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Isolation and Characterization of a Gene, *pmrD*, from *Salmonella typhimurium* That Confers Resistance to Polymyxin when Expressed in Multiple Copies

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We have isolated from *Salmonella typhimurium* a gene, designated *pmrD*, that confers resistance to the membrane-damaging drug, polymyxin B when expressed from the medium-copy-number plasmid pHSG576. The gene maps to 46 min on the standard genetic map, near the *menB* gene, and is therefore distinct from the previously described *pmrA* locus. We have mapped the polymyxin resistance activity to a 1.3-kb *Cla*I-*Pvu*II fragment which contains a small open reading frame that could encode an 85-amino-acid peptide. When an Ω-Tet insertion was made into the putative *pmrD* open reading frame (*pmrD2*::Ω-Tet), the resulting plasmid no longer conferred polymyxin resistance, whereas an Ω-Tet insertion into vector sequences had no effect. Maxicell analysis confirmed that a protein of the expected size is made in vivo. The PmrD protein shows no significant homology to any known protein, but it does show limited homology across the active site of the p15 acid protease from Rous sarcoma virus, indicating that the protein may have proteolytic activity. However, changing the aspartic acid residue at the putative active site to alanine reduced but did not eliminate polymyxin resistance. When *pmrD2*::Ω-Tet replaced the chromosomal copy of *pmrD*, the resulting strain showed wild-type sensitivity to polymyxin and could be complemented to resistance by a plasmid that carried *pmrD*. The *pmrA505* allele confers resistance to polymyxin when present in single copy on the chromosome or when present on a plasmid in *pmrA*$^+$ *pmrD*$^+$ cells. In combination with the *pmrD2*::Ω-Tet mutation, the effect of the *pmrA505* allele on polymyxin resistance was reduced, whether *pmrA505* was present on the chromosome or on a plasmid. Conversely, a strain carrying an insertion in *pmrA* could be complemented to polymyxin resistance by a plasmid carrying the *pmrA505* allele but not by a plasmid carrying *pmrD*. On the basis of these results, we suggest that polymyxin resistance is mediated by an interaction between PmrA or a PmrA-regulated gene product and PmrD.

Nearly 15 years ago Mäkelä et al., in an effort to use polymyxin as a probe for examining the outer membrane properties of gram-negative bacteria, isolated polymyxin-resistant (PM^r) mutants of *Salmonella typhimurium* (18). The mutations in all of the single-step mutants mapped to a single locus, *pmrA* (18). Strains with *pmrA* mutations have unique permeability properties, in that they are more resistant to a variety of permeabilizing treatments (37). Study of these mutants has proved to be quite useful to our understanding of the complex nature of the outer membrane and has provided support for the hypothesis that the initial binding of polymyxin to the outer membrane involves an interaction with the 4′-phosphate of lipid A (37). Despite this, it is still not clear how polymyxin traverses the outer membrane and mediates cell death. Although it is well established that polymyxin does not bind as well to the outer membrane of *pmrA* mutant strains, it is not known whether *pmrA* mutations influence other aspects of the interaction between polymyxin and bacterial cells. Remarkably, until now, no other *S. typhimurium* (or *Escherichia coli*) genetic locus has been identified by using polymyxin resistance as a criterion.

The *pmrA* mutants are also cross-resistant to several cationic antibacterial proteins found in human neutrophils, including CAP37 and CAP57 (together referred to as CAP) (28). Polymyxin and CAP both contain cationic and hydrophobic domains. In addition, the binding of CAP to *S. typhimurium* cells is inhibited by polymyxin (9), indicating that they share a common binding site on the bacterial cell surface. A number of *pmrA* mutants have been found to have a four- to sixfold increase in the amount of 4-aminoarabinose on the lipid A (38), while *Proteus* species, which are highly resistant to polymyxin (34) and CAP (29), have essentially all of the 4′-lipid A phosphates substituted with 4-aminoarabinose (32). Therefore, the binding site for these agents is thought to include the 4′-lipid A phosphates.

The DNA sequence of the *pmrA* locus has been determined and found to encode two proteins, PmrA and PmrB, that share strong similarity with the OmpR/EnvZ class of two-component regulators (23). It was proposed that PmrAB regulates the expression of the genes responsible for the observed lipid A modification and that mutations in *pmrA* lead to polymyxin resistance as a result of increased expression of the target genes (23).

Although it is likely that CAP and polymyxin share a common binding site on lipid A, there is evidence indicating that the final target of these agents is not the outer membrane. A derivative of polymyxin B which lacks the hydrophobic domain and shows no bactericidal activity retains the capacity to disrupt the outer membrane (discussed in reference 37). Veld et al. (39) showed that both polymyxin B and bactericidal/permeability-increasing protein (which is the same as CAP57) inhibit energy-dependent solute uptake in isolated cytoplasmic membrane vesicles, suggesting that the cytoplasmic membrane may be the ultimate target for these agents. In addition,

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TABLE 1. *S. typhimurium* strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
LT2	*pmrA*$^+$ *pmrD*$^+$	S. Maloy
SH7518	*rfaE*	M. Vaara
JKS5	*pmrA505*	23
JKS200	JKS5 carrying *zjd*::Tn*10*	23
JKS1170	LT2 carrying *pmrA505 zjd*::Tn*10d*-Cam	23
JKS1379	LT2 carrying *pmrA508*::Ω-Kan	This study
JKS1417	LT2 carrying *zjd*::Tn*10*	This study
JKS1420	MS1868 carrying *pmrD2*::Ω-Tet/pHSG576	This study
JKS1426	LT2 carrying *pmrD2*::Ω-Tet	This study
JKS1433	ATCC 14028 carrying *pmrD2*::Ω-Tet	This study
JKS1443	JKS1426 carrying *pmrA505 zjd*::Tn*10d*-Cam	This study
TT15258	*cysA1586*::MudQ	3 (via K. Sanderson)
MS1868	*leu-414 hsdL fels2*	T. Elliott
ATCC 14028	*pmrA*$^+$ *pmrD*$^+$	ATCC[a]
Plasmids		
pHSG576	pSC101 derivative, Cam^r	35
pGB2	pSC101 derivative, Spc^r	G. Churchward
pMLB1034	*lacZ* fusion vector, Amp^r	33
pKR100	pHSG576, *pmrA505*	23
pKR300	pHSG576 + 10,000-bp *Sau*3AI fragment from strain JKS5, *pmrD*$^+$	This study
pKR301	pHSG576 + 1,329-bp *Cla*I-*Pvu*II fragment from pKR300, *pmrD*$^+$	This study
pKR302	pUC18 + 1,329-bp *Cla*I-*Pvu*II fragment from pKR300, *pmrD*$^+$	This study
pKR303	pKR300, *pmrD2*::Ω-Tet	This study
pKR304	pKR300, Ω-Tet, *pmrD*$^+$	This study
pKR307	pBHM71 + 886-bp *Pvu*II-*Eco*RI fragment from pKR300, *pmrD*::*lacZ*	This study
pKR308	pKR301, D23A mutation, *pmrD1*	This study
pKR310	pGB2, *pmrD2*::Ω-Tet	This study

