**Effects of regulatory network organization and environment on PmrD connector activity
and polymyxin resistance in *Klebsiella pneumoniae* and *Escherichia coli***

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Running title: PmrD connector activity in polymyxin resistance

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Abstract

Polymyxins are a class of cyclic peptides with antimicrobial activity against Gram-negative bacteria. Resistance to these compounds is often mediated by pathways that also confer resistance to host antimicrobial peptides. In *Enterobacteriaceae*, the PhoQ/PhoP and PmrB/PmrA two-component systems regulate many of the genes associated with these resistance mechanisms. In *K. pneumoniae*, spontaneous polymyxin resistance is frequently acquired through inactivation of the gene *mgrB*, which encodes a negative regulator of PhoQ. However, this resistance mechanism has not been reported in other genera of *Enterobacteriaceae*, despite the presence of *mgrB* among many members of this family. In addition, the frequency of developing spontaneous resistance to the antimicrobial peptide polymyxin through chromosomal mutations is much higher in *Klebsiella* compared to *Salmonella* or *E. coli*. Here we show that in *K. pneumoniae*, PmrD is not required for polymyxin resistance arising from inactivation of *mgrB*. In addition, we show that in *E. coli*, the protein PmrD can activate PmrA under certain conditions. Our results suggest that the importance of PmrD connector activity in polymyxin resistance depends on both the network organization and on the environmental conditions associated with PmrB stimulation.

Introduction

Polymyxins, such as colistin and polymyxin B, have emerged as last-resort antibiotics for many multidrug-resistant Gram-negative bacteria (1). These compounds are cationic antimicrobial peptides that are thought to disrupt the bacterial outer membrane through interactions with negatively charged lipopolysaccharides (LPS) (2-6). In addition to their role in treating infections, polymyxins have also been used in the laboratory as a tool to study pathways in bacteria that mediate resistance to host antimicrobial peptides. Several mechanisms of polymyxin resistance have been identified among Gram-negative bacteria, with the most common mechanism involving modification of the LPS (7, 8).

In many *Enterobacteriaceae*, polymyxin resistance is controlled primarily by the PmrB/PmrA and PhoQ/PhoP two-component systems (9-11). The PmrB/PmrA system regulates the expression of enzymes that covalently modify the lipid A to decrease the negative charge of the outer membrane (9, 12, 13). The sensor kinase PmrB has both kinase and phosphatase activities; activation of PmrB through ferric iron or mild acidity leads to increased levels of phosphorylated PmrA (14, 15). Phosphorylated PmrA activates expression of several genes that encode LPS modification enzymes, including *ugd* and the *arnBCADTEF* operon, whose protein products mediate addition of 4-aminoarabinose to lipid A, and *pmrC*, which encodes an enzyme that modifies the lipid A with phosphoethanolamine (16, 17). PmrA phosphorylation can also be regulated indirectly by the PhoQ/PhoP two-component system (18), which is activated by low magnesium and cationic antimicrobial peptides (19-21). PhoP regulates expression of the gene *pmrD*, which encodes a small protein that inhibits dephosphorylation of PmrA by PmrB (22-25). PmrD is an example of a "connector" protein (because it connects the PhoQ/PhoP and PmrB/PmrA systems) (26), and the indirect regulation of PmrA by PhoP via PmrD is referred to

as the connector-mediated pathway. In *Klebsiella*, the PhoQ/PhoP system regulates *arnBCADTEF* not only indirectly via PmrD but also through a second mechanism, namely direct activation of the *arnB* promoter by PhoP (27). This direct activation is absent in *Salmonella* and *E. coli* (Fig. 1).

Cross-regulation between other two-component systems has also been shown to mediate polymyxin resistance. In some uropathogenic *E. coli*, for example, PmrB can phosphorylate a noncognate response regulator, QseB, which has been shown to play a role in polymyxin resistance (28). In addition, some *K. pneumoniae* strains encode the CrrB/CrrA two-component system, which can activate PmrB through the CrrA-regulated gene *crrC* (29, 30).

Analysis of polymyxin-resistant isolates of *Enterobacteriaceae* in clinical and laboratory settings have identified several mutations that confer polymyxin resistance (7, 31). Curiously, the chromosomal mutations identified in polymyxin-resistant isolates vary among *Enterobacteriaceae*, such as *Klebsiella pneumoniae*, *Salmonella enterica*, and *Escherichia coli*, even though the proteins involved in regulating LPS modifications are largely conserved. In *K. pneumoniae*, mutations in *phoQ*, *phoP*, *pmrB*, and *pmrA* that result in constitutive expression of LPS modifications have been shown to confer polymyxin resistance. Yet the most common mechanism of polymyxin resistance in *K. pneumoniae* is inactivation of the gene *mgrB*, which encodes a negative regulator of PhoQ (32-34). Numerous mutations in *mgrB* have been identified among polymyxin-resistant isolates, including inactivation through insertion sequences, nonsense mutations, or missense mutations. The high frequency of this mechanism of resistance likely reflects the fact that there are many more ways to inactivate a gene compared to the

limited number of mutations in a sensor kinase or response regulator gene that lead to constitutive activation. Since mutations in the polymyxin resistance network, either in *mgrB* or in two-component system proteins, all lead to upregulation of LPS modifications, one might expect to find *mgrB* inactivation as a dominant mechanism of resistance in related bacteria. However, inactivation of *mgrB* has not been observed as a mechanism of resistance among other *Enterobacteriaceae*, even though many other genera have *mgrB* orthologs (34).

In contrast to *K. pneumoniae*, *Salmonella* primarily acquires polymyxin resistance through mutations in the sensor kinase or response regulator of the PmrB/PmrA or PhoQ/PhoP two-component systems (10, 16, 35-37). In *E. coli*, on the other hand, the PmrB/PmrA system alone is the primary target for mutations conferring polymyxin resistance (38-40). Previous reports have shown that stimulation of PhoQ in *E. coli* does not lead to PmrA activation, leading to the conclusion that *E. coli* PmrD cannot function as a connector protein between PhoQ/PhoP and PmrB/PmrA (41). However, the *E. coli* PmrD protein can function as a connector in *Salmonella*, and the *Salmonella* PmrD protein can similarly function as a connector in *E. coli* (41, 42). This difference between the activities of *E. coli* and *Salmonella* PmrD orthologs is apparently due to differences in their ability to inhibit the phosphatase activity of *E. coli* PmrB, which is higher than that of *Salmonella* PmrB (42). These observations raise the question of why *E. coli* has a PmrD protein. However, one study reported an increase in LPS modifications under PhoQ-stimulating conditions in *E. coli* that was dependent on PmrD (43).

