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**Colistin heteroresistance in *Enterobacter cloacae* is regulated by PhoPQ-dependent 4-amino-4-deoxy-L-arabinose addition to lipid A**

**Running Title:** Colistin heteroresistance in *E. cloacae*

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**Keywords:** heteroresistance, antimicrobial resistance, lipid A, outer membrane, colistin, Enterobacteriaceae

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## Summary

The *Enterobacter cloacae* complex (ECC) consists of closely-related bacteria commonly associated with the human microbiota. ECC are increasingly isolated from healthcare-associated infections, demonstrating that these Enterobacteriaceae are emerging nosocomial pathogens. ECC can rapidly acquire multidrug resistance to conventional antibiotics. Cationic antimicrobial peptides (CAMPs) have served as therapeutic alternatives because they target the highly conserved lipid A component of the Gram-negative outer membrane. Many Enterobacteriaceae fortify their outer membrane with cationic amine-containing moieties to prevent CAMP binding, which can lead to cell lysis. The PmrAB two-component system (TCS) directly activates 4-amino-4-deoxy-L-arabinose (L-Ara4N) biosynthesis to result in cationic amine moiety addition to lipid A in many Enterobacteriaceae such as *E. coli* and *Salmonella*. In contrast, PmrAB is dispensable for CAMP resistance in *E. cloacae*. Interestingly, some ECC clusters exhibit colistin heteroresistance, where a subpopulation of cells exhibit clinically significant resistance levels compared to the majority population. We demonstrate that *E. cloacae* lipid A is modified with L-Ara4N to induce CAMP heteroresistance and the regulatory mechanism is independent of the PmrAB<sub>Ecl</sub> TCS. Instead, PhoP<sub>Ecl</sub> binds to the *arnB*<sub>Ecl</sub> promoter to induce L-Ara4N biosynthesis and PmrAB-independent addition to the lipid A disaccharolipid. Therefore, PhoPQ<sub>Ecl</sub> contributes to regulation of CAMP heteroresistance in some ECC clusters.

**Keywords:** heteroresistance, antimicrobial resistance, lipid A, outer membrane, colistin, Enterobacteriaceae

## Introduction

Gram-negative bacteria assemble a highly conserved outer membrane (OM) barrier. Glycerophospholipids comprise the periplasmic monolayer of the asymmetric lipid bilayer, while the surface-exposed monolayer is enriched with lipopolysaccharide (LPS). The LPS glycolipid is organized into three domains; an O-antigen carbohydrate repeat, core oligosaccharide, and the membrane anchor, lipid A (1). The lipid A domain is initially synthesized as a  $\beta$ -1',6-linked glucosamine disaccharide that is both phosphorylated and fatty acylated. Lipid A is the bioactive portion of LPS and robustly activates the human Toll-like receptor 4 (TLR-4) and myeloid differentiation factor 2 (MD-2) immune complex to induce immune reactivity (1–4). Gram-negative pathogens encode highly conserved regulatory mechanisms that modify lipid A to prevent TLR-4/MD-2 recognition and to fortify the OM against immune effectors and antimicrobials, which promotes survival in the host (5).

Lipid A modification enzymes are transcriptionally regulated by two-component systems (TCS) (6, 7). The PmrAB and PhoPQ TCSs are well-studied phosphorelay signaling systems that regulate lipid A modifications in response to specific environmental signals (8–10). PmrAB and PhoPQ are highly conserved among pathogenic Enterobacteriaceae (11). PmrAB responds to high  $\text{Fe}^{3+}$  concentrations, cationic antimicrobial peptides (CAMPs), and slightly acidic pH to directly activate *eptA* (also known as *pmrC*) and *arn* operon expression (12–14), which encode phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) transferases, respectively (15–18). Cationic amine addition to the lipid A domain of LPS neutralizes the surface charge to protect the cell from CAMP-mediated lysis (15, 17).

PhoPQ is activated in response to depletion of divalent cations such as  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  and the presence of CAMPs (8, 10, 19). PhoPQ phosphotransfer directly activates transcription of genes encoding PagL (only in *Salmonella* (5)) and PagP, which add or remove acyl chains from lipid A, respectively (8, 20–22). Additionally, it directly activates

*arn* expression in *Klebsiella* and *Yersinia* spcs. (23, 24). While the PmrAB and PhoPQ TCSs each regulate distinct subsets of genes, the independent signaling pathways also converge through the connector protein, PmrD (25–27). PmrD binds phospho-PmrA, which prevents PmrB-mediated dephosphorylation (25, 28–30). Constitutive PmrA-dependent gene expression increases pEtN and L-Ara4N lipid A modifications.

The *Enterobacter cloacae* complex (ECC) is composed of thirteen closely-related Gram-negative bacterial clusters (designated C-I to C-XIII) (31). ECC are typically associated with the host microbiota. However, many clusters have been associated with hospital-acquired infections, especially in immunocompromised patients (32). Infections manifest in a wide range of host tissues with symptoms including skin, respiratory tract, urinary tract, wound and blood infections (33). ECC have increasingly emerged in nosocomial settings and are problematic because they harbor multidrug resistance (MDR) mechanisms, which limit treatment options (32, 34–36). Alternative last-line therapeutics used to treat MDR Gram-negative infections include the CAMP, colistin (polymyxin E), which binds the lipid A portion of LPS to perturb the outer membrane and lyse the bacterial cell. Despite success as a last-line therapeutic (37, 38), many ECC clusters demonstrate heteroresistance, where a subset of the clonal population is colistin resistant (34, 39–41). We do not fully understand the underlying molecular mechanism(s) that regulate colistin heteroresistance in ECC; further characterization will advance our understanding of antimicrobial resistance and could help inform new treatment strategies.

