 1). A previous study failed to identify single-gene deletion mutants of E. coli that promoted tolerance to RXR-PNA.⁷ We therefore used adaptive laboratory evolution (ALE; see Materials and methods) to generate RXR-PNA-resistant mutants.

Here, we grew wild-type cells in successively increasing concentrations of RXR-PNA, selecting for mutation(s) that enabled survival at high concentration. The ALE experiments were done in two independent lineages (i.e., lineage one and two) and was terminated after 20 successive passages, where clones appeared with a 16-fold increase in MIC (from 0.5 μM to 8 μM) (Table 1), which we define as RXR-PNA resistance.

Five individual clones from each lineage at day 20 (ten clones in total) were isolated and sequenced (Table 2; Tables S1 and S2). From the ten resistant clones, we identified three unique genotypes, which were annotated Evo-1, Evo-2, and Evo-3. Evo-1 and 2 were present in two and three clones of lineage one, respectively, whereas the Evo-3 genotype was found in all five sequenced clones of lineage two. None of the mutations identified in the ALE experiment resulted from adaptation to the growth medium, as they were not present in wild-type cells adapted to the same growth medium without RXR-PNA (Table S3).

All sequenced clones shared an IS5 insertion in the gltF-yhcA intergenic region, IS1 insertions in waaB and waaO, and the rpsL_122N mutation (Table 2). gltF belongs to the gltBDF-operon, where gltB and gltD encode the large and small subunit of glutamate synthase, respectively. The function of GltF and the hypothetical protein YhcA is unknown. The waa-operon encodes enzymes for assembly of the major core oligosaccharide of LPS. The rpsL is the S12 protein, a component of the 30S subunit of the ribosome, where it plays a role in translational accuracy. The role of rpsL_122N in tolerance to RXR-PNA will not be pursued here.
MIC determinations for peptide-PNA are in μM. MICs were determined with broth dilutions (see Materials and methods) at 37°C (no shaking).

Table 1. Minimum inhibitory concentration to peptide-PNA (in μM).

| Compound | CPP | Target gene/protein | Wild type | RXR-PNA resistant strains |
|----------|-----|---------------------|-----------|--------------------------|
| RXR-PNA | arginine-rich RXR | acpF | 0.5 | 8 | 8 | 8 |
| RXR-PNA\textsubscript{mm} | arginine-rich RXR | acpF mismatch<sup>a</sup> | >16 | >16 | >16 | >16 |
| RXR-PNA\textsubscript{ms} | ftsZ | 0.5 | 8 | 8 | 8 |
| RXR-PNA\textsubscript{ms-mm} | ftsZ mismatch<sup>b</sup> | >16 | >8 | >16 | >16 |
| R11-APIM<sup>c</sup> | arginine-rich R11 | [ftsZ clamp [dnaN]<sup>d</sup>] | 1 | N/A | N/A | N/A |
| KFF-PNA | lysine-phenylalanine KFF | acpF | 0.25 | 0.25 | 0.25 | 0.25 |
| KFF-PNA\textsubscript{mm} | acpF mismatch<sup>e</sup> | >16 | >16 | >16 | >16 |

Resistance to RXR-PNA is toward the arginine-rich CPP part and not the PNA part

The Evo-1, Evo-2, and Evo-3 mutants were also resistant to RXR conjugated to a PNA targeting the essential division protein ftsZ; however, they remained sensitive to a PNA conjugated to the lysine-phenylalanine-rich CPP, L((KFF)<sub>3</sub>)<sub>K</sub>, KFF-PNA targeting acpF (Table 1). Thus, the RXR-PNA resistance of Evo-1, Evo-2, and Evo-3 followed the CPP delivery peptide and not the PNA part, supporting the notion of a different uptake mechanism for the RXR peptide compared to KFF, which is transported across the cytoplasmic membrane by SbmA<sup>f</sup>.