Here we investigate the regulation of the *arnB* operon by the PhoQ/PhoP two-component system in *K. pneumoniae* and in *E. coli*. We find that the direct regulation of *arnB* by PhoP in *K.*

102 *pneumoniae* is critical for *mgrB* inactivation to produce high-level polymyxin resistance. We
 103 also find that PmrD can function as a connector between PhoQ/PhoP and PmrB/PmrA in *E. coli*
 104 and further show that the role of PmrD as a connector protein depends on the level of PmrB
 105 stimulation both in *K. pneumoniae* and in *E. coli*. These results indicate that environmental
 106 conditions affect the level of cross-regulation between PhoQ/PhoP and PmrB/PmrA.
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Results

Frequency of spontaneous polymyxin resistance is higher in *K. pneumoniae* than in *Salmonella* or *E. coli* and is consistent with differences in the effect of *mgrB* inactivation

To test whether deletion of *mgrB* could improve polymyxin resistance in other genera of *Enterobacteriaceae* in addition to *Klebsiella*, we compared the minimum inhibitory concentration (MIC) of polymyxin B for wild-type and $\Delta mgrB$ strains of *K. pneumoniae* MGH 78578, *S. enterica* serovar Typhimurium (*S. Typhimurium*) 14028s, and *E. coli* MG1655. As expected, we found that the MIC of polymyxin B was much higher (~16-32 times higher) for a *K. pneumoniae* $\Delta mgrB$ strain compared to wild type (Table 1). In contrast, deletion of *mgrB* in *Salmonella* only increased the MIC by twofold (Table 1), even though activation of PhoQ through low magnesium increases polymyxin tolerance in *Salmonella* (44). Deletion of *mgrB* did not confer any detectable protection against polymyxin in *E. coli* under the conditions of the assay, which is consistent with previous reports that activation of PhoQ does not lead to activation of PmrA in *E. coli* (41).

The above observations suggest that the frequency of spontaneous polymyxin resistance would be higher for *K. pneumoniae* than for *Salmonella* and *E. coli*, since inactivating mutations of *mgrB* are likely to arise with higher frequency than the change-of-function mutations associated with other mechanisms of polymyxin B resistance. To test this hypothesis, we plated overnight cultures of wild-type *K. pneumoniae*, *S. Typhimurium*, and *E. coli* on LB agar containing 10 μ g/mL polymyxin B to select for spontaneous resistance. We found that indeed the frequency of spontaneous polymyxin resistance was much higher for *K. pneumoniae* (Fig. 2). We also tested eight colonies at random and found that all had inactivating mutations in *mgrB* (7

insertion sequences and one nonsense mutation), indicating that inactivation of *mgrB* accounts for the high frequency of spontaneous polymyxin resistance in *K. pneumoniae*.

PmrD and PmrA play a minor role in polymyxin resistance in a *K. pneumoniae* $\Delta mgrB$ strain

In *Klebsiella*, PhoP directly activates transcription of the *arnB* operon, a mode of *arnB* regulation that is not found in *Salmonella* or *E. coli* (Fig. 1) (27). We therefore tested the relative importance of direct regulation and of the connector-mediated pathway in polymyxin resistance in *K. pneumoniae*. We found that deletion of *pmrD* in a $\Delta mgrB$ strain had no effect on the polymyxin B MIC, suggesting that the connector-mediated pathway between PhoQ/PhoP and PmrB/PmrA was not necessary for resistance in this bacterium (Table 1). In addition, deletion of *pmrA* similarly did not affect the MIC of polymyxin B, which is consistent with previous observations (45). On the other hand, resistance was suppressed by deleting *phoP*, indicating that PhoP is necessary for the polymyxin resistance of a $\Delta mgrB$ strain. To detect potentially smaller effects of polymyxin among strains, we also used a polymyxin B sensitivity assay in which exponential-phase cells were treated with polymyxin for one hour, and then plated to determine the survival relative to untreated controls (see Materials and Methods). We found that deleting either *pmrD* or *pmrA* in a $\Delta mgrB$ strain had little effect on survival, whereas deleting *phoP* suppressed polymyxin tolerance of a $\Delta mgrB$ strain (Fig. 3). We also found that the frequency of spontaneous polymyxin resistance was similar for *K. pneumoniae* wild-type, $\Delta pmrD$, and $\Delta pmrA$ strains, and was much higher than that of a $\Delta phoP$ strain (Fig. 2), consistent with the above observations. Taken together, the above results indicate that polymyxin resistance mediated by inactivation of *mgrB* requires PhoP but does not require PmrD or PmrA.

To assess the importance of aminoarabinose and phosphoethanolamine lipid A modification pathways to polymyxin resistance in a *K. pneumoniae* $\Delta mgrB$ strain, we tested the effect of deleting either *arnB* or *pmrC*. Deletion of *pmrC* did not affect polymyxin resistance. On the other hand, deletion of *arnB* in a $\Delta mgrB$ strain lowered the MIC to that of wild type, suggesting that the *arnB* gene is critical for polymyxin resistance mediated by *mgrB* inactivation (Table 1), which is consistent with the results of a previous study (45).

PmrD connector activity in *K. pneumoniae* depends on environmental stimuli

In the assays described above, PmrD did not affect polymyxin sensitivity of a *K. pneumoniae* $\Delta mgrB$ strain. However, a previous study found that deletion of *pmrD* in wild-type *K. pneumoniae* affects polymyxin tolerance (25). We therefore considered the possibility that these different observations reflect differences in growth conditions. We reasoned that if either PhoQ or PmrB is sufficiently activated, then PmrD may not be important for polymyxin resistance, since both PhoP and PmrA can directly activate the *arnB* operon. However, for low or intermediate PmrB and PhoQ stimulation, PmrD may play an important role in limiting PmrA dephosphorylation and promoting polymyxin resistance.