A previous report showed that colistin heteroresistance naturally occurs within clonal ECC clusters (34). Moreover, colistin heteroresistance in *E. cloacae* was induced by innate immune defenses within a murine infection model, which led to treatment failure (39). Transcriptional analysis of susceptible and resistant populations suggested that pEtN and L-Ara4N lipid A modifications contribute to heteroresistance (39) and PhoPQ contributed to

regulation (34, 39), as described in other Enterobacteriaceae (11). However, it was not established that the lipid A modifications actually occur, nor has PhoPQ-dependent, PmrAB-independent regulation of colistin heteroresistance been fully described in *E. cloacae* or other ECC isolates.

Herein, we demonstrate that *E. cloacae* colistin heteroresistance involves PhoPQ<sub>Ecl</sub>, which regulates L-Ara4N modification of lipid A. The PhoP<sub>Ecl</sub> response regulator directly binds to the promoter region of *arnB*<sub>Ecl</sub>, which is the first gene of a seven-gene operon (*arnBCADTEF*<sub>Ecl</sub>). Transcriptomics analysis supports a model of PhoPQ-dependent, PmrAB-independent *arn*<sub>Ecl</sub> regulation. Furthermore, L-Ara4N modification of lipid A increased in response to growth in limiting Mg<sup>2+</sup>, which amplified colistin resistance in a PhoPQ<sub>Ecl</sub>-dependent manner. Lastly, sequencing of twelve ECC colistin-susceptible isolates pinpointed mutations within the PhoPQ-dependent lipid A modification pathway that promoted colistin susceptibility. This study advances our understanding of the molecular mechanisms that mediate colistin heteroresistance in ECC.

## Results

### **Colistin heteroresistance in *E. cloacae* is regulated by PhoPQ<sub>Ecl</sub>, but not PmrAB<sub>Ecl</sub>.**

To elucidate the underlying mechanisms that regulate colistin heteroresistance in ECC, we analyzed a collection of *E. cloacae* subsp. *cloacae* strain ATCC 13047 genetic mutants by calculating the colony forming units (CFUs) during exponential growth in the absence and presence of colistin (Fig 1A). While wild type and all mutant *E. cloacae* strains grew in standard growth media,  $\Delta$ *phoPQ*<sub>Ecl</sub> was not viable when 10  $\mu$ g/ml of colistin was added to the media. Clinical resistance to colistin is defined as >4  $\mu$ g/ml (42). The decrease in  $\Delta$ *phoPQ*<sub>Ecl</sub> cell viability suggested that PhoPQ<sub>Ecl</sub> contributes to colistin heteroresistance. Furthermore, wild type *E. cloacae* grown in colistin demonstrated approximately ten-fold less CFUs at

hour two ( $P < 0.05$ ), suggesting a survival defect in early logarithmic growth phase. However, the fitness defect was no longer significant at hour three. By hour four, CFUs were equivalent to growth without colistin (Fig 1A).

Due to reports of colistin heteroresistance in *E. cloacae* and other ECC strains (34, 39), we subjected wild type,  $\Delta phoPQ_{Ecl}$ ,  $\Delta phoPQ_{Ecl}/pPhoPQ_{Ecl}$ , and  $\Delta pmrAB_{Ecl}$  *E. cloacae* to colistin E-test strip analysis, which provides a convenient method to observe heteroresistance (Fig S1). Squatter colonies within the zone of inhibition indicated colistin heteroresistance in wild type,  $\Delta phoPQ_{Ecl}/pPhoPQ_{Ecl}$ , and  $\Delta pmrAB_{Ecl}$  strains, but not  $\Delta phoPQ_{Ecl}$ . We confirmed colistin heteroresistance by population analysis profiling (PAP) (Table 1) (43). Minimal inhibitory concentration (MIC) values were calculated using the broth microdilution (BMD) method (Table 1). Wild type,  $\Delta phoPQ_{Ecl}/pPhoPQ_{Ecl}$ , and  $\Delta pmrAB_{Ecl}$  *E. cloacae* all demonstrated MICs  $> 256$   $\mu\text{g/ml}$ , while the  $\Delta phoPQ_{Ecl}$ ,  $\Delta phoPQ_{Ecl}/pPhoPQ_{H277A}$ ,  $\Delta phoPQ_{Ecl}/pPhoP_{D56A}Q$  and  $\Delta arn_{Ecl}$  ( $arnBCADTEF_{Ecl}$ ) MIC was  $0.5$   $\mu\text{g/ml}$ . Together, these studies confirm that PhoPQ<sub>Ecl</sub> signal transduction and the *arn<sub>Ecl</sub>* biosynthetic operon (L-Ara4N) contribute to colistin heteroresistance in *E. cloacae*.

Since lipid A modifications induce colistin resistance in pathogenic Enterobacteriaceae (11), we analyzed wild type and mutant *E. cloacae* lipid A for modifications. <sup>32</sup>P-radiolabelled lipid A was isolated and chromatographically separated based on hydrophobicity. As controls, we also analyzed lipid A from *E. coli* strain W3110 (Fig 1B, lane 1), which does not significantly modify its lipid A, and *E. coli* strain WD101 (Fig 1B, lane 9), which constitutively expresses *pmrA* to produce modified lipid A (15). Thin layer chromatography (TLC) analysis indicated that wild type *E. cloacae* produced a mixture of lipid A consistent with modified and unmodified species (Fig 1B, lane 2).  $\Delta phoPQ_{Ecl}$  and the  $\Delta arn_{Ecl}$  strains did not produce modified lipid A (Fig 1B, lanes 3 and 8). PhoPQ complementation fully restored production of modified lipid A in the *phoPQ* mutant (Fig 1B,

lane 4). Furthermore, site-directed mutagenesis to substitute H277 in PhoQ<sub>Ecl</sub> or D57 in PhoP<sub>Ecl</sub> with alanine limited lipid A assembly to only unmodified species (Fig 1B lanes 5 and 6). These results confirm that PhoPQ<sub>Ecl</sub> phosphotransfer and L-Ara4N biosynthesis are essential for lipid A modification in *E. cloacae*. Interestingly, the *pmrAB*<sub>Ecl</sub> mutant assembled a modified lipid A, similar to wild type (Fig 1B, lane 7), and exhibited colistin heteroresistance (Fig 1A, Table 1, Fig S1A), suggesting that PmrAB<sub>Ecl</sub> does not regulate colistin heteroresistance in *E. cloacae*.