No apparent role for Glf or YhcA in tolerance to RXR-PNA

To understand the effect of the conserved IS5 insertion in the glfF-yhcA intergenic region on tolerance to RXR-PNA, glfF and yhcA were individually deleted in the wild-type cell, resulting in ΔglfF and ΔyhcA, respectively. However, neither glfF nor yhcA deficiency had any effect on susceptibility to RXR-PNA compared to wild type (Figure 1A). Additionally, overproduction of glfF or yhcA, in the wild-type cells, led to no changes in MIC toward RXR-PNA (Figure 1A). Hence, the importance of the IS5 insertion in the glfF-yhcA intergenic region to RXR-PNA tolerance is presently unknown.

The outer membrane is not the main barrier for RXR-PNA

During uptake the arginine-rich delivery peptide RXR must first interact with the outer membrane, which is the main barrier for some compounds, including caticnic (antimicrobial) peptides.<sup>g</sup> Evo-1, Evo-2, and Evo-3 mutants all had IS1 insertions in waaB and waaO, expected to result in LPS with a deficient outer membrane. We therefore deleted waaBO in the wild-type cells but observed no changes in susceptibility to RXR-PNA (Figure 1A). Hence, the outer membrane provides a limited or no barrier for RXR-PNA, although the magnitude was smaller (Figure 1C);
The Cpx response is only one of five extracytoplasmic stress response systems known to maintain cell envelope integrity during stress. Whereas the Cpx response can be activated by overproducing nlpE, the four other extracytoplasmic stress responses can be individually activated by overproduction of BaeR for the Bae pathway, RcsB for the Rcs pathway, PspF for the Psp pathway, and RpoE for the σE pathway. Expression of the cloned genes was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). Because overexpression of PspF and RpoE is toxic, we determined the minimal IPTG concentration that resulted in activation of the extracytoplasmic stress responses with minimal cell toxicity. RcsB and BaeR synthesis was induced with 0.1 mM IPTG, whereas PspF and RpoE synthesis was induced with 0.01 mM IPTG (Figure S1). Overexpression of RpoE appeared particularly toxic to the cell (Figure S1), and we cannot exclude that suppressor mutations had formed; nor can we exclude plasmid loss and/or rearrangement. Activation of neither the Bae, Psp, Rcs, nor the σE responses led to an altered susceptibility to RXR-PNA compared to the wild type (Figure 2B), supporting that this is specific to the activated Cpx response. The presence of RXR-PNA at sub-MIC concentrations (0.5 × MIC) did not activate the Cpx response in wild-type cells (no increased cpxP expression; Figure 2A). Hence, although an activated Cpx response confers RXR-PNA tolerance, low-level RXR-PNA treatment in itself does not trigger the extracytoplasmic stress response.

### Cpx-dependent downregulation of respiratory operons leads to tolerance to antimicrobials delivered by arginine-rich CPPs and to aminoglycosides

We determined nuaA (to evaluate expression of the nuoABCDE-GHIJKLM operon) and cyoA (to evaluate expression of the cyoABCDE operon) transcription by qRT-PCR. Both operons were significantly downregulated in cells with a constitutively activated Cpx response: NlpE overproduction: cpxR<sub>20Q</sub>, cpxA<sub>Δ16-17</sub>, and Evo-3 cells (Figure 3A). In agreement, an activated Cpx response has been reported to downregulate aerobic respiratory operons. When the entire nuoABCEFHIJKLM operon or cyoABCD operon was individually deleted in wild-type cells, the resultant Δnuo and Δcyo strains conferred low-level resistance to RXR-PNA, R11-APIM (Figure 3B), as well as the aminoglycosides gentamicin, amikacin, and kanamycin (Figure 3C). This shows that low-level resistance to both arginine-rich CPPs and aminoglycosides in cpx<sup>+</sup> cells results from reducing oxidative phosphorylation through downregulation of NDH-I and cytochrome bo<sub>3</sub> oxidase.