[a] ATCC, American Type Culture Collection.

although both agents interfered with cytoplasmic membrane functions, they did so differentially, indicating that their ultimate targets may be different. In light of these observations, it is possible that altering the expression of gene products that interact with the cytoplasmic membrane also results in PM^r or resistance to CAP (CAP^r). Therefore, although *pmrA* mutants are PM^r CAP^r, it may be possible that altered expression of other gene products will result a PM^r CAP^s or PM^s CAP^r phenotype.

In this work we describe the isolation of a gene designated *pmrD*, which confers the PM^r CAP^s phenotype when expressed on a multicopy plasmid and we present evidence that suggests that PmrD interacts with PmrA or a PmrA-regulated gene product.

MATERIALS AND METHODS

Bacterial strains. The *S. typhimurium* LT2 derivatives used in this study are summarized in Table 1. Strain JKS1417 was constructed in a transductional cross by using strain JKS200 as the donor and strain LT2 as the recipient with selection for tetracycline resistance. Transductants were screened for polymyxin sensitivity, and a Tet^r PM^s isolate was chosen. *E. coli* K-12 strain MV1190, which was used for the maxicell experiments, was obtained from John Battista and has the genotype Δ(*lac-proAB*) *supE thi* Δ(*srl-recA*)*306*::Tn*10*/F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*. *E. coli* 71-18 (41) was the host for bacteriophage M13. P22 transductions were carried out as described previously (19). When appropriate, transductants were determined to be PM^r or PM^s by streaking on polymyxin gradient plates as previously described (23).

Chemicals. Polymyxin B (7,730 U/mg), polymyxin E (colistin methanesulfonate; 13,700 U/mg), and all other antibiotics were purchased from Sigma. Restriction enzymes were purchased from New England Biolabs. Bacterial growth medium was obtained from Difco. Trimethylamine-*N*-oxide (TMAO) was purchased from Sigma.

Medium preparation. Luria-Bertani (LB) broth and LB agar plates were routinely used for cultivation of all strains (2). E plates were prepared as previously described (19) and contained either 0.2% glucose or 1% glycerol as a carbon source. Tryptone broth (pH 5.5) was prepared as previously described (29). Chloramphenicol, tetracycline, and ampicillin were included at 20, 20, and 30 μg/ml, respectively, when required. Kanamycin and spectinomycin were included at 50 μg/ml each. LB-polymyxin E gradient plates were prepared as described previously (23).

Anaerobic growth. To test for anaerobic growth, bacteria were streaked on plates and incubated at 37°C in a GasPak anaerobic jar (BBL Microbiology Systems) under an atmosphere of 95% H_2–5% CO_2 for 48 h. TMAO was included by spreading 0.2 ml of a 5 M solution onto E + glycerol plates prior to inoculation.

DNA preparation and manipulation. Chromosomal DNA was prepared from strain LT2 as described previously (2). The *pmrD* allele from strain LT2 was amplified by PCR with primers PMRD6 and PMRD7, which have the sequence 5′-GAGACGTGAACCTCGCTGAATGG-3′ and 5′-GCTCG GCGTATTGTGTATTATGG-3′, respectively. PCR was performed by using a GeneAmp kit (Perkin-Elmer) as specified by the manufacturer. DNA sequencing of the PCR product was done by using the *fmol* DNA Sequencing System (Promega) and ^{32}P-labeled primers PMRD6 and PMRD7. [γ-^{32}P]dATP was purchased from Amersham. All other procedures for the preparation and manipulation of DNA are as previously described (23).

Bactericidal assays. We performed 1-h bactericidal assays as previously described (23). Crude granule extract from human neutrophils was prepared as described previously (28).

MIC assays were performed with samples in Corning microtiter trays. We used mid-exponential-phase cells grown in LB broth containing the appropriate antibiotic to maintain plasmid selection. Twofold serial dilutions of polymyxin B adjusted in 80 mM NaCl to provide twice the desired final concentration in 100 μl were added to the wells, and 100 μl of bacteria diluted to 10^4 CFU/ml served as the inoculum for each well. The microtiter trays were incubated at 37°C overnight. The MIC was determined to be the lowest concentration at which no visible growth occurred after 20 h.

Plasmid constructions. Plasmid pKR300 is a derivative of plasmid pHSG576 (35) which contains a 10,000-bp *Sau*3AI chromosomal fragment derived from strain JKS5. It was isolated on the basis of its ability to confer resistance to polymyxin E as previously described (23).

Plasmid pKR301 was constructed by purifying the 1,329-bp *Cla*I-*Pvu*II fragment from pKR300 and ligating it to plasmid pHSG576 digested with *Sma*I and *Acc*I. Plasmid pKR302 was constructed in a similar fashion, except that pUC18 was the vector. Plasmid pKR308 is a derivative of plasmid pKR301,

which contains the D23A mutation in *pmrD*. The mutation was introduced by using a uridylated template as described previously (16). Primer PMRD5, which has the DNA sequence 5′-GAACCGCCGC**TT**GCGCACAGCACC-3′ and hybridizes to the coding strand, served to convert codon 23, GAT (Asp), to GCA (Ala). Residues that differ from the wild-type sequence are shown in boldface type. The DNA sequence of the entire *pmrD* open reading frame (ORF), including potential upstream promoter regions, was determined to ensure that no additional mutations had been introduced.