The lipid A anchor of LPS is a pathogen associated molecular pattern (PAMP) that is bound with high affinity by the mammalian host TLR-4/MD-2 complex (44), which activates a proinflammatory response to clear the bacterial infection (45). Structural alterations to lipid A can dramatically alter TLR-4/MD-2-dependent host immune activation (2) and a previous report nicely demonstrated that *E. cloacae* colistin heteroresistance was induced by innate immune effectors (39). Therefore, we examined if *E. cloacae* containing modified or unmodified lipid A would differentially activate TLR-4/MD-2 in a human embryonic kidney reporter cell line (HEK-blue) (2). Wild type and *phoPQ*<sub>Ecl</sub> mutant strains stimulated TLR-4/MD-2-dependent activation equally (Fig S1B), suggesting that lipid A modifications do not significantly alter host immune recognition. Reporter activation by *E. cloacae* lipid A was attenuated compared to *E. coli* lipid A at higher cell densities, suggesting differential recognition by the human TLR-4/MD-2 complex. The Gram-positive *Staphylococcus aureus*, which does not produce lipid A, did not stimulate the TLR-4/MD-2 complex (Fig S1B). Thus, while PhoPQ<sub>Ecl</sub>-dependent lipid A modifications contribute to CAMP resistance in *E. cloacae*, they do not significantly affect innate immune recognition and reactivity.



### Determination of *E. cloacae* lipid A modifications.

In order to define outer membrane modifications, we isolated lipid A from wild type *E. cloacae* grown in media supplemented with 10 µg/ml of colistin and from  $\Delta phoPQ_{Ecl}$ , which was grown without colistin. Purified lipid A was analyzed by direct infusion nanoESI. The MS1 spectra with a range of  $m/z$  750-2000 are shown in Figure S2. The expanded MS1 spectrum ( $m/z$  850-1200) of lipid A isolated from wild type *E. cloacae* demonstrated three distinct modifications: (i) addition of either one or two L-Ara4N moieties (red), (ii) palmitate ( $C_{16:0}$ ) addition (green), and (iii) hydroxylation (Fig 2A). The MS1 spectrum of lipid A isolated from  $\Delta phoPQ_{Ecl}$  did not produce L-Ara4N modified lipid A (Fig 2B). Hydroxyl addition was not labeled for simplicity, but correlates with a  $m/z$  shift of 8 of the doubly-charged molecular ions. Higher-energy collisional dissociation (HCD) and ultraviolet photodissociation (UVPD) MS/MS spectra were obtained for the ions of  $m/z$  1042.68 and 1161.79 from wild type and the ions of  $m/z$  911.62 and 1030.73 from  $\Delta phoPQ_{Ecl}$  *E. cloacae* (Fig S3, S4, S5 and S6). Analysis of the MS/MS spectra from wild type ( $m/z$  1042.68) indicated PhoPQ<sub>Ecl</sub>-dependent addition of L-Ara4N at both the 1- and 4'-phosphates (Fig S3). The MS/MS spectra for the ion of  $m/z$  1161.79 (wild type *E. cloacae*) showed addition of L-Ara4N at both the 1- and 4'-phosphates and palmitate addition to the R-2-hydroxymyristate (Fig S4). Analysis of lipid A from the  $phoPQ_{Ecl}$  mutant ( $m/z$  911.62) completely lacked L-Ara4N modified lipid A (Fig S5) and analysis of the  $m/z$  1030.73 ion from the  $phoPQ_{Ecl}$  mutant demonstrated that palmitate addition at the R-2-hydroxymyristate position of lipid A occurred independent of PhoPQ<sub>Ecl</sub> (Fig S6).

Based on transcriptomics studies, a previous report suggested that *E. cloacae* adds pEtN and L-Ara4N to lipid A to develop colistin heteroresistance (39). However, our genetic and high resolution mass spectrometry analysis demonstrate that only L-Ara4N modifies the

1- and 4'-phosphates of lipid A in a PhoPQ<sub>Ecl</sub>-dependent manner (Fig 2A and B) and this amine-containing modification correlates with colistin heteroresistance (Fig 1A and Table 1).

#### **L-Ara4N lipid A modifications are dependent on PhoPQ<sub>Ecl</sub>, but not PmrAB<sub>Ecl</sub>.**

To further characterize lipid A modifications in the  $\Delta pmrAB_{Ecl}$  mutant, we analyzed purified lipid A from wild type and mutant *E. cloacae* using MALDI-TOF mass spectrometry. Wild type produced a lipid A mixture, which included L-Ara4N modified lipids (Fig S7A and B). In contrast, analysis of  $\Delta phoPQ_{Ecl}$  and  $\Delta arn_{Ecl}$  lipid A indicated that L-Ara4N modified lipids were not present. Expression of PhoPQ<sub>Ecl</sub> *in trans* from an IPTG-inducible promoter restored L-Ara4N modified lipid A in the *phoPQ<sub>Ecl</sub>* mutant. Furthermore,  $\Delta pmrAB_{Ecl}$  produced the L-Ara4N modification, similar to wild type (Fig S7A). The *m/z* of each prominent peak in our MALDI-MS analysis corresponded with the exact mass of an expected structure with only the L-Ara4N-containing structures demonstrating colistin resistance (Fig S7B). Here, we confirmed that L-Ara4N modification of lipid A in *E. cloacae* is not dependent on PmrAB<sub>Ecl</sub>.