To test this hypothesis, we grew cells in different concentrations of FeSO₄, which stimulates PmrB (14), and measured polymyxin B susceptibility. In the absence of iron addition, wild-type *K. pneumoniae* cells showed ~1% survival from treatment with 1 µg/mL polymyxin B. When cells were pre-treated with 15 µM FeSO₄, the survival increased to ~100%, and this increase in survival depended on *pmrD*, *pmrA*, and *phoP* (Fig. 4). When cells were pre-induced

with 100 μ M FeSO₄, the survival of $\Delta pmrD$ cells increased to ~70%, suggesting that high PmrB stimulation decreases the importance of PmrD in promoting polymyxin tolerance. We also found that the survival of a $\Delta phoP$ strain pre-induced with 100 μ M FeSO₄ was much lower than that of wild type. This observation suggests that, even under the strong PmrB-stimulating conditions of high iron, the direct regulation of the *arnB* operon by PhoP, or regulation of other LPS-modifying genes by PhoP, is critical for survival.

Since mild acidity has been shown to stimulate PhoQ and PmrB in *Salmonella* (15, 46-48), we also tested whether pre-induction with mild acidity could increase polymyxin tolerance in *K. pneumoniae*. We found that wild-type *K. pneumoniae* cells grown in pH 5.8 N-minimal medium showed increased polymyxin tolerance compared to those grown at pH 7.5, and deletion of *pmrD* did not affect survival (Fig. 5). Deletion of *pmrA* showed only a slight decrease in survival compared to that of wild type, suggesting that PhoP may play a more dominant role than PmrA under these conditions.

Taken together, the above results suggest that PmrD connector activity in *K. pneumoniae* is critical for polymyxin resistance under conditions with relatively low PhoQ stimulation and intermediate PmrB stimulation, such as growth in 1 mM Mg⁺⁺ and 15 μ M Fe⁺⁺.

Iron enables *E. coli* PmrD to function as a connector between PhoQ/PhoP and PmrB/PmrA

In light of our observations that the role of *K. pneumoniae* PmrD in polymyxin resistance depends on environmental conditions, we revisited the role of PmrD in *E. coli*. As discussed above, previous work indicated that *E. coli* PmrD does not function as a connector between

PhoQ/PhoP and PmrB/PmrA in *E. coli*, and it was suggested that this lack of connector activity is due to the strong phosphatase activity of *E. coli* PmrB compared, for example, to that of *Salmonella* PmrB (41, 42). However, a recent study reported that *E. coli* PmrD plays a role in polymyxin tolerance when PhoQ is stimulated by low magnesium (43), although PmrD connector activity was not addressed directly. The minimal medium used in the latter study contained 15 μM FeSO_4 . We therefore considered the possibility that the apparent discrepancy between these different studies reflects differences in the level of PmrB stimulation. We reasoned that iron might lower PmrB phosphatase activity and thereby enable PmrD to function as a connector in *E. coli*.

To test this hypothesis, we assayed polymyxin sensitivity of wild-type *E. coli* cells growing in N-minimal medium pH 7.5 with high or low magnesium and with varying concentrations of iron. We first tested the effect of iron supplementation for cells growing in 1 mM Mg^{++} , a condition for which *E. coli* PhoQ is only moderately stimulated (49). Addition of FeSO_4 to either 15 μM or 100 μM did not increase tolerance polymyxin B (Fig. 6A). This result contrasts with the behavior of *K. pneumoniae*, for which 15 μM FeSO_4 was sufficient to increase survival to ~100% in these growth conditions.

We next tested cells growing in low magnesium (5 μM Mg^{++}), a condition that strongly stimulates PhoQ. Without added iron, low magnesium did not increase polymyxin tolerance relative to 1 mM Mg^{++} , consistent with previous reports (41, 42) (Fig. 6A). However, addition of 15 μM FeSO_4 to the low magnesium medium increased survival more than 10-fold, and addition of 100 μM FeSO_4 further increased survival. Deletion of *pmrD* abrogated this effect for 15 μM

FeSO₄ but decreased survival only about ~2-fold for 100 μM FeSO₄. This result suggests that the role of *E. coli* PmrD in polymyxin tolerance is most important when there is strong stimulation of PhoQ and intermediate stimulation of PmrB. In addition, the observation that *pmrD* is less important for polymyxin survival under conditions of low magnesium and 100 μM FeSO₄ suggests that this high concentration of iron stimulates PmrB sufficiently strongly to increase polymyxin tolerance on its own.

To determine the effect of the above growth conditions on PmrA activity, we used a fluorescent reporter for the PmrA-dependent *arnB* promoter, *P_{arnB}-gfp*. For wild-type cells growing in high Mg⁺⁺, 100 μM FeSO₄ produced a relatively small increase in *arnB* transcription (Fig. 6B). Under low magnesium conditions, on the other hand, both 15 μM and 100 μM FeSO₄ greatly increased *arnB* transcription, consistent with the results of the polymyxin B sensitivity assays. Furthermore, deletion of *pmrD* decreased the effects of iron on *arnB* transcription at low magnesium (Fig. 6B), although the level of GFP fluorescence from *ΔpmrD* cells with 100 μM FeSO₄ was still higher than that of wild-type cells grown in high magnesium with the same concentration of iron. This latter result is consistent with the observation above that 100 μM iron increases polymyxin tolerance independently of *pmrD* at low magnesium but not at high magnesium and suggests that magnesium exerts both PmrD-dependent and PmrD-independent effects on *E. coli* PmrB/PmrA activity.