For the construction of plasmids pKR303 and pKR304, pKR300 was subjected to partial *Eco*RI digestion. Singly cut molecules were purified, ligated with the *Eco*RI-cut Ω-Tet fragment derived from plasmid pHP45-Ω-Tet (10), and used to transform strain MS1868 with selection for Tet^r. The location of the Ω-Tet insertions was determined by restriction enzyme analysis. Plasmid isolates in which the Ω-Tet fragment disrupted the *pmrD* ORF (pKR303) or was present in vector sequences (pKR304) were moved into strain LT2 by electroporation (2).

Plasmid pBHM71 is a derivative of plasmid pMLB1034 (33) in which the *rrnBt*$_1$ transcriptional terminator has been inserted into the *Bal*I site. *Xma*I-digested pBHM71 and the purified 886-bp *Pvu*II-*Eco*RI fragment from plasmid pKR300 were combined in a fill-in reaction with T4 DNA polymerase (2). The filled in DNA was ligated and used to transform *E. coli* DH5α with selection on LB-ampicillin plates containing 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Transformants yielding blue colonies were picked. The presence and orientation of the insert DNA were confirmed by restriction enzyme analysis, and the predicted in-frame *pmrD*::*lacZ* fusion was verified by DNA sequencing with the universal primer. This plasmid was designated pKR307.

DNA sequence analysis. To obtain the sequence of the *pmrD* gene, we constructed the following four M13 clones. The 1,329-bp *Pvu*II-*Cla*I fragment from plasmid pKR300 was cloned into *Sma*I-*Acc*I-digested M13mp18 and M13mp19 (41) to obtain clones in both orientations. In addition, the 3,900-bp *Hin*dIII-*Eco*RI and 2,800-bp *Pst*I-*Eco*RI fragments from plasmid pKR300 were cloned into appropriately digested M13 mp19. Along with data obtained with the universal primer, we also obtained DNA sequence data with primers PMRD1 (5′-GCGCATCCGCTGGTGCAAC-3′), PMRD2 (5′-CGGC ATCGTCAACCAGCG-3′), PMRD3 (5′-GCCATCACGCCG TAACCTTC-3′), and PMRD4 (5′-GTGTATTATGGCGGG GGTAA-3′), which were purchased from Operon Technologies, Inc. Sequencing was performed with a Sequenase kit (United States Biochemical). [α-^{35}S]dATP was purchased from Amersham.

The DNA sequence comprising portions of the *menB* and *menC* genes was obtained by cloning the 3,150-bp *Pst*I-*Cla*I fragment from plasmid pKR300 into the *Eco*RI site of M13mp19. Both DNA fragments were made blunt ended by treatment with T4 DNA polymerase as described previously (2).

The DNA sequence was read manually and entered into the DNA Strider program for analysis. Location of ORFs, their subsequent translation, and production of hydropathy plots (17) were done with Strider. Data base searches were done by using the BLAST Network Service and the alignment algorithm of Altschul et al. (1).

Construction of chromosomal null mutations in *pmrD* and *pmrA*. To construct a chromosomal insertion mutation in *pmrD*, we subcloned the ca. 12,000-bp *Pst*I fragment from plasmid pKR303 into *Pst*I-cut plasmid pGB2 (6). The resulting plasmid was designated pKR310. Plasmids pKR310 and pHSG576 were used to cotransform strain MS1868, with selection for Cam^r (20 μg/ml) and Tet^r (5 μg/ml). Transformants were screened for sensitivity to spectinomycin. A Spc^s transformant (strain JKS1420) was chosen, and the Tet^r marker was transferred to strain LT2 by transduction. Several transductants were picked, and all were found to be Cam^s and Spc^s, indicating that plasmid sequences were not present. The presence of the Ω-Tet insertion in two of the transductants was confirmed by PCR analysis with primers PMRD6 and PMRD7 (see above). Whereas LT2-derived chromosomal DNA yielded a 565-bp PCR product, DNA derived from the two transductants yielded a 2,700-bp PCR product, as would be expected if the ca. 2.1-kb Ω-Tet element was inserted into the *pmrD* gene. One of these transductants was designated JKS1426.

We constructed a chromosomal null mutation in the *pmrA* gene in which the 670-bp *Stu*I-*Eco*RV fragment from plasmid pKR202 (22), which contains the final 47 codons from ORF1 and the first 177 codons from *pmrA*, was replaced with the 2.1-kb Ω-Kan fragment from plasmid pHPΩ-Kan (10) by similar methods. Complete details of this strain construction will be presented elsewhere. The presence and location of the Ω-Kan insertion were confirmed by Southern hybridization and transductional analysis (22).

β-Galactosidase assays. Cultures were grown in LB medium containing ampicillin. Overnight cultures were diluted 1:1,000 into the same medium, grown to an optical density at 600 nm of 0.5 to 0.6, and harvested. Cells were diluted 1:5 in Z buffer and assayed by the method of Miller (20). Control strains containing vector alone produced less than 1 U of activity. The data described in this work are the average of three independent experiments with less than a 10% variation between assays.

Maxicell analysis. Maxicells were prepared as described previously (24). ^{35}S-labeling of maxicells was performed by using the Express protein labeling mix purchased from New England Nuclear. Labeled proteins were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) gels along with unlabeled rainbow markers (Amersham). Labeled proteins were visualized by autoradiography.

Nucleotide sequence accession number. The DNA sequence presented here has been submitted to GenBank and assigned the accession number UO2281.

RESULTS

In previous work, we described the cloning of the *pmrA* locus of *S. typhimurium* LT2 (23). Using DNA obtained from strain JKS5 (Table 1), a spontaneous *pmrA* mutant, we screened a chromosomal library for plasmids which conferred polymyxin resistance to strain LT2 (23). As expected, we obtained a plasmid, designated pKR100, which carried the *pmrA* gene (23). However, in addition we obtained a plasmid, designated pKR300, which conferred resistance to polymyxin but, as described below, did not contain the *pmrA* locus. We have designated this new locus as *pmrD*.