#### **PhoP<sub>Ecl</sub> directly binds to the *arnB<sub>Ecl</sub>* promoter.**

The *arn* operon is composed of seven genes and expression is driven by a promoter upstream of *arnB* (16). This genetic organization is conserved in *E. cloacae* as illustrated in Fig 3A. *phoP* expression is autoregulated in Enterobacteriaceae, where PhoP binds to the PhoP box to interact with RNA polymerase, which induces transcription (46). The putative PhoP box in the *phoP* promoter region ( $P_{phoP}$ ) is conserved in *E. coli*, *Salmonella*, and *E. cloacae* (Fig 3B). Alignment of the *E. cloacae arnB* promoter region ( $P_{arnB}$ ) with *E. coli*, *Salmonella*, and *E. cloacae P<sub>phoP</sub>* suggested a putative PhoP box region. Importantly, *E. cloacae P<sub>arnB</sub>*, which contains a putative PhoP box, is highly conserved among ECC. However, this feature was not

conserved within *E. coli* P<sub>arnB</sub>, suggesting regulatory mechanisms that control promoter activation are different (Fig 3B).

We performed electrophoretic mobility shift assays (EMSAs) using *E. cloacae* P<sub>arnB</sub> to determine if PhoP<sub>Ecl</sub> directly binds the promoter to activate *arnEcl* transcription. Increasing concentrations of purified PhoP<sub>Ecl</sub> (Fig S8) induced a shift of the biotinylated *arnB<sub>Ecl</sub>* promoter fragment, which contains the putative PhoP box binding motif (Fig 3C). Importantly, PhoP<sub>Ecl</sub> does not bind to *E. coli* P<sub>arnB</sub>, which does not encode the PhoP box motif (Fig 3C). Furthermore, the PhoP<sub>Ecl</sub>-*arnB<sub>Ecl</sub>* promoter interaction was abrogated when unlabeled *E. cloacae* P<sub>arnB</sub> was added in increasing amounts, as a competitive inhibitor. We also show that the interaction is specific because addition of noncompetitive DNA (poly(dI-dC)) did not reduce the PhoP<sub>Ecl</sub> and *E. cloacae* P<sub>arnB</sub> interaction (Fig 3D). Lastly, PhoP<sub>Ecl</sub> bound *E. cloacae* and *E. coli* P<sub>phoP</sub>, which both encode the nucleotide sequence specific to the PhoP box (Fig 3E). Together, these findings suggest that *E. cloacae* encodes a mechanism that enables L-Ara4N biosynthesis to respond directly to PhoPQ<sub>Ecl</sub>.

#### **RNA-sequencing analysis of the *phoPQ<sub>Ecl</sub>* and *pmrAB<sub>Ecl</sub>* mutants.**

To better understand PhoPQ<sub>Ecl</sub> and PmrAB<sub>Ecl</sub> transcriptional regulation, we isolated and sequenced total RNA from wild type and mutant *E. cloacae* strains. A heat map illustrates the fold change of *arnEcl*, *phoPQ<sub>Ecl</sub>*, and *pmrAB<sub>Ecl</sub>* gene expression in the TCS mutants relative to wild type (Fig 4). Expression of the *arnEcl* genes were significantly down regulated in  $\Delta$ *phoPQ<sub>Ecl</sub>* compared to wild type, suggesting that activation of the pathway is dependent on PhoPQ<sub>Ecl</sub>. In contrast, *arnEcl* gene expression was not significantly altered in the  $\Delta$ *pmrAB<sub>Ecl</sub>* mutant relative to wild type. A complete list of the significant up- and down-regulated genes (P <0.05) is included in Table S4.

***E. cloacae* colistin resistance is amplified in response to limiting Mg<sup>2+</sup>.**

Together, these analyses indicate colistin heteroresistance in wild type *E. cloacae* (Fig 1A and Table 1) is mediated by L-Ara4N modification of lipid A, which is regulated in a PhoPQ<sub>Ecl</sub>-dependent manner under standard growth conditions. In *E. coli* and *Salmonella*, PhoPQ is activated by various signals, including low Mg<sup>2+</sup> and CAMPs (8, 19, 21). Here we analyzed if PhoPQ<sub>Ecl</sub> responds to similar physiological cues to induce colistin resistance in *E. cloacae*. Wild type and mutant *E. cloacae* were grown in N minimal medium with high (10 mM) or low (10 μM) Mg<sup>2+</sup> levels. All cultures were exposed to colistin at mid-logarithmic growth. Wild type and complemented *phoPQ<sub>Ecl</sub>* mutant strains grown in high Mg<sup>2+</sup> demonstrated some susceptibility to 5 and 10 μg/ml of colistin (Fig 5A, High Mg<sup>2+</sup>), suggesting colistin-susceptible and -resistant populations were present, which is indicative of heteroresistance. When grown under limiting Mg<sup>2+</sup> conditions, *E. cloacae* cells were more resistant (Fig 5A, Low Mg<sup>2+</sup>). In contrast, *phoPQ<sub>Ecl</sub>* demonstrated a fitness defect in either Mg<sup>2+</sup> concentration when exposed to colistin (Fig 5A). These data suggest that *E. cloacae* PhoPQ<sub>Ecl</sub> amplifies colistin resistance in response to limiting Mg<sup>2+</sup> growth conditions.

**PhoPQ<sub>Ecl</sub> responds to limiting Mg<sup>2+</sup> conditions by inducing L-Ara4N lipid A modification.**

To determine if increased colistin resistance was dependent on L-Ara4N modification of lipid A, we isolated lipid A after growth in either low or high Mg<sup>2+</sup>. TLC analysis demonstrated that wild type and the complemented *phoPQ<sub>Ecl</sub>* mutant primarily produced L-Ara4N-modified lipid A when Mg<sup>2+</sup> concentrations were limiting (Fig 5B, Low Mg<sup>2+</sup>). In contrast, the same strains grown in excess Mg<sup>2+</sup>, produced a mixture of modified and unmodified lipid A, which is indicative of heteroresistance (Fig 5B, High Mg<sup>2+</sup>). Interestingly, growth in excess Mg<sup>2+</sup> does not completely shut-off production of PhoPQ<sub>Ecl</sub>-dependent lipid A modification in *E.*

*cloacae*, as was previously shown in *E. coli* (25). Together, these studies suggest that a subset of the clonal *E. cloacae* population activates PhoPQ<sub>Ecl</sub>-dependent L-Ara4N modification of lipid A under standard growth conditions to promote heteroresistance. However, depletion of Mg<sup>2+</sup> amplifies L-Ara4N modification (Fig 5B) and colistin resistance (Fig 5A) throughout the population.

