The contribution of PmrAB to the virulence of a clinical isolate of *Escherichia coli*

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Previous data from our laboratory suggest a relationship between increased *pmrAB* expression and virulence in an *Escherichia coli* mouse infection model of pyelonephritis. Competitive infections with wild type and *pmrAB* mutants showed that disruption of *pmrAB* caused decreased persistence of *E. coli* within the mouse kidney. These results were confirmed with plasmid-mediated complementation of the *pmrAB* mutant. Additionally, increased expression of *pmrAB* from this complementing plasmid in a previously attenuated *marA-rob-soxS* triple mutant displayed increased bacterial persistence in the infection when compared with the triple mutant alone. These findings suggest a role for this two-component regulatory system in the virulence of *E. coli* in a murine pyelonephritis model.

**Introduction**

Recent findings linking transcriptional regulators of antibiotic resistance to bacterial virulence demonstrate a growing repertoire of regulatory targets which aid in the ability of such factors to be evolutionarily maintained.1,4 The three related transcription factors, MarA, Rob, and SoxS, act in both individual and redundant manners to affect antibiotic resistance and virulence of *Escherichia coli*, through known and unknown mechanisms.5,6 These three transcription factors play a key role in the persistence of *E. coli* in a murine kidney infection model, where deletion of all three genes significantly decreases the bacterial load in the kidney after three days of successful colonization.7 Previous microarray data show that expression levels of genes associated with the *pmrAB* regulon6,7 were decreased in a triple mutant lacking the transcription factors MarA, Rob, and SoxS compared with its parent strain, when extracted from a murine pyelonephritis infection model.6

The *pmrAB* operon encodes a two-component regulatory system present in many bacterial species,8,9 which mediates a wide range of functions, most notably modification of the lipid A moiety of lipopolysaccharide (LPS).10,11 Such modification of LPS alters the host immune response to the bacterial pathogen, and indeed, studies in *Salmonella enterica* serovar Typhimurium12 and *Francisella novicida*13 show that *pmrAB* contributes to virulence in animal models of infection.

*E. coli* is one of the leading causes of urinary tract infections,14 with a significant financial burden to the health care system.15 As such, utilization of mouse models of urinary tract infection is paramount in understanding the genetic basis of this aspect of *E. coli* pathogenicity. Here we used such a model to determine the role of *pmrAB* in *E. coli* virulence, and the contribution of *pmrAB* to our previously described attenuation of persistence seen in a *marA-rob-soxS* triple mutant strain.

**Results**

While the *pmrAB* operon plays a role in virulence for other pathogens,12,16,17 its relationship with virulence in *E. coli* has not been established, nor have there been studies looking at the contribution of this operon to infections of the urinary tract. To address these previous unknowns, and to substantiate the transcriptome data which led to this study, we performed competitive infection studies with strain KM-D and mutant KM-D*pmrAB*. We found no difference in initial colonization at day 1 post-infection (data not shown) but an average competitive index (CI) value of 0.2 at day 4 post-infection (Fig. 1A). In accord with these findings, genetic complementation of the *pmrAB* mutant with pPmrAB showed a significant return to near 1.0 CI values on day 4 post-infection (Fig. 1B). These findings implicate *pmrAB* in the persistence of *E. coli* in a murine pyelonephritis model.

We hypothesized that expression of *pmrAB* may play a role in the decreased persistence of strain PC1012, a *marA-rob-soxS* triple mutant, in the mouse kidney.2 Therefore we performed single strain infections which compared the bacterial loads of groups of mice infected with strain PC1012 alone, or transformed with plasmid pPmrAB. Upon expression of *pmrAB* on this multicopy plasmid in strain PC1012 we observed a significant increase in the bacterial loads of this strain on day 4 post-infection when compared with its parent (Fig. 2). While these increases in colonization do not bring bacterial loads to those of strain KM-D (~10⁴ cfu/g kidney; data not shown), they do show that *pmrAB* is a contributor to the previously observed persistence attenuation seen in a strain lacking all three transcription regulators.9
Discussion

The bacterial pathogen must overcome a variety of challenges in order to colonize and persist within the urogenital tract and kidney. In *E. coli*, *pmrAB* affects the resistance of the cell to antimicrobial peptides, toxic levels of iron, and deoxycholate. It is well established that antimicrobial peptides play a large role in the immune response to bacterial colonization of an otherwise sterile body site. The established role of increased *pmrAB* expression to bacterial resistance to antimicrobial peptides likely explains these findings. Alternatively, a recent study shows in *E. coli* that *pmrAB* (referred to as *bamSR* in that study) controls a much wider range of genes than previously published, including genes responsible for stress response regulation and metabolism. In light of these findings, the decreased stress response may pair with antimicrobial peptide resistance to synergistically cause the observed attenuation in an environment where both challenges are present.

Overexpression of *pmrAB* in the *marA-rob-soxS* triple mutant confirms the importance of the *pmrAB* operon in the murine kidney, by partially rescuing a non-persistent strain (median values of 193 vs. 10³ cfu/g kidney for strains PC1012 + *pPmrAB* and KM-D respectfully). Further, the PC1012 experiments confirm the relationships of *MarA*, *Rob*, and *SoxS* to antimicrobial peptide resistance and/or suggest a linkage of these factors to regulation of the stress response. The characterization of antibiotic resistance factors, such as *marA-rob-soxS*, as contributors to the regulation of non-antibiotic resistance-related phenotypes, reveals new evolutionary pressures to maintain these resistance regulators in the presence or absence of antibiotic pressure.