Characterization of plasmid pKR300. Plasmid pKR300 is a derivative of pHSG576 (35) that contains ca. 10 kb of chromosomal DNA derived from strain JKS5 (Table 1). When this plasmid was introduced into strain LT2, it conferred a 1,000-fold increase in resistance to polymyxin B as measured by the 1-h bactericidal assay (Fig. 1A). Several *pmrA* mutants which are resistant to a similar range of polymyxin B concentrations have been shown to be cross-resistant to the CAPs found in crude granular extracts derived from human neutrophils (23, 28, 29). We tested our transformant for survival in a crude granular extract and found that the plasmid did not confer the

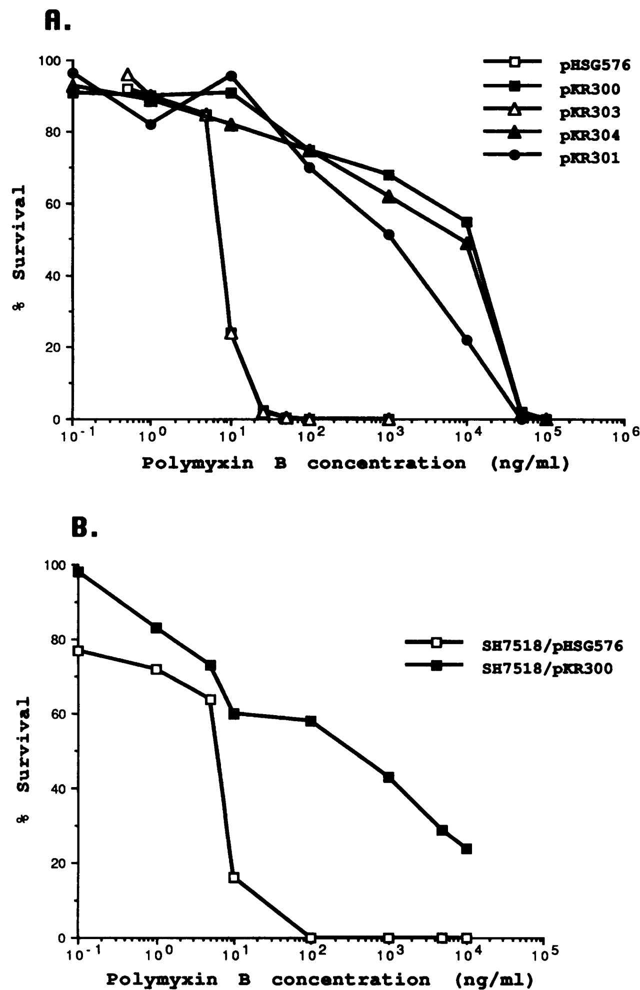

FIG. 1. Effect of multiple copies of *pmrD* on resistance to polymyxin B in *S. typhimurium*. (A) All strains are derivatives of LT2. The 50% lethal doses for strains carrying plasmids pHSG576, pKR300, pKR301, pKR303, and pKR304 were 8, 10,500, 1,200, 9, and 10,600 ng/ml, respectively. (B) The 50% lethal doses for the *rfaE* strain SH7518 carrying plasmids pHSG576 and pKR300 were 8 and 500 ng/ml, respectively.

CAP^r phenotype (data not shown). Therefore, plasmid pKR300 confers a PM^r CAP^s phenotype.

To determine whether full-length lipopolysaccharide was required for the observed phenotype, we examined polymyxin resistance in strain SH7518, which has Re chemotype lipopolysaccharide and therefore lacks the O antigen and the outer core. Strains carrying the Re chemotype lipopolysaccharide are more sensitive to hydrophobic antibiotics (37), and strain SH7518 has been reported to be slightly more sensitive to polymyxin B (36). Plasmid pKR300 conferred polymyxin resistance to this strain (Fig. 1B), although the 50% lethal dose was considerably reduced compared with that for strain LT2. In an MIC assay, strain SH7518 also appeared to be more sensitive to polymyxin B than did strain LT2 (Table 2), but plasmid pKR300 was still able to confer a 10-fold increase in resistance. A 10-fold increase in polymyxin resistance was also conferred by the *pmrA505* mutation, whether it is present on the chromosome (strain JKS1222) or on a multicopy plasmid (strain JKS1187) (Table 2). These data indicate that, like the *pmrA*

TABLE 2. MICs for polymyxin B

Strain	Parent strain/plasmid[a]	MIC (μg/ml)[b]
JKS1184	LT2/pHSG576	1
JKS1187	LT2/pKR100 (*pmrA505*)	10
JKS1276	LT2/pKR301 (*pmrD*$^+$)	10
JKS1391	LT2/pKR308 (D23A)	2.5
JKS1222	JKS5/pHSG576	10
JKS1221	JKS5/pKR300 (*pmrD*$^+$)	10
JKS1217	SH7518/pHSG576	0.6
JKS1215	SH7518/pKR300 (*pmrD*$^+$)	5
JKS1292	LT2/pUC18	1
JKS1298	LT2/pKR302 (*pmrD*$^+$)	10
JKS1305	JKS5/pUC18	10
JKS1404	JKS5/pKR302 (*pmrD*$^+$)	10
JKS1322	MV1190/pUC18	1
JKS1328	MV1190/pKR302 (*pmrD*$^+$)	2.5
JKS1386	JKS1379 (*pmrA508*::Ω-Kan)/pHSG576	1
JKS1387	JKS1379 (*pmrA508*::Ω-Kan)/pKR100 (*pmrA505*)	10
JKS1402	JKS1379 (*pmrA508*::Ω-Kan)/pKR301 (*pmrD*$^+$)	1
JKS1429	JKS1426 (*pmrD2*::Ω-Tet)/pHSG576	1
JKS1430	JKS1426 (*pmrD2*::Ω-Tet)/pKR100 (*pmrA505*)	2.5
JKS1431	JKS1426 (*pmrD2*::Ω-Tet)/pKR301 (*pmrD*$^+$)	10
JKS1434	LT2/pKR100 (*pmrA505*)	10
JKS1170	*pmrA505*	20
JKS1426	*pmrD2*::Ω-Tet	1
JKS1443	*pmrD2*::Ω-Tet, *pmrA505*	5

[a] Relevant genes carried by each plasmid and strain are indicated in parentheses.
[b] MIC assays were performed at least three times with identical results.

phenotype (23), full-length LPS is not a requirement for polymyxin resistance. However, because the MIC for SH7518/pKR300 was still lower than that for LT2/pKR300, the sensitizing effect of the Re chemotype lipopolysaccharide was not completely overcome by *pmrD*.

It was also of interest to determine whether plasmid pKR300 could increase polymyxin resistance in a *pmrA* mutant strain. When the plasmid was introduced into strain JKS5, no change in resistance was observed either in the bactericidal assay (results not shown) or in the MIC (Table 2), indicating that the effects of the two genes are not additive.

