High-level carbapenem tolerance requires antibiotic-induced outer membrane modifications

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Abstract

Antibiotic tolerance is an understudied potential contributor to antibiotic treatment failure and the emergence of multidrug-resistant bacteria. The molecular mechanisms governing tolerance remain poorly understood. A prominent type of β-lactam tolerance relies on the formation of cell wall-deficient spheroplasts, which maintain structural integrity via their outer membrane (OM), an asymmetric lipid bilayer consisting of phospholipids on the inner leaflet and a lipid-linked polysaccharide (lipopolysaccharide, LPS) enriched in the outer monolayer on the cell surface. How a membrane structure like LPS, with its reliance on mere electrostatic interactions to maintain stability, is capable of countering internal turgor pressure is unknown. Here, we have uncovered a novel role for the PhoPQ two-component system in tolerance to the β-lactam antibiotic meropenem in Enterobacteriales. We found that PhoPQ is induced by meropenem treatment and promotes an increase in 4-amino-4-deoxy-L-arabinose [L-Ara4N] modification of lipid A, the membrane anchor of LPS. L-Ara4N modifications likely enhance structural integrity, and consequently tolerance to meropenem, in several Enterobacteriales species. Importantly, mutational inactivation of the negative PhoPQ regulator mgrB (commonly selected for during clinical therapy with the last-resort antibiotic colistin, an antimicrobial peptide [AMP]) results in dramatically enhanced tolerance, suggesting that AMPs can collaterally select for meropenem tolerance via stable overactivation of PhoPQ. Lastly, we identify histidine kinase inhibitors (including an FDA-approved drug) that inhibit PhoPQ-dependent LPS modifications and consequently potentiate meropenem to enhance lysis of tolerant cells. In summary, our results suggest that
PhoPQ-mediated LPS modifications play a significant role in stabilizing the OM, promoting survival when the primary integrity maintenance structure, the cell wall, is removed.

Author summary

Treating an infection with an antibiotic often fails, resulting in a tremendous public health burden. One understudied likely reason for treatment failure is the development of “antibiotic tolerance”, the ability of bacteria to survive normally lethal exposure to an antibiotic. Here, we describe a molecular mechanism promoting tolerance. A bacterial stress sensor (PhoPQ) is activated in response to antibiotic (meropenem) treatment and consequently strengthens a bacterial protective “shell” to enhance survival. We also identify inhibitors of this mechanism, opening the door to developing compounds that help antibiotics work better against tolerant bacteria.

Introduction

The rapid rise of antibiotic treatment failure threatens our ability to prevent and control bacterial infections. Antibiotic resistance, the continued proliferation of bacteria in the presence of the antibiotic, can often explain failure of clinical therapy. However, the response to an antibiotic is oftentimes more nuanced than a simple dichotomy of resistance vs. susceptibility. Bacteria can survive treatment in a non- or slowly-proliferating state, readily reverting to healthy growth after removal of the antibiotic (such as the end of a treatment course), and this is typically referred to as “antibiotic tolerance” [1–3]. Importantly, tolerance to antibiotics has been shown to enhance the evolution of outright resistance mechanisms [4–6], and can thus serve as both a direct and indirect contributor to treatment failure.

β-lactams are the most widely-prescribed antibiotic class used to treat bacterial infections. The β-lactam ring inhibits the activity of the penicillin-binding proteins (PBPs) through covalent modification of a catalytic residue. PBPs are enzymes that synthesize the cell wall, an essential structure composed mainly of the polysaccharide peptidoglycan (PG). In many well-studied model organisms, PBP inhibition induces cell wall degradation and often subsequent lysis through the action of cell wall degrading enzymes (collectively referred to as “autolysins”) in a poorly-understood manner [2]. While lysis is the canonical response of model organisms like Escherichia coli K12, many formally β-lactam susceptible clinical isolates of Gram-negative pathogens (including prominent clinical isolates belonging to the Enterobacterales, like Klebsiella spp. and Enterobacter spp.) exhibit a unique type of β-lactam tolerance [7–9]. Like E. coli, these cells digest their PG upon exposure to β-lactams. However, instead of lysing, these pathogens survive antibiotic-induced cell wall degradation by forming viable, non-dividing, cell wall-deficient spheroplasts, which presumably rely on the outer membrane to counter their internal turgor. Interestingly, spheroplasts do not absolutely require osmotic stabilization and form in diverse types of growth media, including human serum [9]. This cell wall-deficient phenotype is reminiscent of so-called L-forms [10–12], with the notable distinction that spheroplasts do not divide in the presence of the antibiotic.