### **Inactivation of colistin heteroresistance in ECC clinical isolates.**

A previous report showed that while many ECC clinical isolates were colistin heteroresistant, some were susceptible to colistin-mediated lysis. Interestingly, ECC colistin heteroresistance was cluster dependent (34). To determine the genetic basis for colistin sensitivity in ECC clusters, we examined twelve colistin susceptible clinical isolates (CI) and compared them to heteroresistant wild type ATCC 13047. We confirmed colistin susceptibility in all isolates using the BMD method to determine MICs (Table S3) and sequenced each genome. We obtained ~70 X 10<sup>6</sup> reads for each susceptible isolate and the wild type strain, which were mapped to the annotated ATCC 13047 genome (47). The coverage region of each isolate varied between 82% to 99%, where most coverage variation occurred in plasmid DNA regions. Initial analysis of our wild type strain confirmed it did not encode mutations in the *phoPQ*<sub>Ecl</sub>, *pmrAB*<sub>Ecl</sub>, or the *arn*<sub>Ecl</sub> coding sequences (Table S3), consistent with the published annotation. In contrast, multiple mutations were found in the *phoPQ*<sub>Ecl</sub> and *arn*<sub>Ecl</sub> operons of the colistin-susceptible isolates. All of the amino acid changes in these coding regions are listed in Table S3. Interestingly, ten of the twelve isolates encoded single nucleotide polymorphisms (SNPs) in the *phoPQ*<sub>Ecl</sub> promoter sequence immediately upstream of the *phoP*<sub>Ecl</sub> translational start codon (within 8 nucleotides), which indicated the SNPs likely disrupted the ribosome binding site (Fig 6A). To determine if the SNPs attenuated PhoPQ-dependent colistin resistance, clinical isolate-3 (CI-3) (ECC cluster III), which did not encode

amino acid changes in the *phoPQ*<sub>Ecl</sub> or *arn*<sub>Ecl</sub> genetic coding regions, was transformed with pPhoPQ<sub>Ecl</sub>. Complementation restored colistin resistance, where the MIC increased from 0.5 in CI-3 to >256 µg/ml after PhoPQ<sub>Ecl</sub> signaling was restored (Fig 6B). Complementation suggested that SNPs in the *phoPQ*<sub>Ecl</sub> ribosome binding sites contributed to colistin susceptibility. SNP mutations, which abrogate translation of PhoPQ<sub>Ecl</sub>, support a model where colistin heteroresistance is dependent on PhoPQ<sub>Ecl</sub> in diverse ECC isolates.

## Discussion

*E. cloacae* and other ECC members encode PmrAB<sub>Ecl</sub> and PhoPQ<sub>Ecl</sub> homologs. We hypothesized these TCS regulatory systems functioned together in a pathway to control L-Ara4N and pEtN modification of lipid A, based on previous transcriptomics analysis of resistant and susceptible populations (39) and because these lipid A modifications are highly conserved among Enterobacteriaceae (11). However, our genetic and high-resolution mass spectrometry analysis of *E. cloacae* lipid A determined that colistin heteroresistance in *E. cloacae* was mediated by PhoPQ<sub>Ecl</sub>-dependent, PmrAB<sub>Ecl</sub>-independent L-Ara4N lipid A modification. Therefore, we identified a mechanism of ECC colistin heteroresistance that involves the PhoPQ system.

*E. cloacae* and other ECC members do not encode a PmrD homolog, which couples PhoPQ signal transduction to regulation of PmrA-dependent genes in many Enterobacteriaceae (11). Moreover, PmrA<sub>Ecl</sub> shares only 52% identity with *E. coli* PmrA and PmrB<sub>Ecl</sub> shares only 57% identity with *E. coli* PmrB, suggesting the L-Ara4N lipid A modification pathway in *E. cloacae* diverged from *E. coli* and *Salmonella*. We confirmed direct binding of PhoP<sub>Ecl</sub> to the *arnB*<sub>Ecl</sub> promoter, which supports a model where L-Ara4N addition to lipid A and colistin heteroresistance in *E. cloacae* is dependent on PhoPQ<sub>Ecl</sub>, but not PmrAB<sub>Ecl</sub>.

Research from other groups has outlined a complex regulatory network in *E. coli* and *Salmonella* that tightly regulates lipid A L-Ara4N and EptA modifications (15–18, 25). We hypothesize that uncoupling PmrAB<sub>Ecl</sub> regulation from L-Ara4N modification bypasses an important regulatory checkpoint, which likely promotes misregulated PhoPQ<sub>Ecl</sub>-dependent *arn<sub>Ecl</sub>* expression. Furthermore, colistin heteroresistance has also been associated with *Klebsiella pneumoniae* (48), another Enterobacteriaceae family member that activates *arn* expression independently of PmrAB (23). Since selection has driven ECC and other opportunistic pathogens to maintain an altered lipid A modification signaling network, we predict that it is advantageous to maintain a CAMP resistant subpopulation in some environments. Presumably, the alternative regulatory mechanism promotes bacterial fitness in environments specific to their commensal and pathogenic niches.

Colistin heteroresistance is not well-understood at the molecular level in Enterobacteriaceae. Our study indicates that PhoPQ<sub>Ecl</sub> signal transduction contributes to heteroresistance in ECC. However, additional studies are necessary to understand if there is a genetic determinant within the heteroresistant subpopulation that promotes resistance. Alternatively, colistin heteroresistance could be a byproduct of promoter noise-induced bimodality, which has been linked to heteroresistance in other bacteria (49, 50). Despite these two possibilities, our studies demonstrated that PhoPQ<sub>Ecl</sub> phosphotransfer (PhoQ<sub>H227</sub> and PhoP<sub>D56</sub>) is required for colistin heteroresistance (Table 1), suggesting the phenotype is regulated by PhoPQ<sub>Ecl</sub>.