A restriction map of the insert DNA from pKR300 was prepared (Fig. 2). Subsequent subcloning experiments showed that deletion of the ca. 2,500-bp *Hin*dIII fragment had no effect on PM^r. Neither the remaining *Hin*dIII-*Eco*RI$_1$ fragment nor the *Eco*RI$_1$-*Eco*RI$_2$ fragment could confer PM^r. This result indicated that the *Eco*RI$_1$ site was located in sequences required for the PM^r phenotype.

To test this, we constructed an Ω-Tet insertion into the *Eco*RI$_1$ site of plasmid pKR300 (Fig. 2). As a control, we also constructed an Ω-Tet insertion in the EcoRI$_2$ site, which is located in vector-derived sequences and so should not affect PM^r. The resulting plasmids were designated pKR303 and pKR304, respectively (Table 1). When these plasmids were introduced into strain LT2, we found that plasmid pKR303 had lost the ability to confer PM^r (Fig. 1A), confirming that the EcoRI$_1$ site overlapped relevant sequences. The insertion in plasmid pKR304 had no effect on PM^r, indicating that gene expression from the Ω-Tet fragment did not influence the PM^r phenotype.

We then subcloned a 1,329-bp *Cla*I-*Pvu*II fragment from plasmid pKR300 into plasmid pHSG576. This DNA fragment includes the *Eco*RI$_1$ site (Fig. 2). The resulting plasmid, designated pKR301, was introduced into strain LT2. As shown in Fig. 1A, this fragment was sufficient to confer PM^r, although

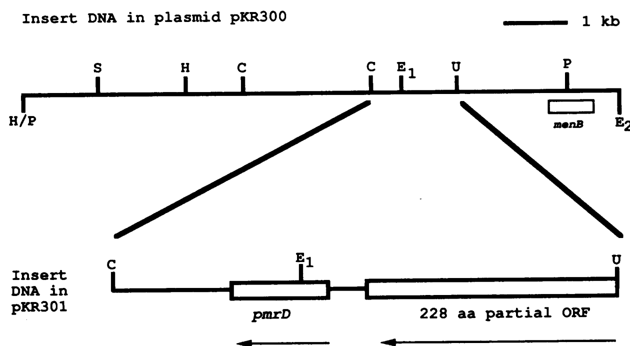

FIG. 2. Restriction map of plasmids carrying the cloned *pmrD* gene. The 10,000-bp insert from pKR300 is shown at the top. The location of the *menB* gene is shown as an open box located below the restriction map. The 1,329-bp insert in plasmid pKR301 is shown below. The locations of the *pmrD* gene and partial ORF are indicated by open regions on the restriction map. The arrows indicate the predicted direction of transcription. Restriction site abbreviations: H, *Hin*dIII; P, *Pst*I; S, *Sal*I, C, *Cla*I; E, *Eco*RI; U, *Pvu*II. aa, amino acid. The two *Eco*RI sites are labeled E_1 and E_2 for clarity. Plasmids pKR303 and pKR304 contain an Ω-Tet insertion in E_1 and E_2, respectively.

the 50% lethal dose was reduced. The reason for the discrepancy between pKR300 and pKR301 is not clear. It may be simply due to differential expression of *pmrD* between the two plasmid constructions, or it is possible that plasmid pKR300 contains another gene which must also be overexpressed to observe the maximum level of resistance. We favor the former explanation because when plasmid pKR301 was present in strain LT2, the increase in the MIC was 10-fold (Table 2), equivalent to the MIC of the *pmrA* mutant, JKS5 (see strain JKS1222 in Table 2).

We also tested whether plasmid pKR301 could confer resistance to other antibiotics. We found that it had no effect on the MICs of nalidixic acid, erythromycin, cycloserine, rifampin, streptomycin, gentamicin, and novobiocin for strain LT2 (data not shown).

Mapping of *pmrD*. We mapped the insert from plasmid pKR300 by using the mapping strains of Benson and Goldman (3). We found that the insert hybridized most strongly to DNA prepared from strain TT15258, indicating a map position between 45 and 50 min. Since the *pmrA* locus maps to 93 min (22, 25), this DNA clearly does not encode *pmrA*. In addition, since the polymyxin resistance phenotype in strain JKS5 has been demonstrated to be 100% linked to the *pmrA* locus (23), we conclude that the *pmrD* gene we have isolated from strain JKS5 is not sufficient to confer an increase in polymyxin resistance when present in a single copy on the chromosome in the $pmrA^+$ background.

We obtained more-refined mapping data by DNA sequence analysis. We cloned the *Cla*I-*Pst*I fragment from pKR300 into M13mp18 and determined the DNA sequence of 220 bp proximal to the *Pst*I site. A data base search of this sequence revealed that it was 95% identical to the *menB* gene of *E. coli* (31). Further, a translation of the DNA sequence gave two partial protein sequences which were 100% identical with the deduced amino acid sequence from the *E. coli menB* (31) and *menC* (30) genes. On the basis of these results, we conclude that plasmid pKR300 contains the *S. typhimurium menB* and *menC* genes. Since these genes have been previously mapped to 46 min on the *Salmonella* chromosome (25), this result confirmed and further refined our mapping data.

DNA sequence analysis of the *pmrD* gene. The DNA sequence for both strands of the 1,329-bp *Cla*I-*Pvu*II fragment from plasmid pKR301 was determined, and the results are shown in Fig. 3. There is only one complete ORF in the fragment, from bp 785 to 1039, which spans the *Eco*RI$_1$ site, consistent with our previous analysis. This ORF encodes an 85-amino-acid protein with a predicted molecular mass of 9,742 Da and a calculated pI of 8.63. A consensus −10 region is located 83 bp upstream of the ORF, although there is no obvious −35 consensus present. There is also a potential ribosome-binding site which is centered 7 bp upstream from the initiation codon. A hydropathy plot (17) of the deduced amino acid sequence indicated that the amino-terminal and carboxy-terminal ends of the protein are highly charged, while the central amino acids are primarily hydrophobic. We have designated this ORF *pmrD*. In addition, there is a partial ORF, corresponding to bp 1 to 684 of the sequenced region (Fig. 3).

To confirm that the *pmrD* allele we cloned from strain JKS5 did not contain a mutation, we amplified the gene by PCR with the primers PMRD6 and PMRD7 (Fig. 3), using chromosomal DNA derived from strain LT2 as the template. The expected 565-bp fragment was obtained, and the PCR product was sequenced directly with the same primers. The DNA sequence was identical to the sequence between the two primers as shown in Fig. 3, indicating that the cloned gene represents the wild-type allele.