Remarkably, spheroplasts formed in response to the carbapenem antibiotic meropenem readily resume growth and revert to wild-type rod shape when the β-lactam is removed from the growth medium [7,9]. Little is known about the molecular mechanisms that facilitate spheroplast formation and survival. In Vibrio cholerae, the two-component system (TCS) VxrAB is
essential for spheroplast recovery by upregulating cell wall synthesis and downregulating iron uptake into the cells, mitigating toxic free iron accumulation induced by β-lactam treatment and allowing the cell to avoid damage by oxidative stress [13,14]. Many questions remain, however, as to how the cell envelope maintains its integrity without a cell wall, the essential structure canonically thought to protect the cell against immense turgor pressure.

In this study, we used Enterobacter cloacae as a model Gram-negative pathogen to investigate genetic factors that contribute to bacterial tolerance to meropenem, which is used as a last-resort β-lactam to treat multidrug-resistant bacterial infections [15–17]. We first show that tolerance is dependent on outer membrane modifications (specifically 4-amino-4-deoxy-L-aminoarabinose [L-Ara4N]) induced by the PhoPQ TCS, an important cell envelope stress sensor that has previously been shown to respond to magnesium limitation, cationic antimicrobial peptide exposure, osmotic challenge, and pH changes [18]. Both PhoPQ regulon transcription and modification of the lipopolysaccharide lipid A domain are induced by meropenem treatment, suggesting a specific response to perturbations of PG synthesis in E. cloacae. These findings represent a novel mechanism of β-lactam tolerance in clinically relevant Enterobacterales, as well as an expanded role for the PhoPQ TCS.

Results

The PhoPQ TCS system regulates carbapenem tolerance

We previously showed that many Gram-negative pathogens are highly tolerant to meropenem. Upon treatment, tolerant cells do not appreciably lyse. Instead, they form viable, enlarged, non-replicating spheroplasts that are devoid of detectable cell wall material [9]. Meropenem-induced spheroplast formation is quantifiable as an OD\textsubscript{600} increase (Fig 1A) with only a moderate concomitant decrease in survival, as measured by colony-forming units (CFU) (Fig 1B). In contrast, non-tolerant bacteria like many E. coli isolates rapidly lyse in the presence of meropenem, indicated by a decrease in both OD\textsubscript{600} and survival (Fig 1A and 1B). Since spheroplast integrity is presumably maintained by the outer membrane, rather than the cell wall, we hypothesized that the strength of the outer membrane might correlate with tolerance. To test this, we repeated the killing experiments in the presence of the known outer membrane fortifying agents Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, which link adjacent lipopolysaccharide molecules by forming ionic bridges between phosphate groups on the lipid A domain [19,20]. Addition of either divalent cation (Mg\textsuperscript{2+} or Ca\textsuperscript{2+}) supported spheroplast formation with a concomitant reduction in lysis during meropenem treatment in a concentration-dependent manner (S1A and S1B Fig), particularly at very high concentrations. Furthermore, combinatorial addition of excess Ca\textsuperscript{2+} and Mg\textsuperscript{2+} completely prevented lysis (S1A Fig). Thus, divalent cations prevent lysis during meropenem treatment, potentially through increasing the mechanical load-bearing capacity of the outer membrane through lipopolysaccharide crosslinking to protect the spheroplast structure.