### The cytoplasmic membrane potential correlates with RXR-PNA, R11-APIM, and aminoglycoside activity

We determined the cytoplasmic membrane potential (ΔΨ) at the intracellular pH 7.6 based on the distribution of the lipophilic tetraphenylphosphonium ion (TPP⁺), using a TPP⁺-selective electrode. At this pH value, ΔpH is zero and the PMF is equal to ΔΨ. Wild-type

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**Table 2. RXR-PNA resistant genotypes**

| Mutation type<sup>a</sup> | Region/gene<sup>b</sup> | Strain | Lineage<sup>c</sup> | No. | Evo-1 | Evo-2 | Evo-3 |
|--------------------------|--------------------------|--------|----------------------|-----|-------|-------|-------|
| IS DEL SNP               |                          |        |                      |     |       |       |       |
| IS1                      | IG: chbb-oomE            | x      |                      | 2/5 | 3/5   | 5/5   |       |
| IS5                      | IG: gcf-yhcA             | x      |                      |     | x     |       |       |
| IS1                      | rapA                     | x      |                      |     | x     | x     |       |
| IS1                      | waaB                     | x      |                      |     | x     |       |       |
| IS1                      | waaO                     | x      |                      |     | x     | x     |       |
| x                       | yfaP<sub>Y357G</sub>     | x      |                      |     |       |       |       |
| x                       | yfID<sub>AD30</sub>      | x      |                      |     |       |       | x     |
| x                       | bdcA<sub>G37T</sub>      | x      |                      |     |       |       | x     |
| x                       | cpxRL<sub>20Q</sub>      | x      |                      |     |       |       | x     |
| x                       | Δrac                    | x      |                      |     |       |       | x     |

<sup>a</sup>Mutation type; IS, IS insertion; DEL, deletion; SNP, single-nucleotide polymorphism.

<sup>b</sup>Mutated genomic region; IG, intergenic region between two genes; Δrac, excised rac prophage.

<sup>c</sup>Lineage from which the evolved clones were isolated within the ALE experiment with RXR-PNA.

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To assess whether the cpxA<sub>Δ16-17</sub> and cpxR<sub>20Q</sub> mutations were dependent on a functional Cpx system for low-level resistance to RXR-PNA, cpxA was deleted in the cpxR<sub>20Q</sub> strain and cpxR was deleted in the cpxA<sub>Δ16-17</sub> strain. In either case, the MIC for RXR-PNA was reduced to wild-type level (Figure 1B). Thus, the substitution to glutamine in position 20 in CpxR does not make it constitutive. This con

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**The Cpx response is activated in cpxA<sub>Δ16-17</sub> and cpxR<sub>20Q</sub> and is the only extracytoplasmic stress response conferring tolerance to RXR-PNA**

We used cpxP transcription as a readout for activation of the cpx response, and both cpxR<sub>20Q</sub> and cpxA<sub>Δ16-17</sub> and Evo-3 all had significant increased expression of cpxP compared to wild-type (Figure 2A), albeit not to the level achieved by overproduction of NlpE (full activation of the Cpx response; Figure 2A). This confirmed that the Cpx response was indeed activated by the cpxR<sub>20Q</sub> and cpxA<sub>Δ16-17</sub> mutations. Accordingly, NlpE overproduction also provided tolerance to RXR-PNA to the same level (compare Figure 1B to Figure 2B). Both cpxR<sub>20Q</sub> and cpxA<sub>Δ16-17</sub> are therefore gain-of-function mutations leading to a constitutively active Cpx response, which previously have been denoted cpx<sup>+</sup>.
cells had a $\Delta \Psi$ of approximately $-140$ mV, in accordance with previous observations \cite{29} (Figure 4A). The $\Delta \Psi$ was significantly reduced by the $\text{cpx} \alpha_{120Q}$ as well as the $\text{cpxA}_{16-17}$ mutation (Figure 4A). $\Delta\text{cyo}$ and $\Delta\text{nuo}$ cells also had a significantly decreased $\Delta \Psi$ compared to wild type, with values comparable to $\text{cpx} \alpha_{120Q}$ and $\text{cpxA}_{16-17}$ (Figure 4A). Thus, it is conceivable that the decreased $\Delta \Psi$ of $\text{cpx} \alpha_{120Q}$ and $\text{cpxA}_{16-17}$ cells results from downregulation of the respiratory operons.