A data base search of the deduced amino acid sequence of PmrD revealed no striking homology with any known proteins. One of the best matches obtained (P = 0.51) was with the p15 *gag* protease of Rous sarcoma virus (27). The homology was over a 36-amino-acid stretch which included the active site of the protease (21). This result indicated that the PmrD protein may be a protease, raising the possibility that its proteolytic activity was required for polymyxin resistance. To investigate this, we introduced the mutation D23A, which changes the putative active-site Asp residue to Ala, into plasmid pKR301, thereby creating plasmid pKR308. The MIC for LT2/pKR308 was fourfold lower than that for LT2/pKR301 (Table 2), indicating that Asp-23 is required for optimal activity of the PmrD protein. However, the MIC was reproducibly twofold higher than that for the control strain containing vector alone, indicating that *pmrD* activity is not abolished completely, as might be expected if Asp-23 was the catalytic center of a protease. Although these data serve to further confirm our assignment of the *pmrD* ORF and demonstrate that Asp-23 is important for PmrD activity, they are not consistent with the model that Asp-23 is the catalytic center of an acid protease required for *pmrD*-mediated polymyxin resistance.

The best match we obtained for the partial ORF protein (P = 0.0003) was with the FcbA protein of *Arthrobacter globiformis*, an enzyme that dehalogenates 4-chlorobenzoic acid (26), probably via a covalently attached acetyl coenzyme A intermediate. The ORF also had a high degree of homology with the recently defined superfamily of adenylate-forming enzymes described by Guilvout et al. (13). Because the partial ORF in our sequence is ca. 1,600 bp downstream of *menB*, we speculate that it encodes the *menE* gene. The *menE* gene encodes *o*-succinyl benzoic acid-coenzyme A synthetase (4), which might be expected to be homologous with this family of enzymes (13).

Maxicell analysis. To confirm our assignment of the small ORF, we performed maxicell analysis of our clone. The 1,329-bp *Cla*I-*Pvu*II fragment from pKR300 was cloned into pUC18. The resulting plasmid, pKR302, conferred PM^r to strain LT2 as judged by growth on polymyxin E gradient plates and by increased MIC (Table 2). Like plasmid pKR300, this

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         .         .         .         .         .         .         .         .         .         .
CTGTGGCGGCTGCTGGCGAATCGGGCGGCGGTGACGCTGAAAGCCGTTTTGCTCGGCGGCGCGGTGATTCCGGTTGAACTCACCGACCAGGCCAGCAAAC  100
L  W  R  L  L  A  N  R  A  A  V  T  L  K  A  V  L  L  G  G  A  V  I  P  V  E  L  T  D  Q  A  S  K  Q
'orf ->

AAGGGATTCGCTGCTGGTGCGGGTATGGGCTCACGGAGTTCGCGTCTACGGTATGCGCCAAAGAGGCCGATGGTTCGGATGATGTTGGCGCGCCGCTTCC  200
 G  I  R  C  W  C  G  Y  G  L  T  E  F  A  S  T  V  C  A  K  E  A  D  G  S  D  D  V  G  A  P  L  P

CGGCAGAGAGATCAGAATCGTCGATAATGAAGTCTGGCTGCGCGCCGCCAGTATGGCGGAAGGTTACTGGCGTGATGGCAAACTGATCCCGCTGGTTAAT  300
 G  R  E  I  R  I  V  D  N  E  V  W  L  R  A  A  S  M  A  E  G  Y  W  R  D  G  K  L  I  P  L  V  N

GACGAAGGCTGGTTTGCCACGCGCGATCGCGGCGAGCTGAACCACGGCAGGCTAACCATCGCCGGGCGGCTGGACAACCTGTTCTTTAGCGGCGGCGAAG  400
D  E  G  W  F  A  T  R  D  R  G  E  L  N  H  G  R  L  T  I  A  G  R  L  D  N  L  F  F  S  G  G  E  G

GCATTCAGCCGGAGGAAGTCGAACGCGTTATTAATGCGCATCCGCTGGTGCAACAAGCCTTCGTCGTGCCGGTAGAAGATAAAGAGTTTGGTCATCGTCC  500
 I  Q  P  E  E  V  E  R  V  I  N  A  H  P  L  V  Q  Q  A  F  V  V  P  V  E  D  K  E  F  G  H  R  P

                   PMRD6--------------------->
CGTTGCGGTTGTGGAGTATGCATCGCAGGCCGGAGACGTGAACCTCGCTGAATGGGTTCGTGATAAGCTGGCGCGTTTTCAACAGCCGGTTCGCTGGTTG  600
 V  A  V  V  E  Y  A  S  Q  A  G  D  V  N  L  A  E  W  V  R  D  K  L  A  R  F  Q  Q  P  V  R  W  L

ACGATGCCGTCAGAACTGAAAAACGGAGGGATTAAAATATCTCGCCGTGCGCTCCAGCAGTGGGTGTGTGAGAACTGTAAAAATTAATGTTAGGTTAATA  700
T  M  P  S  E  L  K  N  G  G  I  K  I  S  R  R  A  L  Q  Q  W  V  C  E  N  C  K  N

TACAACCATTCCATCGCTATTGCCGTTTTGTTTATCCGTTGATGTGTAACGTGTTATGAACAATCAGCGTGAAACGGGGGCGCTATGGAATGGTTGGTTA  800
                                                                                    M  E  W  L  V  K
                                                                                   pmrD ->
                                                                                       ▽
AGAAATCGCATTATGTCAAAAAGAGGGCGTGCCATGTTCTGGTGCTGTGCGATAGCGGCGGTTCGCTAAAAATGATCGCCGAGGCGAATTCCATGATATT  900
 K  S  H  Y  V  K  K  R  A  C  H  V  L  V  L  C  D  S  G  G  S  L  K  M  I  A  E  A  N  S  M  I  L

ACTGAGTCCCGGCGATATCCTGTCGCCTTTACAGGATGCGCAGTATTGTATTAATCGGGAAAAACACCAGACCTTAAAAATCGTTGATGCACGCTGTTAT 1000
 L  S  P  G  D  I  L  S  P  L  Q  D  A  Q  Y  C  I  N  R  E  K  H  Q  T  L  K  I  V  D  A  R  C  Y