***E. coli* PmrD also functions as a connector at low pH or when PhoQ is strongly stimulated**

Since low pH is another PmrB stimulus (in addition to stimulating PhoQ), we tested whether this condition also enables PmrD to function as a connector. We found that growth at

pH 5.8 greatly increased GFP fluorescence from P_{arnB} -*gfp* relative to cells grown at pH 7.5, and this increase depended on *phoP*, *pmrD*, and *pmrA* (Fig. 7). Thus, *E. coli* PmrD connects PhoQ/PhoP with PmrB/PmrA under two conditions that are known to stimulate PmrB.

We also wondered whether PmrD could function as a connector even in the absence of iron or low pH if PhoQ were stimulated to a sufficiently high level. We reasoned that strongly stimulated PhoQ/PhoP might produce enough PmrD to effectively overpower the strong phosphatase activity of *E. coli* PmrB. To achieve high levels of PhoQ stimulation, we grew a $\Delta mgrB$ strain in low magnesium (34, 50). We found that P_{arnB} -*gfp* transcription increased with decreasing concentrations of magnesium, and this increase in transcription required *pmrD* (Fig. 8). These results suggest that very high stimulation of PhoQ achieved through the combination of low magnesium and deletion of *mgrB* enables PmrD to function as a connector between PhoQ/PhoP and PmrB/PmrA in *E. coli*.

Discussion

This work extends previous observations concerning natural variation in the organization of the polymyxin resistance network mediated by the PhoQ/PhoP and PmrB/PmrA two-component systems. Our results indicate that differences in the organization of this network account for the fact that *mgrB* inactivation leads to high levels of polymyxin resistance in *Klebsiella* but not in the closely related *Enterobacteriaceae* *E. coli* and *Salmonella*. Specifically, the direct regulation of the *arnB* operon by PhoP, which occurs in *Klebsiella* but not in *E. coli* and *Salmonella*, is required for this resistance mechanism. This direct regulation is not unique to *Klebsiella* and also appears, for example, in *Yersinia pestis*. It would therefore be interesting to test whether *mgrB* inactivation similarly confers polymyxin resistance in other bacteria with this type of regulation of *arnB*.

The above results also revise our understanding of the polymyxin resistance network in *E. coli*. We find that PmrD functions as a connector between PhoQ/PhoP and PmrB/PmrA in *E. coli*, but this activity depends on appropriate levels of PhoQ and PmrB stimulation. This latter observation explains why *E. coli* connector activity was not observed in previous studies and reconciles results that appeared to be in conflict, but instead likely reflect different levels of PmrB stimulation due to the presence or absence of 15 μ M iron in the growth medium (41, 43). As discussed above, we hypothesized that PmrB stimulation lowers the otherwise strong phosphatase activity of *E. coli* PmrB, enabling PmrD to function as a connector. In this sense, our results do not contradict and in fact provide further support for previous observations regarding the differences in the activity of PmrD and PmrB in *E. coli* and *Salmonella* (41, 42). In *Klebsiella*, we also find that PmrD activity depends on the levels of PhoQ and PmrB stimulation,

and is most important for polymyxin resistance when PhoQ stimulation is low and PmrB stimulation is intermediate.

For growth in mild acidity, we found that a *K. pneumoniae* $\Delta phoP$ strain was more sensitive to polymyxin than a $\Delta pmrA$ strain. We also found that under high iron conditions, deletion of *phoP* showed a significant decrease in survival compared to wild type, even though survival was higher than that of a $\Delta phoP$ strain grown without added iron. This latter result may indicate that the direct regulation of *arnB* by PhoP contributes to polymyxin resistance even under the strong PmrB-stimulating conditions of high iron. It may also indicate that, despite high PmrB stimulation, additional LPS modification genes regulated by PhoP are needed for full protection against polymyxin (45). Overall, at least under the conditions tested here, PhoP appears to play a dominant role in polymyxin resistance in *Klebsiella*.

The regulatory architecture in *K. pneumoniae*, which consists of direct activation and indirect activation of *arnB* through a connector protein, is an example of a feedforward connector loop. This network topology is characterized by fast activation and slow deactivation of transcription, properties that may be important for bacterial fitness (27, 51, 52). Our results highlight another feature of this architecture: the ability to tune the output in response to multiple environmental signals that act at different points in the network—in this case, signals that stimulate PhoQ (low magnesium), PmrB (iron), or both (pH). Thus, this architecture enables complex environmental control of *arnB* transcription in *Klebsiella* that is distinct from the pattern found in *E. coli* and *Salmonella*. Future work may uncover additional signals that also control these networks *in vivo* or in other environments.

Materials and Methods

Strains and Plasmids

Strains, plasmids, and primers are listed in Tables 2, 3, and 4, respectively. *E. coli* strains were derived from *E. coli* K-12 strain MG1655 (CGSC #7740), *S. Typhimurium* strains were derived from *S. Typhimurium* 14028s, and *K. pneumoniae* strains were derived from *K. pneumoniae* MGH 78578 (ATCC 700721).

Media and growth conditions

Cultures were grown aerobically at 37 °C in LB medium, low salt LB medium (5 g/L yeast extract, 10 g/L tryptone, and 5 g/L NaCl adjusted to pH 8.0 with NaOH), Mueller-Hinton broth (MHB), cation-adjusted MHB (MHB with 20-25 mg/L Ca^{+2} and 10-12.5 mg/L Mg^{+2}), or in N-minimal medium (0.1 M Bis-Tris, pH 7.5 or 5.8, 5 mM KCl, 7.5 mM $(\text{NH}_4)_2\text{SO}_4$, 0.5 mM K_2SO_4 , and 1 mM KH_2PO_4) containing 0.2% glucose, 0.1% casamino acids, and the indicated concentrations of MgCl_2 and FeSO_4 . Low salt LB was only used for growing cultures with hygromycin. LB (Miller) agar was used for growth on solid medium. Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 2 mM KH_2PO_4 . The antibiotics streptomycin, kanamycin, ampicillin, chloramphenicol, hygromycin, and apramycin were used at concentrations of 100 µg/mL, 25 µg/mL, 100 µg/mL, 25 µg/mL, 100 µg/mL, and 50 µg/mL.

Strain Construction

E. coli

Gene deletions in *E. coli* were transferred to strains by transduction with P1_{vir} (53). Antibiotic resistance cassettes flanked by FLP recombinase target (FRT) sites were removed using pCP20 (54).