## **Experimental Procedures**

### **Bacterial Strains and Growth**

*E. cloacae* subsp. *cloacae* ATCC 13047 and ECC strains were initially grown from freezer stocks on Luria-Bertani (LB) agar. Isolated colonies were used to inoculate LB broth or N

minimal medium (0.1M Bis-Tris, pH 7.5 or 5.8, 5 mM KCl, 7.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 M K<sub>2</sub>SO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.10% casamino acids 0.2% glucose, 0.0002% thiamine, 15 μM FeSO<sub>4</sub>, 10 μM or 10 mM MgSO<sub>4</sub>) at 37° C. Strains were grown into mid-logarithmic growth (OD<sub>600</sub> = 0.6) before analysis. Kanamycin was used at 25 μg/ml for selection and colistin was used at 5 μg/ml or 10 μg/ml where indicated.

All strains and plasmids used in this study are listed in Table S1. Briefly, *E. cloacae* subsp. *cloacae* 13047 mutant strains were constructed as previously described using recombineering with the plasmid pKOBEG (51). Linear PCR products were introduced in to the *E. cloacae* ATCC 13047/pKOBEG strain by electroporation and plated on selective media. Selected clones were transformed with pCP20 to cure the antibiotic resistance cassette.

To complement *E. cloacae* mutants, the coding sequence from *phoPQ*<sub>Ecl</sub> was cloned into the Sall and KpnI sites in pMMBKn (3). To generate point mutants in PhoQ<sub>H277A</sub> and PhoQ<sub>D56A</sub>, site directed mutagenesis was performed using Pfu Turbo using primers that incorporated the associated alanine-encoded nucleotide replacements. All constructs were validated using Sanger sequencing. IPTG inducible constructs were transformed into the *phoPQ* mutant and grown in 2.0 mM IPTG to induce expression.

### **Broth Microdilution assays**

MICs of colistin were determined in triplicate by the broth microdilution (BMD) method. Briefly strains were inoculated from overnight cultures when OD<sub>600</sub> = 0.1. Various concentrations (0 - 256 μg/ml) of colistin were added to each well and cultures were incubated overnight. Growth was measured by reading the OD<sub>600</sub>. The lowest concentration



at which growth was inhibited was recorded as the MIC. *E. coli* W3110 and WD101 were used as control strains. In some cases, 'skip wells' were observed suggesting a heteroresistance phenomenon and the MIC was determined disregarding the clear wells (34).

### **Population Analysis**

Population analysis profiling was performed by plating  $1 \times 10^{10}$  CFU onto LB agar containing 1 to 64  $\mu\text{g/ml}$  colistin (in 2-fold increments). Plates were incubated overnight at  $37^\circ\text{C}$  and frequency of the subpopulation was determined by dividing by the total number of cells (43).

### **Isolation of Lipid A**

Isolation of lipid A for TLC analysis involved  $^{32}\text{P}$ -radiolabeling of whole cells was performed as previously described with slight modifications (52). In brief, 12.5 ml of *E. cloacae* was grown at  $37^\circ\text{C}$  to  $\text{OD}_{600} = 1.0$ . Bacteria were harvested by centrifugation at  $10,000 \times g$  for 10 min. Lipid A extraction was carried out by mild-acid hydrolysis as previously described (53).

### **Mass Spectrometry**

MS1 spectra of lipid A in Figure S7 were collected on a MALDI-TOF/TOF (Axima Performance, Shimadzu) mass spectrometer in the negative mode. All other spectra were collected in the negative mode on a Thermo Scientific Orbitrap Fusion Lumos mass spectrometer (San Jose, CA, USA) modified with a Coherent ExciStar XS ArF excimer laser (Santa Clara, CA), as previously described (54). HCD was performed with the normalized collision energy (NCE) of 25%. UVPD was performed with the laser emitting 193 nm photons at 5 mJ per laser pulse with 5 pulses per scan. The laser pulse repetition rate was 500

Hz. The instrument was operated at 120000 resolving power with a precursor isolation window of 3  $m/z$ . All samples were dissolved in 50:50 MeOH:CHCl<sub>3</sub> and directly infused into the mass spectrometer via a static nano-electrospray ionization source. The presented spectra are an average of 50 scans.

### **TLR-4 Signaling Assays**

HEK-Blue hTLR4, cell line was maintained according to the manufacturer specifications (Invivogen). Overnight bacterial cultures in stationary phase were serially diluted for assays as previously described (2, 3). At least two biological replicates were each done in triplicate and one representative set was shown.

### **Colony Forming Unit Counts and Colistin Survival Assays**

For colony forming unit counts (CFUs), *E. cloacae* subsp. *cloacae* 13047 and mutant strains were initially grown from freezer stocks on Luria-Bertani (LB) agar. Isolated colonies were resuspended and used to inoculate LB broth with 10 µg/ml or without colistin at an OD<sub>600</sub> = 0.01. Cells were plated at designated time points on LB agar. Plates were grown overnight at 37° C and colony forming units (CFU) were counted and reported.

Colistin survival assays were performed as previously described with slight modifications (25). Briefly, wild type and mutant *E. cloacae* strains were grown overnight on LB agar. The following day, N minimal media pH = 7.5 containing either 10 µM MgSO<sub>4</sub> (low Mg<sup>2+</sup>) or 10 mM MgSO<sub>4</sub> (high Mg<sup>2+</sup>) were inoculated at OD<sub>600</sub> = 0.05 with bacteria from overnight cultures after cells were washed with N minimal media without Mg<sup>2+</sup>. Cultures were grown until OD<sub>600</sub> = 0.6, when they were split and treated with 0, 5 or 10 µg/ml of colistin (Polymyxin E). Cultures were incubated for 1 h at 37° C and then colony-

forming units were plated on LB, grown, and calculated. Percent survival was calculated by dividing the number of bacteria after treatment with colistin relative to those incubated in the absence of colistin and then multiplied by 100.