When divalent cations are limiting, many Gram-negative pathogens induce outer membrane modifications (hyperacylation and/or increasing the positive charge of lipid A) to functionally substitute for divalent ionic bridges between lipopolysaccharide molecules [21]. In many Enterobacterales, these modifications are controlled by the well-studied PhoPQ two-component system (TCS) [18]. As shown in E. cloacae [22] and other Enterobacterales [23,24], the PhoPQ TCS directly regulates expression of the \textit{arn} operon, which encodes enzymes that synthesize and transfer positively charged L-Ara4N moieties to lipid A, and partially regulates expression of \textit{pagP} (coding for an outer membrane acyltransferase) [25]. In our strain of \textit{E. cloacae}, the PhoPQ system is required to sustain “heteroresistance”, i.e. background resistance of a small subpopulation of cells against therapeutic antimicrobial peptides like colistin [22,26,27]. To test whether the PhoPQ TCS contributes to meropenem tolerance, we measured
spheroplast formation and stability over 24 hours in ΔphoPQ. Strikingly, OD₆₀₀ declined sharply in ΔphoPQ relative to wild type, which could be fully complemented by ectopic expression of phoPQ (Fig 2A). The decline in spheroplast formation correlated with a robust 10-fold decrease in ΔphoPQ viability (measured by CFU/mL) relative to wild type (Fig 2B). Step-wise titration of Ca²⁺ and/or Mg²⁺ markedly enhanced ΔphoPQ tolerance (S1C and S1D Fig), suggesting decreased tolerance (spheroplast formation) in ΔphoPQ is due to its inability to stabilize adjacent lipopolysaccharide molecules in the outer membrane [28]. To corroborate the involvement of PhoPQ, we sought to either enhance or reduce its activity and measured the effect of such perturbations on tolerance. PhoQ is antagonized by the small periplasmic MgrB protein; mgrB overexpression is thus expected to result in suppression of PhoPQ induction [29]. Indeed, mgrB overexpression from a plasmid reduced spheroplast formation (proxied by OD₆₀₀ measurements) (Fig 2C) in the wild type, closely resembling the ΔphoPQ phenotype (Fig 2A). We next tested a strain deleted in mgrB. Modified lipid A structures in ΔmgrB were confirmed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (S3A and S3B Fig), and we also phenotypically validated this mutant. Since the PhoPQ system mediates colistin resistance and heteroresistance, the ΔmgrB mutant (where PhoPQ regulon baseline levels are elevated) should be more resistant against colistin. As expected, colistin MIC was higher in the mutant (≥128 μg/mL) than in the wild type (16 μg/mL). Exposing ΔmgrB to meropenem resulted in an increase in OD₆₀₀ (S2A Fig) that coincided with an approximately 1000-fold increased survival to meropenem relative to wild

Fig 1. Enterobacter cloacae ATCC 13047 is highly meropenem tolerant. (A) Representative experiment demonstrating changes in OD₆₀₀ measurements following meropenem treatment in E. cloacae relative to E. coli. Error bars represent the average of 3 technical replicates +/- standard deviation. (B) Survival was calculated as CFU/mL from the experiment depicted in (A). The red line denotes the limit of detection. Phase images from the same experiment show cells before and after meropenem exposure to illustrate spheroplast formation in E. cloacae and lysis in E. coli (only cell debris is visible in phase image). Scale bars, 2 μm.

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type (Fig 2B). Lastly, two fully colistin-susceptible (non-heteroresistant) clinical isolates of *E. cloacae* from our collection exhibited a low-tolerance phenotype compared to ATCC 13047 (S2C Fig), consistent with the idea that the same OM modifications that cause colistin resistance also contribute to meropenem tolerance.

To dissect individual contributions of PhoPQ-regulated genes to tolerance, we created mutants in the *pagP* and *arn* loci. Specifically, we deleted *arnT*, which encodes the L-ara4N transferase that is necessary for aminoarabinose addition to lipid A [30]. While Δ*pagP* displayed spheroplast formation levels similar to wild type (Fig 2D), Δ*arnT* exhibited a drop in OD$_{600}$ reminiscent of Δ*phoPQ* (Figs 2D and S2B) and a concomitant 100-fold decrease in CFU/mL relative to wild type (Fig 2B). Notably, the survival defect, as measured by CFU/mL, was consistently more pronounced in Δ*arnT* vs. Δ*phoPQ* (Fig 2B), suggesting either that residual L-Ara4N modification is retained in the absence of PhoPQ through basal expression
of \textit{arn}, or that \textit{phoPQ} induction in the absence of \textit{arn} is detrimental for an unknown reason.

**Meropenem exposure induces the PhoPQ regulon**

Expression of the \textit{arn} operon in \textit{E. cloacae} is directly regulated by phosphorylated PhoP [22]. Because we observed that PhoPQ was necessary for \textit{arn}-mediated tolerance, we asked whether meropenem induced \textit{arn} transcription in a PhoPQ-dependent manner. To test this, \textit{E. cloacae} cells were exposed to meropenem for 30 minutes, after which \textit{arnB} (the first gene in the \textit{arn} operon and thus the most direct readout of promoter activity [22,31]), \textit{pagP}, and \textit{phoP} transcript levels were quantified (Fig 3A). Relative expression was calculated using 16s rRNA as an internal control. \textit{phoP}, which is autoregulated [32], showed 2.5-fold higher expression in meropenem-treated cells relative to untreated. Additionally, \textit{pagP} expression was 5-fold higher, and \textit{arnB} expression was 6-fold higher after meropenem treatment. These results support a