S. Typhimurium

The *mgrB* gene in *S. Typhimurium* was deleted and replaced with a FRT-*cat*-FRT DNA segment by lambda red recombineering using the helper plasmid pKD46 and the plasmid pKD3 as the template for FRT-*cat*-FRT, as described previously (55). The deletion was transferred to a fresh 14028s background by transduction with P22 phage. Transductants were streaked on green indicator plates (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar, 0.25% glucose, 0.5% K₂HPO₄, 0.00125% methylene blue, and 0.0025% sodium fluorescein) to identify phage-free colonies (56).

K. pneumoniae

Gene deletions in *K. pneumoniae* were constructed by replacing the gene of interest with an apramycin resistance cassette FRT-*apra*-FRT by recombineering using pACBSR-Hyg as described in (57). Plasmid pMDIAI was used as the template for the FRT-*apra*-FRT segment for the $\Delta mgrB$ strain. For all other deletions, plasmid pAC25 was used as the template for the FRT-*apra*-FRT segment. Antibiotic resistance cassettes flanked by FLP recombinase target (FRT) sites were removed using pFLP-Hyg.

Plasmid Construction

Plasmid pAC25 was constructed by replacing the kanamycin resistance gene in pKD13 with the apramycin resistance gene using FLP recombinase expressed from pFLP-Hyg. The plasmid was confirmed by sequencing using the primer oriR6Kseqprim1.

Fluorescence quantification

Strains were grown overnight in N-minimal medium pH 7.5 with 1mM MgCl₂ to saturation at 37 °C. Cultures were diluted 1:1000 into fresh medium at the indicated pH and with the indicated concentrations of MgCl₂ and FeSO₄ in plastic culture tubes and grown to an OD₆₀₀ ~ 0.3-0.4. Streptomycin was added to a final concentration of 100 µg/mL, and cultures were put on ice. GFP fluorescence was measured in a fluorometer (Photon Technology International Quantamaster). Fluorescence was then divided by OD₆₀₀ to normalize by cell density, and background fluorescence from non-fluorescent cells, measured from the strain MG1655 containing pWKS130, a control plasmid without *gfp*, was subtracted from each measurement.

Minimum inhibitory concentration assay

Minimum inhibitory concentrations (MIC's) of polymyxin B were determined by a broth microdilution assay as described previously (58). Briefly, cells were grown overnight in MHB to saturation at 37 °C. Cultures were diluted 1:1000 into cation-adjusted MHB and grown to OD₆₀₀ ~ 0.3-0.4. Cells were diluted 1:150 in cation-adjusted MHB, and 50 µL was added to a 96-well microtiter plate containing two-fold dilutions of polymyxin B. The microtiter plate was incubated on a shaker at 37 °C overnight, and the MIC was determined based on turbidity.

Polymyxin B susceptibility assay

Susceptibility assays were performed essentially as described previously (41). Strains were grown overnight in N-minimal medium pH 7.5 with 1 mM MgCl₂ to saturation at 37 °C. Cultures were diluted 1:1000 into fresh medium at the indicated pH and concentrations of MgCl₂

and FeSO₄ in plastic culture tubes and grown to an OD₆₀₀ ~ 0.3-0.4. Cultures were diluted 1:200 into Eppendorf tubes (pre-incubated at 37 °C for at least one hour) containing 1 mL PBS + 1 mM MgCl₂ with and without 1 µg/mL polymyxin B and incubated at 37 °C for one hour. Serial dilutions in PBS were then plated in triplicate on LB + 40 mM MgCl₂ and incubated overnight at 30 °C. Percent survival was calculated by dividing the number of colony forming units (CFUs) for samples treated with polymyxin by the corresponding CFUs of the untreated samples and multiplying by 100.

Frequency of spontaneous polymyxin B resistance

Strains were grown in LB overnight at 37 °C. A volume of 50 µL was spread on LB containing 10 µg/mL PMB and incubated at 37 °C overnight. The initial number of CFUs plated was calculated by plating serial dilutions of the overnight culture in triplicate on LB agar. The frequency was calculated as the number of spontaneous polymyxin-resistant colonies divided by the initial number of CFUs plated. To determine whether resistant colonies of *K. pneumoniae* had mutations in *mgrB*, the *mgrB* locus was amplified using primers mgrB-extF and mgrB-extR. The DNA fragment that did not appear to have an insertion based on gel electrophoresis was sequenced using the primer mgrB-extR.

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Figure Captions

Figure 1. Schematic of polymyxin resistance network in different genera of

Enterobacteriaceae. **A.** In *S. enterica*, PmrD functions as a connector between PhoQ/PhoP and PmrB/PmrA, and PmrA also negatively regulates transcription of PmrD (24, 59). **B.** Previous studies did not detect PmrD connector activity in *E. coli* (41). Since in this study we report that PmrD can function as a connector in *E. coli* under certain conditions, we indicate the connector activity with a dotted line. **C.** In *K. pneumoniae*, PmrD can function as a connector, and PhoP can also directly regulate transcription of the *arnB* operon (27).

Figure 2. Frequency of spontaneous polymyxin resistance. Cells were grown in LB and plated on LB with 10 µg/mL polymyxin B. Dilutions of the culture were also plated on LB. The frequency of spontaneous polymyxin resistance was determined by dividing the number of resistant CFUs (colony forming units) on the LB + polymyxin plate by the number of CFUs on the LB plate. Each dot represents a biological replicate. The dotted line denotes the limit of detection. Strains used: MGH 78578, ACK25, ACK24, ACK26, 14028s, MG1655.

Figure 3. Relative roles of PmrD, PmrA, and PhoP in polymyxin tolerance of a *K.*

pneumoniae Δ *mgrB* strain. Strains were grown in N-minimal medium pH 7.5 with 1 mM MgCl₂ to exponential phase and diluted into PBS + 1 mM MgCl₂ with or without 1 µg/mL polymyxin B and incubated for 1 hour before plating. Percent survival was calculated based on CFUs after treatment with polymyxin relative to those of untreated samples. In some cases, the calculated survival may have been over 100% due to pipetting error or the completion of a round of replication during the killing assay. Horizontal lines represent the medians of the pooled data

from two independent experiments, and each dot represents a biological replicate. Strains used: ACK9, ACK21, ACK20, and ACK28.