When wild-type cells are grown anaerobically or in acidic medium (pH 6), the $\Delta \Psi$ part of the PMF is decreased \cite{10} (Figure 4B). This is also the case when cells are treated with carbonyl cyanide $m$-chlorophenyl hydrazine (CCCP) (Figure 4C), which uncouples the proton gradient because of its ability to act as a ionophore. \cite{32} All treatments that reduced $\Delta \Psi$ resulted in decreased sensitivity to RXR-PNA, R11-APIM, and aminoglycosides (Figures 4B and 4C). On the other hand, when the $\Delta \Psi$ part of the PMF is increased by growing in an alkaline medium (pH 8.0) \cite{10} (Figure 4D), by deletion of the $\text{F}_{1}$-$\text{F}_{0}$ ATPase \cite{33} (Figure 4D), or if growth is supplemented with alanine \cite{34} (Figure 4E), sensitivity to RXR-PNA, R11-APIM, and aminoglycosides was increased. Moreover, sensitivity to KFF-PNA was not affected by changes of the $\Delta \Psi$ across the cytoplasmic membrane (Figures 4B and 4D), in agreement with a different uptake mechanism for the KFF peptide.

**Neither $\text{cpx} \alpha_{120Q}$ nor the $\text{cpxA}_{16-17}$ mutations conferred resistance to cationic antimicrobial peptides**

Cationic AMPs represent the biggest class of AMPs, and the majority of these are amphiphilic. Here, Cap11, Cap18, cecropin P1, apidaecin 1B, indolicidin, protamine, and the most well-known polypeptide antibiotic, colistin, were tested against wild type, $\text{cpx} \alpha_{120Q}$, $\text{cpxA}_{16-17}$, and Evo-3 (Table 3). Colistin, \cite{35} Cap11, \cite{26} Cap18, \cite{26,36} protamine, \cite{37} and cecropin P1 \cite{38} are believed to target the cell envelope (disruption of bilayers), whereas indolicidin (disruption of bilayers and DNA synthesis) \cite{39} and apidaecin 1B (ribosomes) \cite{40} have intracellular targets. An activated Cpx response and a decrease in $\Delta \Psi$ did not confer tolerance to any of the tested AMPs (Table 3). Interestingly, the evolved strain Evo-3 was more susceptible to cecropin P1, Cap11, Cap18, and colistin than wild type (Table 3), most likely due to the presence of IS1 insertions in $\text{waaB}$ and $\text{waaO}$ in the Evo-3 (Table 2). \cite{26} Accordingly, $\Delta\text{waaBO}$ cells also became more susceptible to cecropin P1, Cap11, Cap18, and colistin.

**RXR-PNA resistance is associated with a fitness cost**

The doubling times of $\text{cpx} \alpha_{120Q}$ and $\text{cpxA}_{16-17}$ mutants were 28 min and 27 min, respectively, when grown aerobically in Müller-Hinton I Broth (MHBI) at 37°C, somewhat slower than that of wild-type cells (25 min). Of interest, the evolved mutant, Evo-3 (58 min), which conferred the highest resistance to RXR-PNA, grew more than twice as slowly as both the wild type and the $\text{cpx} \alpha_{120Q}$ and $\text{cpxA}_{16-17}$ mutants. This shows that resistance to RXR-PNA came with a high fitness cost.

**DISCUSSION**

We have isolated and characterized mutants resistant toward (R-Ahx-R$_4$-Ahx-(bAla))-PNA. Obtaining these proved difficult, most likely because it required additive mutations, and came with a high fitness cost, showing promise for the possible future medical use of CPP-PNA. The increased resistance to RXR-PNA was in part due to a constitutively active Cpx response, which mediates a decrease in $\Delta \Psi$ across the cytoplasmic membrane. This phenotype conferred cross-resistance to aminoglycosides and R11-APIM, indicating a similar uptake between the antibacterial compounds. These observations highlight the importance of extracytoplasmic stress response in modulating the cytoplasmic membrane to avoid growth cessation by AMPs with intracellular targets.