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**Fig 3. Expression of \textit{arnB} in response to meropenem treatment is dependent on PhoPQ.** (A) Relative expression reverse-transcription quantitative PCR (qRT-PCR) of PhoPQ regulon transcripts after meropenem exposure. Each experiment was independently replicated three times (individual data points of the three experiments are reported here). (B) Strains carrying transcriptional P\textit{arnB}:msGFP fusions were exposed to indicated conditions and analyzed on a C6 Accuri flow cytometer. Statistical difference between populations was determined with a one-way ANOVA followed by Tukey’s correction for multiple comparisons (ns, not significant; \textit{***}, \(p \leq 0.0001\)).

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model where PhoPQ signaling, as well as transcription of its regulon, is induced in response to meropenem treatment.

To corroborate the qRT-PCR findings, we also constructed a fluorescent transcriptional reporter, fusing the \textit{arnB} promoter with msfGFP, followed by fluorescence measurements upon exposure to meropenem (Fig 3B). As a control, we first exposed cells to EDTA, which chelates divalent cations to destabilize the outer membrane and consequently activates PhoPQ \cite{33}. As expected, \( P_{\text{arnB}} \)-msfGFP was induced by EDTA treatment in a \( \text{phoPQ} \)-dependent way (Fig 3B). Interestingly, meropenem treatment also robustly activated the \( P_{\text{arnB}} \)-msfGFP reporter, where a significant 3-fold fluorescence increase was measured, comparable with the effect of EDTA treatment. In contrast, meropenem only slightly (but reproducibly) increased \( \text{arnB} \) expression in the \( \Delta \text{phoPQ} \) mutant under the same conditions. Thus, \( \text{arn} \) transcription is induced by meropenem in a primarily PhoPQ-dependent manner.

**Meropenem treatment promotes formation of L-Ara4N-modified lipid A species in a PhoPQ-dependent manner**

The lipopolysaccharide lipid A domain is modified with L-Ara4N in a PhoPQ-dependent manner when Mg\(^{2+}\) is limiting \cite{22}. To determine if the \textit{E. cloacae} lipid A structure is modified with L-Ara4N in response to meropenem treatment, we isolated lipid A from treated and untreated cultures, which we then analyzed using MS and thin-layer chromatography (TLC). A distinct shift in lipid A structures was evident following 3 hours of meropenem treatment, where an increase in L-Ara4N modified vs. unmodified forms was observed (Fig 4A and 4B). Notably, the lipid A species that dominated before treatment (hexa-acylated, bis-phosphorylated, \( m/z = 1825.25 \)) decreased in abundance in favor of Arn- and PagP-modified lipid A.

![Image](https://doi.org/10.1371/journal.ppat.1010307.g004)
Doubly Arn/PagP-modified lipid A (m/z = 2114.14) was also produced following treatment. Notably, while L-Ara4N modification of lipid A was PhoPQ-dependent, PagP-dependent lipid A acylation was not (see below for discussion). Quantitative TLC supported the MS results and revealed a 12.21 (+/- 1.13)-fold increase in single-modified L-Ara4-N lipid A in a PhoPQ-dependent manner (Fig 4C).

\textit{E. cloacae} encodes the principal putative PagP acyltransferase Ecl_03072 (henceforth “PagP”). Lipid A extracted from \textit{ΔpagP} lacked the acyl chain induced upon meropenem treatment (S4A Fig), suggesting that PagP removes palmitate (C\textsubscript{16:0}) from surface-exposed glycerophospholipids and transfers it to lipid A, as previously shown [34,35]. We also confirmed that meropenem-induced L-Ara4N modification was dependent on the \textit{arn} operon (S4B Fig). Furthermore, MS analysis of \textit{ΔphoPQ ΔpagP} (S4C Fig) and \textit{ΔarnT ΔpagP} (S4D Fig) lipid A revealed that the mutants produced lipid A structures lacking all modifications following meropenem treatment, confirming that PagP and the \textit{arn} operon products coordinate \textit{E. cloacae} lipid A modifications in response to meropenem treatment. Interestingly, meropenem-induced hyperacylation was absent in \textit{ΔmgrB} cells (S3A Fig). One potential explanation is that meropenem-induced hyperacylation is a result of outer membrane glycerophospholipid accumulation in the outer leaflet of spheroplasts, a condition known to activate PagP enzymatic activity post-translationally [33,36]. This model is also consistent with our observation that while PagP is partially under genetic control of PhoPQ [22], meropenem-induced hyperacylation is independent of PhoPQ (Fig 4A and 4C). The absence of PagP-dependent modification in \textit{ΔmgrB} might indicate increased outer membrane strength (and concomitant reduction in glycerophospholipid accumulation in the outer leaflet of the outer membrane) in this background. Further analysis of spheroplast membrane composition is necessary to support this model.