Figure 4. The effect of iron on *K. pneumoniae* polymyxin B tolerance. Polymyxin

susceptibility assays were performed as in Figure 3 except that the indicated concentrations of FeSO₄ were added to the growth medium. Horizontal lines represent the medians of the pooled data from two independent experiments, and each dot represents a biological replicate. Strains used: MGH 78578, ACK25, ACK24, ACK26, and ACK51.

Figure 5. The effect of mild acidity on polymyxin tolerance in *K. pneumoniae*. Cells were

grown in N-minimal medium pH 5.8 with 1 mM MgCl₂ to exponential phase and then polymyxin B assays were performed as described in Figure 3. Horizontal lines represent the medians of the pooled data from two independent experiments, and each dot represents a biological replicate. Strains used: MGH 78578, ACK25, ACK24, ACK26, and ACK51.

Figure 6. Effect of iron on polymyxin resistance and *arnB* transcription in *E. coli*. A. *E. coli*

wild-type and $\Delta pmrD$ strains were grown in N-minimal medium pH 7.5 with the indicated concentrations of MgCl₂ and FeSO₄, and polymyxin B assays were then performed as in Figure

3. B. Fluorescence of a P_{arnB}-gfp reporter for cells grown as described in Fig. 6A. Horizontal lines represent the medians of the pooled data from two independent experiments, and each dot represents a biological replicate. Strains used: A) MG1655 and AIC241; B) MG1655/pWKS130, MG1655/pP_{arnB}-gfp, and AIC241/pP_{arnB}-gfp.

Figure 7. Effect of mild acidity on *arnB* transcription in *E. coli*. *E. coli* were grown in N-minimal medium pH 7.5 or pH 5.8 with 1 mM MgCl₂ to exponential phase. PmrA activity was measured using a plasmid fluorescent reporter for the *arnB* promoter. Horizontal lines represent the medians of the pooled data from three independent experiments, and each dot represents a biological replicate. Strains used: MG1655/pWKS130, MG1655/pP_{*arnB*}-gfp, AIC241/pP_{*arnB*}-gfp, AIC204/pP_{*arnB*}-gfp, and TIM136/pP_{*arnB*}-gfp.

Figure 8. Effect of high PhoQ stimulation on *arnB* transcripton in *E. coli*. Cells were grown in N-minimal medium pH 7.5 with the indicated concentrations of MgCl₂. PmrA activity was measured using a plasmid fluorescent reporter for the *arnB* promoter. Horizontal lines represent the medians of the pooled data from three independent experiments, and each dot represents a biological replicate. Strains used: MG1655/pWKS130, MG1655/pP_{*arnB*}-gfp, MMR155/pP_{*arnB*}-gfp, and AIC242/pP_{*arnB*}-gfp.

633 Table 1. MIC of polymyxin B ($\mu\text{g/mL}$)

Strain	MIC of polymyxin B ($\mu\text{g/mL}$)
<i>E. coli</i> MG1655	1
<i>E. coli</i> $\Delta mgrB$	1
<i>S. Typhimurium</i> 14028s	2
<i>S. Typhimurium</i> 14028s $\Delta mgrB$	4
<i>K. pneumoniae</i> MGH 78578 (Kp WT)	2
Kp $\Delta mgrB$	32-64
Kp $\Delta mgrB \Delta pmrD$	32-64
Kp $\Delta mgrB \Delta pmrA$	32-64
Kp $\Delta mgrB \Delta phoP$	2
Kp $\Delta mgrB \Delta arnB$	2
Kp $\Delta mgrB \Delta pmrC$	32-64
Kp $\Delta pmrD$	2
Kp $\Delta pmrA$	2
Kp $\Delta phoP$	2

Table 2. Strains used in this study

Strain	Relevant Genotype	Reference or Source
<i>E. coli</i>		
MG1655(Seq)	F ⁻ λ <i>ilvG rfb-50 rph-1 crl::IS1</i>	<i>E. coli</i> Genetic Stock Center (CGSC no. 7740) (60)
AFS135	MG1655 $\Delta pmrA::$ (FRT-Kan-FRT)	Gouliau lab stock
AIC204	MG1655 $\Delta pmrA::$ FRT	This study (removal of antibiotic resistance cassette from AFS135 with pCP20)
AIC234	MG1655 $\Delta pmrD::$ (FRT-Kan-FRT)	This study P1 _{vir} (JW2254) X MG1655
AIC235	MG1655 $\Delta mgrB::$ FRT $\Delta pmrD::$ (FRT-Kan-FRT)	This study P1 _{vir} (JW2254) X MMR155
AIC241	MG1655 $\Delta pmrD::$ FRT	This study (removal of antibiotic resistance cassette from AIC234 with pCP20)
AIC242	MG1655 $\Delta mgrB::$ FRT $\Delta pmrD::$ FRT	This study (removal of antibiotic resistance cassette from AIC235 with pCP20)
MMR155	MG1655 $\Delta mgrB::$ FRT	Gouliau lab stock
TIM136	MG1655 $\Delta phoP::$ FRT	Gouliau lab stock
JW1116	$\Delta phoP::$ (FRT-Kan-FRT)	(61)
JW2254	$\Delta pmrD::$ (FRT-Kan-FRT)	(61)
JW4074	$\Delta pmrA::$ (FRT-Kan-FRT)	(61)
<i>K. pneumoniae</i>		
MGH 78578	wild type	ATCC 700721
ACK7	MGH 78578 $\Delta mgrB::$ (FRT-Apramycin-FRT)	This study
ACK9	MGH 78578 $\Delta mgrB::$ FRT	This study (removal of antibiotic resistance cassette from ACK7 with pFLP-Hyg)
ACK13	MGH 78578 $\Delta mgrB::$ FRT $\Delta pmrA::$ (FRT-Apramycin-FRT)	This study