**Reduced arginine-rich CPP uptake arises in multiple steps**

The finding that three RXR-PNA-resistant mutants arose across the two lineages in the ALE experiment indicates either multiple ways to confer resistance or that they all confer a similar resistance phenotype. Nonetheless, resistance to RXR-PNA is complex, requiring multiple mutations in independent loci (Table 2). This is in stark contrast to the straightforward route to restrict KFF translocation, by a loss-of-function mutation in the E. coli $\text{sbmA}$ gene, \cite{7} which inhibits uptake across the cytoplasmic membrane. We show that the $\text{cpx}^*$ phenotype...
The $cpx^+$ phenotype relies on a functional Cpx-system

We show that alterations in the first transmembrane domain of CpxA can produce a $cpx^+$ phenotype. Several gain-of-function mutations in cpxA have been reported, but most cluster to and around the second transmembrane domain of CpxA. We suggest that the two-amino acid deletion in the first transmembrane domain leads to a conformational change in the periplasmic part of CpxA, which either disallows CpxP regulation or simulates the stress signal that activates the Cpx response, i.e., a constitutive active CpxA. To the best of our knowledge, $cpxR_{L20Q}$ is the first reported mutation in cpxR resulting in a $cpx^+$ phenotype. The activated Cpx response in the $cpxR_{L20Q}$ mutant was CpxA dependent; i.e., the $cpxR_{L20Q}$ mutation does not result in a constitutive phosphorylated CpxR. Therefore, it is likely that the $cpxR_{L20Q}$ mutation inhibits/diminishes the ability of CpxA to dephosphorylate CpxR-P to CpxR, which in turn results in a constitutive active Cpx response. CpxA and CpxR-P both function as dimers. Thus overproducing the respective wild-type allele in the two mutants restored sensitivity to RXR-PNA by either hetero-dimerization of a mutated and a wild-type protein, with the wild type being dominant to the mutant, or by homo-dimerization of either mutated or wild-type proteins but with wild-type dimer domination due to abundance.

The cytoplasmic membrane is the main barrier for arginine-rich CPP translocation

Loss of waaBO and gltF, not all mutations from the ALE experiment on their own resulted in low-level resistance to PNA delivered by arginine-rich CPPs. Either these mutations require another mutation to synergistically enable low-level resistance to RXR-PNA or they were selected as mutations that compensate the fitness cost observed in Evo-1 to 3. Indeed, strains with the highest MIC observed to RXR-PNA have increased doubling time, highlighting that this comes with a cost.
previously found to determine aminoglycoside uptake across the cytoplasmic membrane in *Bacillus subtilis* and *E. coli* but has never been reported for arginine-rich CPP translocation in bacteria. In wild-type cells, RXR-PNA (and possibly also aminoglycosides and R11-APIM) does not significantly activate the Cpx response. When delivered to the inside of the bacterial cell, the antimicrobial part arrests growth; aminoglycosides by interacting with the 30S subunit of ribosomes, PNA by preventing *acpP* translation, and APIM by preventing *β*-clamp function (Figure 5A). We propose that *cpx*-dependent low-level resistance to RXR-PNA, R11-APIM, and aminoglycosides relies on a decreased ΔΨ component of the PMF, resulting from downregulation of respiratory operons resulting in a reduction in the electric potential. The latter also explains cross-resistance between antimicrobials delivered by arginine-rich peptides and aminoglycosides. Two newly identified Cpx mutations give further insight into sensing and activation of the Cpx system.

**MATERIALS AND METHODS**

Additional materials and methods are found in Supplemental materials and methods.

**Growth conditions**

All strains are listed in Table S4. Cells were grown in Luria-Bertani Broth (LB) medium or Müller-Hinton I Broth (MHBI) at 37°C with aeration unless stated otherwise. When necessary, antibiotics were added to the following concentrations: chloramphenicol, 20 μg/mL; ampicillin, 150 μg/mL; kanamycin, 50 μg/mL; tetracycline, 10 μg/mL.