Based on the structural studies and our genetic evidence, we suggest PhoPQ-dependent tolerance is primarily mediated via L-Ara4N addition to lipid A. Presumably, L-Ara4N lipid A modification increases the structural integrity of the outer membrane through stabilization of lateral lipopolysaccharide interactions [28], which protects spheroplasts from high levels of internal turgor.

\textbf{Colistin exposure primes \textit{E. cloacae} for meropenem tolerance}

Cationic antimicrobial peptides (CAMPs) are known inducers of the PhoPQ TCS [37]. Since our data above suggest that PhoPQ induction promotes tolerance, we hypothesized that pre-exposure to the CAMP colistin “primes” \textit{E. cloacae} for tolerance to meropenem, potentially by inducing PhoPQ or by selecting for cells that have a higher baseline level of PhoPQ induction. To test this, we measured the extent to which \textit{E. cloacae} was killed by meropenem with and without prior growth in medium containing supra-MIC colistin; this is expected to enrich specifically for the heteroresistant (more heavily OM modified) subpopulation in \textit{E. cloacae} ATCC 13047 [22]. Interestingly, after pre-exposure to colistin, the fraction of cells surviving meropenem treatment was reproducibly (and statistically significantly) 3.5-fold greater (Fig 5). This suggests that CAMPs have the potential to induce tolerance to β-lactam antibiotics, but that the temporal conditions of treatment may determine the extent to which this effect is significant.

\textbf{Outer membrane modifications are conserved β-lactam tolerance determinants in other Enterobacterales}

We next sought to establish whether outer membrane modifications might promote tolerance in other Enterobacterales. We first turned to \textit{Klebsiella pneumoniae} and used a hypertolerant
clinical isolate (Kp 1084) and its ΔphoPQ derivative for killing experiments. We observed a striking, 10- (6 hours) to 10^5-fold (24 hours) decrease in viability in the presence of meropenem, which could be fully complemented by expressing PhoP in trans (Fig 6A). We also analyzed a well-characterized E. coli K12 variant, WD101, engineered to constitutively upregulate the PmrAB two-component system [38] (which induces L-Ara4N modification of lipid A in E. coli) to test the hypothesis that outer membrane modifications increase tolerance in E. coli (Fig 6B). WD101 exhibited a dramatic, 10,000-fold increase in survival after 24 hours of meropenem exposure compared to the wild type parental strain, further supporting a role for outer membrane modifications in meropenem tolerance beyond E. cloacae ATCC13047.

A small molecule histidine kinase inhibitor synergizes with meropenem and colistin in vitro

Tolerance is likely an under appreciated contributor to antibiotic treatment failure. Antibiotic adjuvants that promote killing of tolerant cells thus have the potential to find a prominent place in our antibiotic armamentarium. Since histidine kinases like PhoQ are in principle targetable by small molecules, we tested whether his-kinase inhibitors synergized with meropenem. To this end, we turned to a previously developed suite of small molecules with potent histidine kinase inhibitory activity [39] and tested them in combination with meropenem. The anti-Amyotrophic Lateral Sclerosis (ALS) drug Riluzole, as well as its derivative Rilu-2,
exhibited potent, concentration-dependent synergy in combination with meropenem to rapidly lyse tolerant *E. cloacae* cells in vitro (Figs 7A and S5A). We also verified that Rilu-2 inhibited the formation of the L-ara4N lipid A structure using MALDI-TOF MS (Fig 7B). The peak corresponding to this modification (*m/z* 1876.29) was completely absent in Rilu-2-treated and ΔphoPQ cells. Since the PhoPQ system is primarily recognized for its contribution to CAMP resistance in many Enterobacterales, we next tested the Rilu compounds’ ability to synergize...
with colistin. As expected, Rilu-2 and Riluzole indeed potentiated colistin-mediated killing (S5B Fig), lending additional support to a PhoQ-inhibitory role of these compounds and also confirming previous results in *Salmonella* [40]. Importantly, Riluzole is an FDA-approved treatment for ALS and could thus readily serve as an adjuvant against both meropenem-tolerant and colistin-resistant Enterobacterales.