Materials and Methods

Bacterial strains and growth conditions. All strains were cultured in LB-broth or LB-agar at 37 °C. Strain KM-D is a clinical *E. coli* isolate described previously. Antibiotics were added to media when indicated at the following concentrations: 100 μg ampicillin (Ap) ml⁻¹, 50 μg kanamycin (Km) ml⁻¹ and 40 μg chloramphenicol (Cm) ml⁻¹.

Genetic constructs. Isogenic insertion mutants were generated using the suicide vector pSR47s as described utilizing *E. coli* genome sequence (NC_000913). The *pmrAB* operon and 500 bp of upstream and downstream flanking DNA were amplified by PCR with primers (pmrABfor [CGACGGATCC GACCTATTAC AA] and pmrABrev [TGTAGGATCC GACCTATTAC AA]) and ligated with linearized plasmid pGEM-Easy (Invitrogen). This construct was transformed into DH5α and *Ap*² transformants were confirmed for the presence of the *pPmrAB* plasmid by PCR sequencing. Plasmid *pPmrAB* was digested with restriction enzyme BsIl and ligated to a blunt-ended cat cassette PCR amplified from plasmid pKD3 described previously, creating plasmid *pPmrAB-Cm*. This plasmid was selected for its *Ap*/Cm⁷ phenotype and confirmed with PCR. The disrupted operon was isolated from the pGEM vector by digestion with NotI and ligated into the NotI site of the suicide vector pSR47s, and successful ligations were selected for by transformation into strain S17Apr. Transformants were subsequently selected on LB-agar plates containing Cm and Km.

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** The role of *pmrAB* in pyelonephritis. Competitive mouse infection data are expressed as competitive indices as described in Methods. Each data point represents the kidney homogenate from a single mouse cultured on day 4 post-infection from one of 3 separate experiments. The horizontal bars represent the geometric means of the data. Mice were infected with a mixture of strains KM-D and KM-D *pmrAB* (A) or a mixture of strains KM-D and KM- KPmrAB + *pPmrAB* (B). The distribution of values was compared using a Mann–Whitney Test, yielding a *P* value of ≤ 0.001.

![Figure 2](https://via.placeholder.com/150)

**Figure 2.** The role of *pmrAB* in the persistence of strain PC1012. Mice were infected with single strain inoculum of strain PC1012 or strain PC1012 + *pPmrAB*. Each point represents a mouse from one of 3 separate experiments, and the median bacterial loads at day 4 post-infection (cfu/gram kidney) are expressed as horizontal bars. A Student t test was used to determine the significance of the differences in data. *P* = 0.006.

After large-scale screening, 635 PC1012 + *pPmrAB* transformants were screened by PCR for the presence of the *pmrAB* operon, and 635 transformants were subsequently selected on LB-agar plates containing Cm and Km. Cm⁷/Km⁷ transformants were screened by PCR, before being used as donors in conjugations with strain KM-D as described previously. KM- *DpmrAB* candidates were screened by PCR for the presence of the disrupted operon, and were confirmed phenotypically by characterization of growth in iron or deoxycholate. Plasmid *pPmrAB* was transformed into strains KM-D *DpmrAB* and PC1012 via chemical transformation, and successful transformants were cultured on media supplemented with ampicillin.

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Complemented strains were also confirmed phenotypically by growth in iron or deoxycholate. All strains and constructs are summarized in Table 1.

Mouse infection. Six- to eight-week-old female CD-1 mice (Charles River) were treated with 5% (w/v) glucose and limited food for 5 d pre-infection to help visualize the bladder during inoculation. Bacterial strains, grown 18 h at 37 °C in LB-broth the night before infection, were centrifuged and washed three times with phosphate-buffered saline (PBS), and resuspended at a final concentration of ~109 cfu/ml. Twenty-five microliters of the inoculum (~5 × 107 cfu) were injected into the bladder of ketamine-anesthetized mice as described previously.5 Mice at a final concentration of ~109 cfu/ml. Twenty-five microliters was centrifuged and washed three times with phosphate-buffered saline (PBS), and resuspended with either strain PC1012 or PC1012 bearing plasmid pPmrAB.

Competitive index (CI) studies were performed and analyzed as described previously.6 Briefly, a total of 5 × 107 cfu consisting of a 1:1 mixture of independently grown strains were used to inoculate 7–12 mice. The competitive indices were determined by culturing kidney homogenates on LB-agar with and without Cm. No potential conflicts of interest were disclosed.

Table 1. Strains and plasmids used in this study

| Strain   | Description                  | References |
|----------|------------------------------|------------|
| KM-D    | Clinical E. coli isolate     | 21         |
| PC1012  | Km-D Δsox5, rob, marA        | 5          |
| DH5x    | fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Δ(800 lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | Invitrogen |
| S17 Δpir| lamB F′ supE44 thi-1 thr-1 leuB6 lacY1 tonA21 hsdR hsdM recA pro (RP4; 2-Tc: Mu: Km: Tn7) Δpir | 22         |
| KM-DpmrAB| This study                    |            |

Plasmid | Description | References |
|---------|-------------|------------|
| pGEM    | (Amp® cloning vector) | Promega |
| pSK73  | Km® R6KoriV RP4oriT sacB | 23 |
| pPmrAB | (Amp®) | This study |
| pPmrAB::Cm | (Amp® Cm®) | This study |
| pSKpmpAB::Cm | (Cm® Km®) | This study |
| pKD3   | Origen of cat cassette (Cm®) | 24 |

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