### **Protein Purification**

To purify the PhoP<sub>Ecl</sub> protein, the coding sequence was cloned into pT7-7Kn, as previously described (55). Briefly, the *phoP<sub>Ecl</sub>* coding sequence was amplified from *E. cloacae* cDNA with primers that added a C-terminal His<sub>8X</sub> tag. From an overnight starter culture, 1 Liter of LB broth containing 25 µg/ml of kanamycin was inoculated at 1:50 and grown at 37° C until the OD<sub>600</sub> = 0.5. IPTG was added to a final concentration of 1mM, and the culture was incubated at 37° C for an additional 4 h. Bacteria were recovered by centrifugation at 10,000 x g for 10 min, and the bacteria were resuspended in lysis buffer. Bacteria were lysed using sonication and the soluble fraction was recovered by centrifugation at 10,000 x g for 30 min. PhoP<sub>Ecl</sub>-His<sub>8X</sub> was purified on a Ni-nitrilotriacetic acid (NTA) beads according to the manufactures instructions (Qiagen).

### **Electrophoretic Mobility Shift Assay**

PhoP<sub>Ecl</sub>-His<sub>8X</sub> proteins were purified as described above. EMSAs were performed based on a modified protocol (56). 250-bp DNA fragments of *phoP<sub>Ecl</sub>* and *arnB<sub>Ecl</sub>* spanning -230 to +20 relative to the translational start site were amplified from *E. cloacae* or *E. coli* cDNA using 5'-biotinylated primers. PhoP<sub>Ecl</sub>-His<sub>8X</sub> proteins were incubated with biotinylated DNA at 25° C for 20 min. For competition experiments, unlabeled *E. cloacae* P<sub>arnB</sub> and poly(dI-dC) were added at 1:1, 2:1, or 5:1 ratios relative to biotin-labeled P<sub>arnB</sub> DNA. 0.1 - 10 µM of PhoP<sub>Ecl</sub>-His<sub>8X</sub> proteins were used. After electrophoresis at 4° C, protein/DNA was transferred onto a

positively charged nylon membrane. Blots were blocked in 5% milk in TBS for 20 min and streptavidin conjugated HRP was used at a 1:300 dilution.

### **Nucleic Acid Extraction**

Total RNA was extracted using the Direct-Zol RNA MiniPrep Kit (Zymo Research) from *E. cloacae* grown to a final  $OD_{600} = 0.6$ . Isolated RNA was treated with DNA-free DNA removal kit (Thermo-Fisher Scientific) to eliminate genomic DNA contamination. DNase-depleted RNA was used for qRT-PCR and RNA-seq.

### **RNA-sequencing**

RNA-sequencing was performed as previously described (57). Briefly, DNA-depleted RNA was processed for Illumina sequencing using the NEB Next Ultra Directional RNA Library Prep kit for Illumina as described by the manufacturer (NEB). Sequencing was performed using Illumina HiSeq. Sequencing data was aligned to the *E. cloacae* subs. *cloacae* ATCC 13047 published genome annotations (47) using CLC genomic workbench software (Qiagen) and RPKM expression values were determined. The weighted proportions fold change of expression values between samples was determined and a Baggerley's test on proportions was used to generate a false discovery rate corrected P-value. We then used a cut-off of 2-fold weighted proportions absolute change with a false-discovery rate corrected P-value of  $\leq 0.05$  to identify significantly differentially regulated genes between samples. The sequencing data for the clinical isolates has been deposited in the Nation Center for Biotechnology's Gene Expression Omnibus (GSE127802).

## **Genomic-sequencing**

Genomic sequences were analyzed as previously done (3). Briefly, samples were processed for Illumina sequencing using the NEB Next Ultra DNA Library Prep kit (NEB). Sequencing was performed using Illumina HiSeq. Reads were aligned to *E. cloacae* strain ATCC 13047 published genome annotations using CLC genomic workbench software (Qiagen) with 90% length fraction and 90% similarities parameters. Mapped reads were locally realigned and fixed ploidy detection identified low and high frequency variants. *E. cloacae* variant tracks were compared to identify mutations. Mutations not present in strain ATCC 13047 were called if 95% of aligned reads contained the variant. The sequence data have been submitted to the GenBank under accession number SUB4176618.

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## **Author Contributions**

KNK, JMB: Design of the study; KNK, DRK, MIK, FG, VC, JSB, JMB: Acquisition analysis and interpretation of the data; KNK, JMB: Writing the manuscript.

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## Table

Table 1. MICs of colistin and PAP analysis for each *E. cloacae* mutant

Isolate	MIC of colistin (µg/ml) by BMD <sup>b</sup>	MIC of colistin (µg/ml) by Etest	Frequency of appearance of subpopulations (PAPs <sup>a</sup> )						
			Concentration of colistin						
			1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml
Wild type	≥256*	0.125**	9.5 X 10 <sup>-3</sup>	6.3 X 10 <sup>-2</sup>	5.2 X 10 <sup>-3</sup>	7.5 X 10 <sup>-3</sup>	7.6 X 10 <sup>-2</sup>	6.3 X 10 <sup>-3</sup>	9.3 X 10 <sup>-3</sup>
ΔphoPQ <sub>Ecl</sub>	0.5	0.125	2.6 X 10 <sup>-3</sup>	0	0	0	0	0	0
ΔphoPQ <sub>Ecl</sub> +pPhoPQ <sub>Ecl</sub>	≥256*	0.125**	3.6 X 10 <sup>-2</sup>	1.3 X 10 <sup>-2</sup>	4.3 X 10 <sup>-3</sup>	1.6 X 10 <sup>-2</sup>	4.1 X 10 <sup>-3</sup>	1.7 X 10 <sup>-3</sup>	4.5 X 10 <sup>-4</sup>
ΔphoPQ <sub>Ecl</sub> +pPhoPQ <sub>H277AEcl</sub>	0.5	0.125	3.1 X 10 <sup>-2</sup>	0	0	0	0	0	0
ΔphoPQ <sub>Ecl</sub> +pPhoP <sub>D56AQ<sub>Ecl</sub></sub>	0.5	0.125	7.4 X 10 <sup>-2</sup>	0	0	0	0	0	0
ΔpmrAB <sub>Ecl</sub>	≥256*	0.125**	2.9 X 10 <sup>-2</sup>	1.8 X 10 <sup>-3</sup>	7.7 X 10 <sup>-2</sup>	9.2 X 10 <sup>-3</sup>	5.5 X 10 <sup>-2</sup>	1.3 X 10 <sup>-3</sup>	2.3 X 10 <sup>-4</sup>
ΔarnT <sub>Ecl</sub>	0.5	0.125	4.0 X 10 <sup>-2</sup>	0	0	0	0	0	0