**Discussion**

While much work has been done in Enterobacterales to elucidate mechanisms of antibiotic resistance and persistence, the genetic and molecular determinants of tolerance, and especially spheroplast formation, have remained poorly understood. In contrast to resistance (continued growth) and persistence (dormancy), carbapenem-tolerant populations are initially susceptible to treatment (i.e., lose their cell wall). This phenotype is reminiscent of so-called "L-forms" [10], with the notable difference that spheroplasts do not replicate in this state, while L-forms do. This is likely a consequence of L-forms being able to "escape" the outer membrane to then proliferate through stochastic membrane blebs [41,42].

The remarkable ability of spheroplasts to survive without their PG layer lends support to the recent realization that the outer membrane has load-bearing capabilities [43] and prompted us to interrogate the molecular mechanism of outer membrane stabilization during antibiotic exposure. Our data suggest that L-Ara4N addition to lipid A is a key factor in spheroplast integrity. We propose that lipid A molecules with a positive charge increase outer
membrane stability due to the enhancement of electrostatic interactions between adjacent lipopolysaccharide molecules on the surface-exposed face of the outer membrane [28]. Of note, the L-Ara4N modification has been previously implicated in resistance to CAMPs, such as colistin [22]. In this context, it is worrisome that colistin therapy can select for mgrB mutations [44–46], which we demonstrate here also confers high meropenem tolerance in addition to colistin resistance. Thus, treatment with antimicrobial peptide analogs (and we speculate that this may potentially apply to innate AMPs as well) can select for bacteria that are stably tolerant to subsequent therapy with a β-lactam. Our data also suggest that colistin (and, by extension, potentially AMPs of the innate immune system) can induce transient β-lactam tolerance, e.g., through induction of the PhoPQ system and perhaps other protective stress responses.

However, the relationship between PhoPQ induction and tolerance is not absolute; a significant proportion of both colistin-pretreated and mgrB-deleted cells still lyse in the presence of meropenem. It is thus likely that colistin heteroresistant subpopulations are not the same as carbapenem tolerant cells. In our pre-treatment experiments with colistin, the PhoPQ system should be induced in all cells to similar degrees, yet only a fraction (0.1%) of these cells was also meropenem tolerant. We speculate that β-lactam tolerance and colistin resistance, while relying on the same basic outer membrane modification, may each require a specific fraction of lipopolysaccharide molecules to be modified. Thus, within a sample, there may be a limited subset of cells exhibiting the correct amount of modification to enable both colistin resistance and meropenem tolerance (or even just optimal meropenem tolerance). This would explain why we observe only partial overlap between these two phenomena.

In summary, this work demonstrates a novel genetic determinant of carbapenem tolerance in clinically relevant Enterobacterales. Despite being a well-known regulator of polymyxin resistance, the PhoPQ two-component system was not previously known to respond or mediate tolerance to carbapenem treatment. As tolerance (and spheroplast formation in particular) is a possible culprit for antibiotic treatment failure [2,3,47], our results suggest a potential for combination therapies with histidine kinase inhibitors to increase the efficacy of carbapenems.

Materials and methods

Bacterial strains and growth

All strains/plasmids and primers used in this study are listed in S1 and S2 Tables. All strains were initially grown from freezer stocks on solid agar at 37°C. Isolated colonies were used to inoculate Luria-Bertani (LB), Brain heart infusion (BHI) or N-minimal medium (0.1M Bis-Tris, pH 7.5, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 M K₂SO₄, 1 mM KH₂PO₄, 0.10% casamino acids 0.2% glucose, 0.0002% thiamine, 15 µM FeSO₄, 10 mM MgSO₄) at 37°C. Where required, kanamycin was used at 50 µg/mL, meropenem was used at 10 µg/mL (300 x MIC; MICATCC13047 = 0.03 µg/mL) and colistin was used at 20 µg/mL (1.25 x MIC; MICATCC13047 = 16 µg/mL), unless noted otherwise. K. pneumoniae 1084 is an isolate (AR0080) from the CDC AR isolate bank (https://wwwn.cdc.gov/ARisolateBank/Search), cured of its imipenemase via spontaneous loss.