ACK14	MGH 78578 $\Delta mgrB::FRT$ $\Delta pmrD::(FRT-AprA-FRT)$	This study
ACK17	MGH 78578 $\Delta pmrA::(FRT-AprA-FRT)$	This study
ACK18	MGH 78578 $\Delta pmrD::(FRT-AprA-FRT)$	This study
ACK19	MGH 78578 $\Delta phoP::(FRT-AprA-FRT)$	This study
ACK20	MGH 78578 $\Delta mgrB::FRT$ $\Delta pmrA::FRT$	This study (removal of antibiotic resistance cassette from ACK13 with pFLP-Hyg)
ACK21	MGH 78578 $\Delta mgrB::FRT$ $\Delta pmrD::FRT$	This study (removal of antibiotic resistance cassette from ACK14 with pFLP-Hyg)
ACK24	MGH 78578 $\Delta pmrA::FRT$	This study (removal of antibiotic resistance cassette from ACK17 with pFLP-Hyg)
ACK25	MGH 78578 $\Delta pmrD::FRT$	This study (removal of antibiotic resistance cassette from ACK18 with pFLP-Hyg)
ACK26	MGH 78578 $\Delta phoP::FRT$	This study (removal of antibiotic resistance cassette from ACK19 with pFLP-Hyg)
ACK27	MGH 78578 $\Delta mgrB::FRT$ $\Delta phoP::(FRT-AprA-FRT)$	This study
ACK28	MGH 78578 $\Delta mgrB::FRT$ $\Delta phoP::FRT$	This study (removal of antibiotic resistance cassette from ACK27 with pFLP-Hyg)
ACK51	MGH 78578 $\Delta phoP::FRT$ $\Delta pmrA::(FRT-AprA-FRT)$	This study
<i>Salmonella enterica</i> serovar Typhimurium		
DMS1445	wild type (14028s)	From D. Schifferli (62)
ACS8	DMS1445 $\Delta mgrB::(FRT-cat-FRT)$	This study

Table 3. Plasmids used in this study

Plasmid	Genotype	Reference or Source
pAC25	Contains FRT-Apra-FRT, oriR6K	This study
pACBSR-Hyg	Contains arabinose-inducible lambda red recombinase genes, Hyg ^R , p15A origin	(57)
pCP20	λ cI857(ts) <i>repA101</i> (ts) <i>oriR101</i> λ pR-FLP <i>bla cat</i>	(54)
pFLP-Hyg	p15A replicon, contains FLP recombinase, Hyg ^R	(57)
pKD3	Contains FRT-cat-FRT, oriR6K	(55)
pKD13	Contains FRT-Kan-FRT, oriR6K	(55)
pKD46	Contains arabinose-inducible lambda red recombinase genes, Amp ^R , pSC101 origin	(55)
pMDIAI	Contains FRT-Apra-FRT, ColE1 origin	(63)
pP _{arnB} -gfp	Reporter for <i>E. coli arnB</i> promoter	(64)
pWKS130	Control plasmid without <i>gfp</i> , Kan ^R	(65)

641 Table 4. Primers used in this study

Primer	Sequence	Reference or Source
Kp-arnB-check-F	5' GTGGTCGAGATACCCTGAAATGG 3'	This study
Kp-arnB-check-R	5' CTGCTGCCATCGTCGATCAACAG 3'	This study
Kp-arnB-LRed-F	5' ATATTTTATTCTAACGACCCAGATTTCGTGCGT GGGTCATCTACAGAACAGGATGATGATGATT CCGGGGATCCGTCGACC 3'	This study
Kp-arnB-LRed-R	5' TCGTTATAAACC GGGGATAACCACCGAGACC TTCTTCACGGGAGGGTAAGTAAGCATATCAT GTAGGCTGGAGCTGCTTC 3'	This study
Kp-mgrB-check-F	5' GATGCCTCATTATTTGTATGATCCCTG 3'	This study
Kp-mgrB-check-R	5' CAACAGACCGACAAGCAGCAGCGC 3'	This study
Kp-mgrB-LRed-F	5' GCGGTTTAAGAAGGCCGTGCTATCCTGGCGA CATTGCGTACTGATGCGGAGAGTGGAGTGAT TCCGGGGATCCGTCGACC 3'	This study
Kp-mgrB-LRed-R	5' AAGGCGTTCATTCTACCACCCGCGGCGCAGA AGGAAATCAGTCGGCAGAAAAATGTCTTATG TAGGCTGGAGCTGCTTC 3'	This study
Kp-phoP-check-F	5' GCATCCTGCCGCTGGGCGTTTACT 3'	This study
Kp-phoP-check-R	5' GTCAATAGCGCCGTTTTCCCATCTCG 3'	This study
Kp-phoP-Lred-F	5' TATTTTGCGTATATAATGAGCTTCAGACTACT ATCGAAAATGCTAGGGAGAACAGCCATGATT CCGGGGATCCGTCGACC 3'	This study
Kp-phoP-Lred-R	5' GCGAACCCGTAATGACAGCGGGAAGATAT GCCGCAACAGTCCCTTCATCAGCGCAATTCG TG TAGGCTGGAGCTGCTTC 3'	This study
Kp-pmrA-check-F	5' GAAACATGTGCCGATGGCGCTGTG 3'	This study
Kp-pmrA-check-R	5' CTGGATCTGCTCTTTGCTCTCATGCC 3'	This study