                                                                          <---------------------PMRD7
TCCTGCGACGAATGGCAGCGGTTGACGCGCAAGCCATCATGAATCTGGCGGGCAGAAAAATCAGCATTACCCCCGCCATAATACACAATACGCCGAGCATC 1100
S  C  D  E  W  Q  R  L  T  R  K  P  S

GCTTTTAGGCTGAAAGCGCCTTGCAAACCGGGTAACAGCATTGAGGCTACCCAGACAAGCACATAGCTGAGACTCAGTAGCGCATAGGCTTTGCTGAGCG 1200
CGAGCGTGTGTAATGTTTTCTGCCAGCAGAAGACGGAAACCAGATACCCCGCCAAACCGGCGAATAGCGCCAGGGTCGCGGCATTAAATGCGCCCAGCCC 1300
GGAAATAAACGCCAGTGGATGCGCTATCG
```

FIG. 3. DNA sequence of the 1,329-bp *Cla*I-*Pvu*II fragment from pKR300. The noncoding strand is shown. The deduced amino acid sequence of PmrD and the partial ORF are shown below the DNA sequence. Putative stop and start codons are shown in boldface type. A potential promoter −10 sequence is shown in boldface italics and underlined, and a potential ribosome-binding site is underlined. The *Eco*RI$_1$ site is indicated by a double underline. The dashed arrows indicate the positions of the two primers, PMRD6 and PMRD7, used for PCR. The inverted triangle indicates the location of the Ω-Tet insertion discussed in the text.

higher-copy-number derivative failed to increase the MIC for strain JKS5 (Table 2). *E. coli* MV1190 transformed with plasmid pKR302 was more resistant to polymyxin than was a pUC18 control strain (Table 2), although the increase was not as great as in strain LT2. Maxicells prepared from the *E. coli* transformant expressed a protein of the predicted size (Fig. 4). This result is consistent with our finding that a *pmrD*::*lacZ* translational fusion is expressed from a multicopy plasmid, producing 800 Miller units when assayed at mid-exponential phase.

Construction and characterization of a chromosomal insertion mutation. To assess the biological role of *pmrD*, we constructed a chromosomal insertion mutation by transferring the insertion in plasmid pKR303 (Table 1) to the chromosome (see Materials and Methods). The resulting strain, JKS1426, contained an insertion of the appropriate size by PCR analysis with the primers shown in Fig. 3 (data not shown). Strains JKS1426 and LT2 had a similar growth rate when grown in LB medium and produced the same-sized colonies on LB plates or

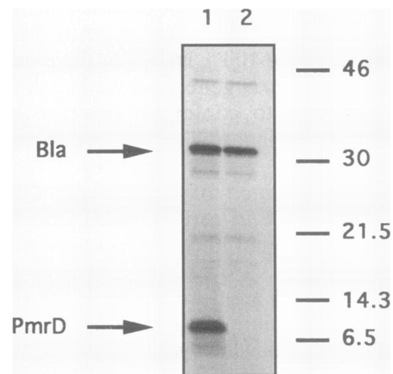

FIG. 4. Expression of *pmrD* in maxicells. Arrows indicate the positions of β-lactamase (Bla) and PmrD. The numbers on the right indicate the position of rainbow marker proteins. [^{35}S]methionine-labeled protein expressed in maxicells containing plasmid pKR302 (lane 1) or pUC18 (lane 2) is shown.

on minimal media containing either glucose or glycerol as the sole carbon source.

Because *pmrD* is just downstream of the *menB* and *menC* genes, we investigated the possibility that it is required for menaquinone biosynthesis. A number of *men* mutants which do not utilize TMAO as an electron acceptor during anaerobic growth have been isolated (4). Therefore, we asked whether the insertion in *pmrD* would have any effect on TMAO-dependent anaerobic growth. Unfortunately, we found that neither strain JKS1426 nor its parent strain LT2 could grow anaerobically on glycerol minimal medium containing TMAO. However, strain ATCC 14028 grew well under these conditions, so the *pmrD2*::Ω-Tet mutation was transferred to that strain by P22 transduction. We found that the insertion had no effect on TMAO-dependent growth. In addition, all strains grew well anaerobically on both LB and glucose minimal medium.

Strain JKS1426 was no more sensitive to polymyxin than was strain LT2 (compare strains JKS1184 and JKS1429 in Table 2), and plasmid pKR301 conferred the same 10-fold increase in the MIC for this strain as it did to the MIC for strain LT2 (Table 2).

Interdependency of *pmrD* and *pmrA* for the polymyxin-resistant phenotype. To test the effect of a *pmrA* mutation in strain JKS1426, we introduced plasmid pKR100. Plasmid pKR100 contains the *pmrA505* allele (25) and confers a 10-fold increase in the MIC of polymyxin for strain LT2 (Table 2). However, when this plasmid was introduced into strain JKS1426, only a twofold increase in the MIC was observed (Table 2). To confirm that the plasmid present in the transformant of strain JKS1426 (JKS1430) contained a functional *pmrA505* gene, we prepared plasmid DNA from strain JKS1430 and used it to transform strain LT2. Eight of the resulting LT2 transformants were tested and found to be resistant to polymyxin as judged by growth on gradient plates. One of these isolates, JKS1434, was tested in the MIC assay and found to show the expected level of resistance (Table 2), confirming that the plasmid present in strain JKS1430 is fully functional.

We examined this effect further by crossing the *pmrA505* allele into the chromosome of strain JKS1426 by P22 transduction and determining the MIC for two of the transductants that were judged to be polymyxin resistant on gradient plates. The data for one of these, strain JKS1443, are given in Table 2. The MIC for both of these strains was 5 μg/ml, a value intermediate between the MICs for strains JKS1426 (*pmrD2*::Ω-Tet) and JKS1170 (*pmrA505*) (Table 2). These results are consistent with the data we obtained with pKR100. We conclude from these observations that a functional *pmrD* gene is required for optimal PmrA-mediated polymyxin resistance.

To determine whether a functional *pmrA* allele was required for PmrD-mediated polymyxin resistance, we constructed a null mutation in *pmrA* in which the first 177 codons of the *pmrA* gene was replaced by an Ω-Kan cassette (see Materials and Methods). The resulting strain was designated JKS1379. The MIC of polymyxin was not affected by this mutation (see strain JKS1386 in Table 2). The introduction of plasmid pKR100 into strain JKS1379 resulted in a 10-fold increase in the MIC (Table 2). Conversely, the presence of plasmid pKR301 had no effect on the MIC for strain JKS1379 (see strain JKS1402 in Table 2). Plasmid DNA from JKS1402 was isolated and introduced into strain LT2, and eight LT2 transformants were tested and found to be polymyxin resistant as judged by streaking onto polymyxin gradient plates, indicating that the plasmid present in strain JKS1402 contains a functional *pmrD* gene. We conclude from this observation that a functional *pmrA* gene is required for *pmrD*-mediated polymyxin resistance. Taken together, these results suggest that there is an interaction between PmrD and either PmrA or the product of a gene regulated by PmrA.