Meropenem killing experiments

Unless noted otherwise, killing experiments were conducted in 100-well honeycomb plates in a Bioscreen C growth curve analyzer (Growth Curves USA, Piscataway NJ). Overnight cultures were diluted 10-fold into fresh LB medium containing meropenem (10 µg/mL, 300x MIC) and transferred to honeycomb plates (200 µL volume/culture). OD₆₀₀ was measured by plate reader; at indicated timepoints, the experiment was paused and an aliquot was removed for
CFU/mL determination or microscopy. Rilu compounds were dissolved in DMSO as 50 mM (Rilu-2) or 500 mM (Riluzole) stocks and added directly to the LB medium containing meropenem at the indicated concentrations.

For *K. pneumoniae* killing experiments, we employed our standard tolerance assay as described previously [9], but with 0.2% arabinose present for induction of plasmid-borne PhoP.

**Colistin MIC experiment**

Cultures were grown overnight at 37˚C shaking, then diluted 1000-fold into fresh LB. Subcultures were grown for 1 hour at 37˚C shaking before being diluted 1000-fold again into fresh LB to create a "seed culture". 100 μL of seed culture was subsequently diluted 2-fold into a 96-well plate containing colistin concentrations ranging 0.25–128 μg/mL. Reported values are medians of 4 technical replicates.

**qRT-PCR**

Strains were grown in 5 mL LB overnight at 37˚C. 500 μL of overnight cultures was added to 4.5 mL pre-warmed BHI broth, either water or 10 μg/mL meropenem was added and cultures were incubated statically at 37˚C for 0 and 30 mins. Following incubation, cells were harvested via centrifugation and resuspended in 500 μl RNA later and stored in -80˚C prior to RNA extraction. Relative-abundance quantitative PCR (qPCR) was performed as previously described [48,49]. In brief, the Sybr Fast One-Step qRT-PCR kit (Kapa Biosystems) was used with 16S rDNA as the internal reference. The PCR was performed using the Bio-Rad CFX Connect Real-Time PCR System. Relative expression levels were calculated using the ΔΔCt method [50], with normalization of gene targets to 16S rDNA signals.

**Flow cytometry GFP measurements**

Cultures of strains harboring transcriptional *P*arnB:msfGFP fusions were grown overnight in LB supplemented with 10 mM MgSO₄. Overnight cultures were then washed 2x in fresh LB before 10-fold dilution into fresh LB medium containing MgSO₄ (10 mM), Ethylenediaminetetraacetic acid (1 mM), or meropenem (10 μg/mL). Cultures were incubated statically for 3 hours at 37˚C. Then, 500 μL of culture was harvested and run through a C6 Accuri flow cytometer (BD Biosciences) until 100,000 events (cells) had been analyzed. Mean green fluorescence as measured by the FL1-A channel was used as a readout for GFP.

**Mutant construction**

*E. cloacae* subsp. *cloacae* 13047 mutant strains (*phoPQ*, *arn* and *arnT*) were constructed as previously described using recombineering with the plasmid pKOBEG [22,51]. Briefly, linear PCR products were amplified from pKD3 and transformed into *E. cloacae* ATCC 13047/pKOBEG strain by electroporation and plated on chloramphenicol selective media. Selected clones were transformed with pCP20 to cure the antibiotic resistance cassette. All mutants were verified by PCR.

*pagP* was deleted using the Wanner method as described previously [22]. Briefly, the chl resistance cassette was amplified from pKD3 using primers TDP1532/TDP1533, which contain 75 bp flanking homology overhangs. The resulting PCR product was electroporated into *E. cloacae* ATCC13047 expressing lambda red recombaine from pACBSR-hyg [52] (a hygromycin-resistant derivative of pKD46 [53]). Mutants were selected on chloramphenicol (100 μg/mL) and verified by PCR.
Other mutants were constructed using either lambda red recombinase [53] or the suicide vector pTox [54]. The mgrB gene was deleted using the suicide plasmid pTox5 as described in [54]. ~700 bp upstream and downstream flanking homology regions were amplified from ATCC13047 using primers TDP1767/68 and TDP1769/70, and cloned into pTox5 (digested with EcoRV) using isothermal assembly [55]. Successful pTox5ΔmgrB were conjugated into ATCC13047 using the E. coli donor strain MFD lambda pir; successful recombinants were selected on plates containing 100 μg/mL chloramphenicol. Upon single colony purification, colonies were directly streaked out on an M9 minimal medium plate containing 0.2% casamino acids and 1% rhamnose, followed by incubation at 30˚C for 24–36 hours. Mutants were tested using primers TDP1771/72.