Kp-pmrA-LRed-F	5' CACCCGCGAGTACCAGGCGGCAGATGATATC TTAACGCCATGTAGAGAGGCTGGTTGATGAT TCCGGGGATCCGTCGACC 3'	This study
Kp-pmrA-LRed-R	5' GGTCAGCAGCAGCCGGTGGCGCATGGTCCAG GTTTCAGTTGCAAACAGAGCCATGGTCTATG TAGGCTGGAGCTGCTTC 3'	This study
Kp-pmrC-check-F	5' CGGTTGGTGATCCAGACGCCGCTGGA 3'	This study
Kp-pmrC-check-R	5' CACGATCAGGCTGTAATGATTGCTGG 3'	This study
Kp-pmrC-LRed-F2	5' CCTTAAAATCTTAAGGTTGTCTTAAAATAGG GCTGGCACTATAACCCTCATCTTTTCTGTGGG ATTCCGGGGATCCGTCGACC 3'	This study
Kp-pmrC-LRed-R	5' TCGCCAGAATTAACCCCTGCAACAGCAGCGC ATCGTCTTCAATGACTAAGATTTTCATCATGT AGGCTGGAGCTGCTTC 3'	This study
Kp-pmrD-check-F	5' CAAATTAAACCCGGCAGATCATTGCC 3'	This study
Kp-pmrD-check-R	5' GTCAGACTGGCGGCCTTGATATTC 3'	This study
Kp-pmrD-LRed-F	5' GAATTTATCTGTCAAATGCGACGATACTCA GACCATAACCAGTAAAAGGAGCGGGCTCGTG TTATGTAGGCTGGAGCTGCTTC 3'	This study
Kp-pmrD-LRed-R	5' GGTTGTCTTAAAATAGGGCTGGCACTATAACC CTCATCTTTTTCTGTGGGTGTGCATTATGATT CCGGGGATCCGTCGACC 3'	This study
mgrB_14028_LRed_u1	5' GTAATCATGATAATTTAGCGACATAAGATG TAAGATCGGAGAGTGGAGTGTAGGCTGGAGC TGCTTC 3'	This study
mgrB_14028_LRed_l1	5' GACGTCATTCTACCACTGCTACACGGGAAGG AAATCTCTGGTGTAAAACGTCATATGAATAT CCTCCTTAG 3'	This study

mgrB_14028_LRe dcheck_u1	5' GAGTAGCGTGTACATTGATTGAAACAAGTGA ATC 3'	This study
mgrB_14028_LRe dcheck_l1	5' CCACCAGAACCACCATTGATACTATTAAAGA TGC 3'	This study
mgrB-extF	5' AAGGCGTTCATTCTACCACC 3'	(66)
mgrB-extR	5' TTAAGAAGGCCGTGCTATCC 3'	(66)
oriR6Kseqprim1	5' GACACAGGAACACTTAACGGC 3'	Goulian lab stock

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Figure 1. Schematic of polymyxin resistance network in different genera of *Enterobacteriaceae*. **A.** In *S. enterica*, PmrD functions as a connector between PhoQ/PhoP and PmrB/PmrA, and PmrA also negatively regulates transcription of PmrD (24, 59). **B.** Previous studies did not detect PmrD connector activity in *E. coli* (41). Since in this study we report that PmrD can function as a connector in *E. coli* under certain conditions, we indicate the connector activity with a dotted line. **C.** In *K. pneumoniae*, PmrD can function as a connector, and PhoP can also directly regulate transcription of the *armB* operon (27).

Figure 2. Frequency of spontaneous polymyxin resistance. Cells were grown in LB and plated on LB with 10 μ g/mL polymyxin B. Dilutions of the culture were also plated on LB. The frequency of spontaneous polymyxin resistance was determined by dividing the number of resistant CFUs (colony forming units) on the LB + polymyxin plate by the number of CFUs on the LB plate. Each dot represents a biological replicate. The dotted line denotes the limit of detection. Strains used: MGH 78578, ACK25, ACK24, ACK26, 14028s, MG1655.

Figure 4. The effect of iron on *K. pneumoniae* polymyxin B tolerance. Polymyxin susceptibility assays were performed as in Figure 3 except that the indicated concentrations of FeSO₄ were added to the growth medium. Horizontal lines represent the medians of the pooled data from two independent experiments, and each dot represents a biological replicate. Strains used: MGH 78578, ACK25, ACK24, ACK26, and ACK51.

Figure 5. The effect of mild acidity on polymyxin tolerance in *K. pneumoniae*. Cells were grown in N-minimal medium pH 5.8 with 1 mM $MgCl_2$ to exponential phase and then polymyxin B assays were performed as described in Figure 3. Horizontal lines represent the medians of the pooled data from two independent experiments, and each dot represents a biological replicate. Strains used: MGH 78578, ACK25, ACK24, ACK26, and ACK51.

Figure 6. Effect of iron on polymyxin resistance and *armB* transcription in *E. coli*. **A.** *E. coli* wild-type and $\Delta pmrD$ strains were grown in N-minimal medium pH 7.5 with the indicated concentrations of MgCl₂ and FeSO₄, and polymyxin B assays were then performed as in Figure 3. **B.** Fluorescence of a P_{armB}-gfp reporter for cells grown as described in Fig. 6A. Horizontal lines represent the medians of the pooled data from two independent experiments, and each dot represents a biological replicate. Strains used: A) MG1655 and AIC241; B) MG1655/pWKS130, MG1655/pP_{armB}-gfp, and AIC241/pP_{armB}-gfp.

Figure 7. Effect of mild acidity on *arnB* transcription in *E. coli*. *E. coli* were grown in N-minimal medium pH 7.5 or pH 5.8 with 1 mM MgCl₂ to exponential phase. PmrA activity was measured using a plasmid fluorescent reporter for the *arnB* promoter. Horizontal lines represent the medians of the pooled data from three independent experiments, and each dot represents a biological replicate. Strains used: MG1655/pWKS130, MG1655/pP_{arnB}-gfp, AIC241/pP_{arnB}-gfp, AIC204/pP_{arnB}-gfp, and TIM136/pP_{arnB}-gfp.

Figure 8. Effect of high PhoQ stimulation on *arnB* transcripton in *E. coli*. Cells were grown in N-minimal medium pH 7.5 with the indicated concentrations of MgCl₂. PmrA activity was measured using a plasmid fluorescent reporter for the *arnB* promoter. Horizontal lines represent the medians of the pooled data from three independent experiments, and each dot represents a biological replicate. Strains used: MG1655/pWKS130, MG1655/pP_{*arnB*}-gfp, MMR155/pP_{*arnB*}-gfp, and AIC242/pP_{*arnB*}-gfp.