**Peptide conjugates**

PNA-peptide conjugates were synthesized as described previously. R11-APIM (RWLVK* with the complete sequence Ac-MD-RWLVK-GILQWRKI-RRRRRRRRRR) has been described previously.

**Adaptive laboratory evolution experiment**

Wild type was evolved for 20 days to CPPRXR-PNA, with two lineages evolved in parallel for each drug. Here, 1 × 10⁵ colony-forming units (CFU)/mL of cells were inoculated in 2-fold peptide-PNA gradient in 8 dilutions in 100 μL of MHBI in a 96-well polystyrene microtiter plate.
and grown for 18–24 h at 37°C. The following day, the population grown in the highest concentration of peptide-PNA were re-diluted to 1 × 10^5 CFU/mL and inoculated in a fresh 2-fold peptide-PNA gradient as above. After 20 successive passages, five peptide-PNA-resistant single clones were isolated from each lineage and whole genome sequenced.

As control, E. coli MG1655 was evolved to the MHBI in four independent biological replicates without the presence of peptide-PNA. Here, two clones were isolated and whole genome sequenced for each lineage.

**Whole genome sequencing and data analysis**

Reads were mapped to MG1655 NC_000913.3 with BWA-MEM. Variants were extracted with Freebayes (only variants with >50% frequency were retained). For location of IS insertion, mapped reads with <60 mapQuality were selected and paired reads (CIGAR-Left-clip/Right-Clip, CIGAR-D and CIGAR-I) were aligned to NC_000913.3 by using NCBI Blast to confirm deletion/insertion.

**Antimicrobial susceptibility testing**

MIC values were determined by broth microdilution according to the standard protocol with a few modifications. Briefly, an overnight bacterial cell culture was diluted to ~5 × 10^5 CFU/mL in MHBI. Peptide-PNAs were dissolved in 0.02% acetic acid containing 0.4% bovine serum albumin (BSA), and dilutions were performed in 0.01% acetic acid containing 0.2% BSA, while gentamicin, amikacin, kanamycin, cecropin P1, Cap11 (GenScript), Cap18 (GenScript), apidaecin 1B (GenScript), colistin, indolicidin, and protamine (Sigma-Aldrich) were diluted according to vendors’ specifications. To determine MICs under acidic and alkaline conditions, MHBI was
buffered to pH 6.0 or pH 8.0 with citrate buffer (pH 3.0) and carbonate-bicarbonate buffer (pH 10.0), respectively. Anaerobic growth for MIC determinations was performed in a double-sealed bag, using anaerobic atmosphere generation bags (Becton Dickinson, Franklin Lakes, NJ, USA). MHBI was supplemented with 0.1 mM IPTG when tested strains harbored pCA24n-based plasmids, except for pCA24n:pspF and pCA24n:rpoE, which were induced with 0.01 mM IPTG.

Antibiotic bacteriocidal assay using L-alanine
The effect of alanine on tolerance to RXR-PNA and aminoglycosides was tested as previously described by Peng et al.,44 with a few modifications. Briefly, overnight cultures were collected by centrifugation at 8,000 rpm for 5 minutes, washed twice in 0.9% NaCl solution, and resuspended to an OD600 of 0.5 in ABT minimal medium. Samples were then diluted to 5 × 10⁵ CFU/mL in 10 mL of the same medium supplemented with or without 100 mM alanine and RXR-PNA or gentamicin. After 10 h of incubation at 37°C, an aliquot was periodically removed, serially diluted in 0.9% NaCl, spotted onto LB agar plates, and incubated at 37°C overnight, and CFU were calculated. The data were normalized by dividing the CFU obtained from a treated sample by the CFU obtained from the control sample. The study was performed in a biological triplicate.