The K. pneumoniae ΔphoPQ mutant was constructed using pTox5. Upstream and downstream homology regions were amplified using primers TC120/TC344 and TC345/TC127. Allelic exchange was conducted as described above. Mutants were tested using flanking primers TC166/167. For the complementation construct, the PhoP open reading frame was synthesized as a gene block (gBlock TC391) with a 3x Flag tag (Twist Biosciences, South San Francisco, USA), and cloned into pBAD33 using isothermal assembly.

**Lipid A isolation and mass spectrometry**

Isolation of lipid A for analysis was performed as previously described [56] with slight modifications. To analyze lipid A after meropenem treatment, overnight cultures grown in BHI broth were diluted 1:10 in pre-warmed media with or without meropenem statically for 3 h. To assess Rilu-dependent modification of lipid A, 12.5 mL of E. cloacae was grown to OD₆₀₀ 1.0. Rilu-2 was used at a final concentration of 200 μM. Bacteria were harvested and lipid A extraction was carried out by mild-acid hydrolysis as previously described [57]. For mass spectrometry (MS), data were collected on a MALDI-TOF (Axima Confidence, Shimadzu) mass spectrometer in the negative mode, as previously done [22].

For quantification of lipid A, cultures were grown with 2.5 μCi/mL of ³²P ortho-phosphoric acid (³²P) (Perkin Elmer) and lipid A was extracted. Thin layer chromatography was done in a pyridine, chloroform, 88% formic acid, aqueous (50:50:16:5 v/v) tank for 3 hours. Plates were exposed to a phosphor screen, imaged, and densitometry was used to calculate the percentage of each lipid species. Reported densitometry was calculated using 2 replicates +/- standard deviation. For lipid A structural comparisons, purified ³²P-lipid A from E. coli W3110, WD101 [58] were used in each experiment. These structures were comparable to previously published E. cloacae structures [26] and validated by mass spectrometry.

**Supporting information**

**S1 Fig. Addition of divalent cations prevents spheroplast lysis.** Wild type (WT) (A-B) or its ΔphoPQ derivative (C-D) were treated as described in Fig 1A with addition of the indicated concentrations of (A,C) MgSO₄ (Mg²⁺) or (B,D) CaCl₂ (Ca²⁺). Data represent the average of 3 replicates +/- standard deviation. (TIF)

**S2 Fig. Independent biological replicates of experiments shown in Fig 1.** (A) An mgrB mutation promotes a moderate increase in mass increase during meropenem exposure. (B) Experiments were conducted as described in Fig 1A legend; each graph represents experiments conducted on a different day. In addition, data in each graph represent the average of 3 biological replicates +/- standard deviation. (C) Fraction of colistin-susceptible populations surviving after 24 hours of 10 μg/mL meropenem exposure. Statistical significance determined
by one-way ANOVA of log transformed data, followed by Tukey’s correction for multiple comparisons (ns, not significant; ***, p ≤ 0.001).

(TIF)

S3 Fig. Analysis of lipid A from ΔmgrB. (A) MALDI-MS analysis of lipid A isolated from E. cloacae ΔmgrB. m/z corresponding with L-Ara4N modifications are illustrated in red. Each experiment was independently replicated three times, and one representative data set was reported. (B) Relevant lipid A chemical structures are shown.

(TIF)

S4 Fig. Analysis of lipid A from E. cloacae mutants. (A) MALDI-MS analysis of lipid A isolated from ΔpagP, (B) Δarn (full operon deletion), (C) ΔphoPQ ΔpagP and (D) ΔarnT ΔpagP. m/z corresponding with L-Ara4N modifications are illustrated in red, while structures with altered acyl chain patterns are illustrated in green. Each experiment was independently replicated three times, and one representative data set was reported.

(TIF)

S5 Fig. Rilu synergizes with colistin to enhance killing. (A) Rilu compounds do not cause lysis, but (B) potentiate colistin mediate killing. Experiments were conducted as described in Fig 1A legend. Data represent the average of 3 replicates +/- standard deviation.

(TIF)

S1 Table. Strains, plasmids and peptides used in this study.

(DOCX)

S2 Table. Oligos used in this study.

(DOCX)

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