To further test this hypothesis, we attempted to complement the *pmrA* defect in strain JKS1402 (JKS1379/pKR301) with several plasmid constructions that contain *pmrA*$^+$, including the previously described pUC18 derivative pKR202 (23) and a pACYC184 derivative (22). Neither plasmid could complement strain JKS1402 to PM^r. Further, we found that when plasmid pKR202 was introduced into strain JKS1276 (LT2/pKR301), the transformants became sensitive to polymyxin (22). These results indicate that the relative copy number of each gene is important for the polymyxin resistance phenotype.

We were, however, able to transduce strain JKS1402 to polymyxin resistance. In this cross, the donor strain, JKS1417, contained a Tn*10* 50% linked to *pmrA*$^+$. Of the 32 Tet^r transductants examined, 16 had become Kan^s. We infer that the loss of Kan^r by the transductants resulted in the acquisition of *pmrA*$^+$. In every case in which strain JKS1402 lost Kan^r, it became resistant to polymyxin. We conclude from this experiment that a functional *pmrA* locus is required for the *pmrD*-mediated polymyxin resistance phenotype.

DISCUSSION

We present here the first example of a locus other than *pmrA* which can mediate resistance to polymyxin. It is composed of a small gene, *pmrD*, which confers polymyxin resistance when expressed from a relatively low-copy-number plasmid. The effect is dependent upon *pmrA*, since a functional *pmrA* locus is required for *pmrD*-mediated resistance and a functional *pmrD* gene is required for optimal *pmrA*-mediated resistance (Table 2). We have previously argued that the *pmrA505* allele results in increased expression of at least two genes involved in lipid A modification (23), since the lipid A in *pmrA* strains has an increase in the substitution of two different modifying groups (38). However, it is possible that overexpression of a single *pmrA*-regulated gene is sufficient to express the polymyxin resistance phenotype. Although we have not addressed the regulation of *pmrD* expression (see below), we can infer from our data that PmrD interacts with PmrA or a PmrA-regulated gene product. Therefore, we suggest that *pmrD* is a component of the pathway that leads to polymyxin resistance in *pmrA* mutant strains. Furthermore, there must be another, *pmrD*-independent component to this pathway because *pmrA*-mediated polymyxin resistance is not completely abolished by the *pmrD* insertion (Table 2).

The biological role of *pmrD* is not clear. Because the only known target of polymyxin is the bacterial membrane, the PmrD protein must either interact with a membrane or membrane precursor directly or mediate changes in membrane structure indirectly by interacting with other proteins. Because of its small size, it is possible that PmrD is part of a larger protein complex. In a recent survey of small proteins (<100 amino acids), White (40) found that proteins known to be peripherally associated with a membrane correlated with an Arg and Lys frequency of 4.2 and 9.0%, respectively, as opposed to a frequency of 15 and 6.4%, respectively, for DNA-associated proteins. PmrD contains 5.8% Arg and 9.3% Lys, suggesting that this protein is membrane associated, a location that would be consistent with a role in a coordinated multiprotein complex, perhaps involved in lipid A modification. Alternatively, since the ultimate target of polymyxin B is apparently the cytoplasmic membrane, PmrD may affect that

stage of the interaction. The *pmrA* null mutation used in this study also truncates the ORF1 gene, which is located just upstream of *pmrA*, is part of the same operon, and encodes a putative membrane protein (23). The null mutation is probably polar to *pmrB* as well. Therefore, if PmrD is a peripheral membrane protein, it is also possible that PmrD interacts with either PmrB or the ORF1 protein. These possibilities warrant further study.

The ability of the cloned *pmrD* gene to confer resistance to polymyxin B is not unique, because overexpression of chromosomal genes has been shown to confer antibiotic resistance in several other systems. The *bacA* gene of *E. coli* encodes a 151-amino-acid protein which confers high levels of resistance to bacitracin when expressed in multiple copies (5). As with *pmrD* (Table 2), the level of resistance was the same whether the gene was amplified by its inclusion in a relatively low-copy-number pACYC177-based plasmid or in a high-copy-number pUC19-based construction (5). It is thought that this phenotype is due to the overproduction of an enzyme that competes with bacitracin for its target molecule, undecaprenyl diphosphate. Another example of gene dosage-related drug resistance has been described for *Bacillus subtilis*. Ives and Bott (14, 15) identified a chromosomal gene originally isolated from a Tet^s strain that conferred Tet^r when the chromosomal copy was amplified to greater than 100 copies per cell. In this case, resistance was associated with a 72-kDa protein that they believed increased the efflux of tetracycline from the cell. Perhaps the best-characterized example of this type of resistance is the *mar* locus of *E. coli*, which was originally defined by mutations that resulted in increased levels of resistance to a number of unrelated antibiotics (12). Subsequent DNA sequence analysis revealed a pattern of mutations that could result in overproduction of MarA, the putative positive regulator of the *mar* locus (7). Gambino et al. (11) showed that overexpression of MarA on a multicopy plasmid is sufficient to mimic the *mar* mutant phenotype. This locus is widespread among enteric bacteria, including *S. typhimurium*, and it has been found to be overexpressed in a number of multidrug-resistant clinical isolates of *E. coli* (8), indicating that this locus may play a role in human disease.

Although PmrA is required for *pmrD*-mediated resistance, an important question that our data do not address is whether *pmrD* expression is regulated by PmrA. Preliminary experiments with a multicopy *pmrD::lacZ* fusion plasmid indicate that PmrA is not required for *pmrD* expression (22), although this type of data can be misleading because of the titration of regulatory components. A more detailed analysis of this question will be carried out by using a single-copy fusion.

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