Antibiotic bacteriocidal assay using carbonyl cyanide m-chlorophenyl hydrazone
Time-kill curves were performed for RXR-PNA and gentamicin, as previously described,45 with minor modifications. Shortly, overnight buffered to pH 6.0 or pH 8.0 with citrate buffer (pH 3.0) and carbonate-bicarbonate buffer (pH 10.0), respectively. Anaerobic growth for MIC determinations was performed in a double-sealed bag, using anaerobic atmosphere generation bags (Becton Dickinson, Franklin Lakes, NJ, USA). MHBI was supplemented with 0.1 mM IPTG when tested strains harbored pCA24n-based plasmids, except for pCA24n:pspF and pCA24n:rpoE, which were induced with 0.01 mM IPTG.

**Table 3.** cpXRLR_{20Q} and cpxA_{Δ16-17} do not provide tolerance to cationic antimicrobial peptides

| AMP/strain | Wild type | cpxA_{Δ16-17} | cpXRLR_{20Q} | ΔnuoBO | Evo-3 |
|------------|-----------|---------------|--------------|--------|------|
| RXR-PNA | 0.5 | 4 | 4 | 0.5 | 8 |
| Cecropin P1 | 32 | 32 | 32 | 16 | 16 |
| Cap11 | 2 | 2 | 2 | 1 | 1 |
| Cap18 | 2 | 2 | 2 | 1 | 1 |
| Indolicidin | 4 | 4 | 4 | 4 | 4 |
| Apidaecin 1B | 8 | 8 | 8 | 8 | 8 |
| Colistin | 0.125 | 0.125 | 0.125 | 0.0625 | 0.0625 |
| Protonem | 16 | 16 | 16 | 16 | 16 |

MIC values for peptide-PNA (in μM) and AMPs (in μg/mL) for wild type and indicated mutants. MICs were determined with broth dilutions (see Materials and methods) at 37°C (no shaking). Peptide-PNA sequences are listed in Table 1.

**Figure 5.** RXR-PNA, R11-APIM, and aminoglycoside uptake in wild-type and cpx* cells
A schematic model of RXR-PNA, R11-APIM, and aminoglycoside (AG) internalization across the cytoplasmic membrane and action in wild type (A) and a cpXR− mutant (B). (A) In the wild type, RXR-PNA, R11-APIM, and aminoglycoside crossing of the cytoplasmic membrane from the periplasmic space and into the cytosol correlates with the magnitude of the ΔΨ. The respiratory complexes NADH ubiquinone oxidoreductase complex I (NDH-I) transcribed from the nuoABCEFGLHJKLMN operon (nuo) and cytochrome bo ubiquinol oxidase (Cyo) transcribed from the cyoABCD operon (cyo) are shown. RXR-PNA, AG, and R11-APIM in the cytosol lead to growth arrest by steric hindrance of ribosome binding to acpP mRNA for RXR-PNA (Aa), binding to the 30S subunit of ribosomes impairing translational accuracy (Ab), and binding to the β-clamp, respectively. (B) In cpXR− mutant, RXR-
cultures were diluted to 5 × 10^5 CFU/mL in MHBI. Samples were pre-treated with 50 μM CCCP for 5 min. CCCP was dissolved in dimethyl sulfoxide (DMSO), and the same amount of DMSO was added to the control sample. After CCCP treatment RXR-PNA or gentamicin was added at 4 × MIC concentration (time zero). A 100 μL aliquot was periodically removed, serially diluted down to 1,000-fold in 0.9% NaCl, and spotted onto LB agar plates, followed by incubation at 37°C overnight, and CFU/mL was determined. The study was performed in a biological triplicate.

qRT-PCR
For qRT-PCR experiments, after genomic DNA digestion, 1 μg of total RNA was retrotranscribed with the QuantiTect Reverse Transcription Kit (QIAGEN). Primers designed to amplify cpxP, nuoA, and cyoA (Table S5) were targeted to regions of unique sequences within the genes in wild-type E. coli MG1655. The qRT-PCR was performed with TB Green Premix Ex Taq II (Takara Bio, Shiga, Japan) on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). All data were normalized to the reference genes hctA and cysG. These data were transformed to log2 to obtain a change difference (n-fold) between strains.