<sup>a</sup>PAP : Population Analysis Profile using an initial culture of 10<sup>10</sup> CFU/mL

<sup>b</sup>BMD : Broth microdilution method

\* Presence of skip wells

\*\* Presence of squatter colonies inside the zone of inhibition

## Figure Legends

**Figure 1: Survival of colistin heteroresistant *E. cloacae* is dependent on PhoPQ<sub>Ecl</sub>, but not PmrAB<sub>Ecl</sub>-regulated lipid A modifications.** (A) *E. cloacae* logarithmic phase growth over time as measured by colony forming units (CFUs). At two hours, the growth rate between wild type grown in LB was significantly (\*) different from cells grown in LB + colistin (P value <0.05). (B) <sup>32</sup>P-radiolabeled lipid A was isolated from wild type and mutant *E. cloacae* strains and separated based on hydrophobicity using thin layer chromatography. Lipid A species are labeled as unmodified or modified as determined by *E. coli* W3110 (lane 1) and WD101 (lane 9) lipid A, respectively.

**Figure 2: Expanded MS1 spectra of lipid A isolated from (A) wild type *E. cloacae* grown in media supplemented with 10 µg/ml colistin and (B) Δ*phoPQ*<sub>Ecl</sub>, which was grown in media without antibiotics.** The chemical structures associated with the MS1 spectra are illustrated on the right. The presence of aminoarabinose groups are denoted by L-Ara4N (red), while addition of palmitoyl groups are denoted by +C<sub>16:0</sub> (green). Hydroxylation is not illustrated, but is indicated by an *m/z* shift of 8 relative to doubly-charged lipid A ions in the spectra.

**Figure 3: PhoP<sub>Ecl</sub> binds to the *arnB* promoter of *E. cloacae* (*Ecl*), but not *E. coli* (*Ec*).** (A) Illustration of the *arn* operon organization. (B) Sequence alignment of the *phoP* promoter (P<sub>phoP</sub>) region in *Ec*, *Salmonella* (*St*), and *Ecl*, which each contain a PhoP box. The *arnB* promoter (P<sub>arnB</sub>) of *Ecl* contains a putative PhoP box binding site that is not present in *Ec*. The putative PhoP boxes have been boxed, while the -10 region is underlined. There were no putative PmrA boxes in the *Ecl arnB* or *phoP* promoter regions, so they were not included for simplicity. (C) Electrophoretic mobility shift assay (EMSA) of *Ecl* P<sub>arnB</sub> with increasing

concentrations of PhoP<sub>Ecl</sub>. PhoP<sub>Ecl</sub> was used at concentrations of 0, 0.1, 1.0, 5.0 and 10.0  $\mu$ M. EMSA using *Ec* P<sub>arnB</sub> in the absence or presence of PhoP<sub>Ecl</sub>, respectively. (D) EMSA competition experiments where increasing concentrations (1:1, 2:1, 5:1) of unlabeled P<sub>arnB</sub> competes with biotin-labeled P<sub>arnB</sub>, but nonspecific unlabeled poly(dI-dC) (2:1, 5:1) does not. (E) PhoP<sub>Ecl</sub> binds to both the *Ecl* and *Ec* *phoP* promoters.

**Figure 4: RNA-sequencing analysis of *E. cloacae* genes.** Heat map illustrating the altered expression of select operons in  $\Delta$ *phoPQ*<sub>Ecl</sub> and  $\Delta$ *pmrAB*<sub>Ecl</sub> mutants. Expression is shown as a ratio of mutant to wild type expression ( $P < 0.05$ ).

**Figure 5: PhoPQ<sub>Ecl</sub>-dependent activation of L-Ara4N addition induces colistin resistance in low Mg<sup>2+</sup>.** (A) Wild type and mutant *E. cloacae* strains were grown in N minimal medium with high (10mM, top) or low (10  $\mu$ M, bottom) Mg<sup>2+</sup>. Strains were challenged with 0, 5, or 10  $\mu$ g/ml of colistin for 1 h and plated for survival. Two biological replicates were each analyzed in triplicate with data from one representative set reported.  $P$  value  $< 0.05$ . (B) <sup>32</sup>P-radiolabeled lipid A was isolated from wild type and mutant *E. cloacae* strains and separated based on hydrophobicity using thin layer chromatography. The associated lipid A structures (right) are illustrated with black circles indicating L-Ara4N Addition. Lipid A species were labeled as determined by *E. coli* W3110 (unmodified) and WD101 (modified) lipid A.

**Figure 6: PhoP<sub>Ecl</sub> single nucleotide polymorphisms that attenuate colistin heteroresistance in ECC clinical isolates.** (A) Sequence alignment of *E. cloacae* and other ECC *phoP* promoter (P<sub>phoP</sub>) regions. The region includes 25 nucleotides upstream of the start codon (boxed). Grey boxes indicate single nucleotide replacement mutations, while grey

triangles indicate single nucleotide insertion mutations. **(B)** E-test strips of CI-3 and CI-3/pPhoPQ<sub>Ecl</sub> to visualize heteroresistant colonies. The black arrow indicates colistin resistant colonies.