Determination of ∆Ψ by measuring TPP⁺ uptake with the TPP⁺-selective electrode method
The ∆Ψ was determined by measuring the uptake of the permeating lipophilic cationic probe TPP⁺ (Merck, Germany). An overnight culture was diluted (5,000-fold) in MHBI, and cells from 25 mL of culture were harvested when OD₅₉₅ reached 0.2. Cells were washed twice in 0.1 M Tris-HCl (pH 8.1) under the conditions mentioned above, and the pellets were gently resuspended in 1 mL of the same buffer. To generate cells permeable to TPP⁺, after a first incubation for 6 min at 36°C with occasional agitation, bacteria were treated with K⁺-EDTA to a concentration of 10 mM and then incubated at 36°C for 3 more minutes. Cells were diluted 10-fold in ice-cold 0.1 M potassium phosphate buffer (pH 7.5) containing 200 mM NaCl, 0.14 mM CaCl₂, 0.1 mM MgSO₄, and 0.01 mM MnCl₂. The cells were kept on ice until use.

To form TPP⁺ uptake, after a second incubation for 30 min, the control sample was added to the wells of the plate. The plates were then incubated at 37°C for 30 min. After the incubation, the wells were removed, serially diluted down to 1,000-fold in 0.9% NaCl, and spotted onto LB agar plates, followed by incubation at 37°C overnight, and CFU/mL was determined. The study was performed in a biological triplicate.

Bacteria were added to an OD₅₉₅ of 4.0 in 10 mL of the same buffer supplemented with glucose and TPP⁺ to a concentration of 5 mM and 10 μM, respectively, and TPP⁺ uptake was measured with the TPP⁺-selective electrode method described by Hosoi et al.⁴⁶ TPP⁺ concentration in the external medium was determined with a Kwik-Tip Ag/AgCl half-cell electrode, which was constructed according to the instructions provided by the manufacturer (World Precision Instruments, Sarasota, FL, USA). Both the TPP⁺-selective electrode and a Flexible Dri-Ref reference electrode (World Precision Instruments, Sarasota, FL, USA) were connected to a Jenway 3510 ion meter (Cole-Parmer, Staffordshire, UK) and the LabTrax 4-Channel Data Acquisition system (WPI). Finally, TPP⁺ uptake measurements were carried out in a 15 mL closed tube at 30°C and pH 7.5 for 10 min, and data were recorded with iWorx LabScribe recording and analysis software (iWorx Systems, Dover, NH, USA).

Statistical analysis
Statistical analyses for all experiments were performed with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Data from three independent replicates of all groups and controls were compared with one- or two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple-comparison post-test. Between groups compared, differences were considered significant at a p value of <0.05.

Data and material availability
All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplemental information. Additional data are available from authors upon request.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.06.009.

ACKNOWLEDGMENTS
This paper is dedicated to the memory of Mads Christian Guldbæk (1991–2019). This research was funded by grants from the Danish National Research Foundation (DNRF120), the Novo Challenge Center for Peptide-Based Antibiotics (NNF16OC0021700), A.P. Møller Lægefonden, Augustinus Fonden, Aase og Ejnar Danielsen’s Fond, Brødrene Hartmanns Fond, the program NTNU Health at Norwegian University of Science and Technology (NTNU), and the Trond Mohn foundation, Norway. We are grateful for the acquisition of strains TR530, MC3, GEB658, and CAG16037 from Philippe Bouloc. All figures were created with BioRender.com.

AUTHOR CONTRIBUTIONS
Conceptualization: J.F.-M., P.E.N, and A.L.-O.; data curation: J.F.-M. and A.K.; formal analysis: J.F.-M., A.K., and A.L.-O.; investigation: J.F.-M. and A.K.; methodology: J.F.-M., A.K., G.C., and A.L.-O.; project administration: J.F.-M. and A.L.-O.; resources: J.F.-M., P.E.N., and A.L.-O.; bioinformatics: G.C.; supervision: P.E.N and A.L.-O.; manuscript writing: J.F.-M.; manuscript editing: J.F.-M., A.K., G.C., M.O., P.E.N., and A.L.-O.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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