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Spontaneous *pmrA* Mutants of *Salmonella typhimurium* LT2 Define a New Two-Component Regulatory System with a Possible Role in Virulence

KENNETH L. ROLAND,* LARRY E. MARTIN, CHARLES R. ESTHER, AND JOHN K. SPITZNAGEL

Department of Microbiology and Immunology, 3152 Rollins Research Center, Emory University, Atlanta, Georgia 30322

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We isolated spontaneous mutations (*pmrA*) in the smooth strain *Salmonella typhimurium* LT2 that show increased resistance to the cationic antibacterial proteins of human neutrophils and to the drug polymyxin B. The mutation in one strain, JKS5, maps to 93 min on the *S. typhimurium* chromosome, near the *proP* gene and the *melAB* operon. The mutation, designated *pmrA505*, confers a 1,000-fold increase in resistance to polymyxin B and a 2- to 4-fold increase in resistance to neutrophil proteins. We cloned both the *pmrA505* and *pmrA*$^+$ alleles and found that the *pmrA*$^+$ gene is partially dominant over *pmrA505*. DNA sequence analysis of the *pmrA505* clone revealed three open reading frames (ORFs). The deduced amino acid sequences indicated that ORF1 encodes a 548-amino-acid (aa) protein with a putative membrane-spanning domain and no significant homology to any known protein. ORF2 and ORF3, which encode 222- and 356-aa proteins, respectively, show strong homology with the OmpR-EnvZ family of two-component regulatory systems. ORF2 showed homology with a number of response regulators, including OmpR and PhoP, while ORF3 showed homology to histidine kinase-sensor proteins EnvZ and PhoR. Genetic analysis of the cloned genes suggested that ORF2 contained the *pmrA505* mutation. Comparison of the *pmrA505* and *pmrA*$^+$ ORF2 DNA sequences revealed a single G-A transition, which would result in a His-to-Arg substitution at position 81 in the ORF2 mutant protein. We therefore designate ORF2 PmrA and ORF3 PmrB. The function of ORF1 is unknown.

Mutations in the *pmrA* locus of *Salmonella typhimurium* confer an increase in resistance to cationic, hydrophobic agents, including the antibiotic drug polymyxin B (22) and two of the cationic, antibacterial proteins (CAP) from human neutrophils: CAP37 (33, 40, 42) and CAP57 (41, 46). In addition, *pmrA* mutants survive better in human neutrophils (46), suggesting that this locus plays a role in virulence.

We and others have investigated the role of CAP as an O_2-independent killing mechanism in neutrophils. CAP37, also called azurocidin (7), is multifunctional, showing both bactericidal activity (40, 41, 42) and chemotactic activity toward monocytes (34). N-terminal amino acids 20 to 44 are responsible for at least part of its bactericidal activity (33). The bactericidal action of CAP57, also referred to as bactericidal permeability-increasing protein, has been extensively studied as well (15, 25, 40, 41, 43, 44, 46, 54). As in CAP37, the bactericidal activity resides in the amino-terminal portion of the protein (31). Both proteins bind to lipid A and lipopolysaccharide (LPS) (25, 33, 40), are active against a wide range of gram-negative bacteria (41, 42), and have both cationic and hydrophobic domains (16, 29, 36). Despite their similarities, these proteins lack any significant homology at the DNA or amino acid sequence level (16, 29, 36). It is thought that the LPS of the outer membrane is the initial target for binding of CAP to bacteria, presumably because of interactions driven by the net positive charge of CAP and the net negative charge of the outer membrane.

Polymyxin B, like CAP, has both a cationic and a hydrophobic domain, binds lipid A, and is bactericidal to a similar range of gram-negative bacteria (52). The *pmrA* mutants, isolated by selection on plates containing polymyxin B (22), have an increased level of substitution by 4-aminoarabinose at the lipid A phosphoryl groups (53). This is thought to reduce the net negative charge on the LPS which, in turn, results in decreased binding of polymyxin B to the cell (52). Binding of CAP57 to bacterial cells is also decreased in *pmrA* mutants (15).

Although the original *pmrA* mutants were isolated 15 years ago (22) and their phenotype was extensively studied in various rough backgrounds (52), very little is known about the gene responsible or its impact on resistance in a smooth background. In this study, we isolated spontaneous *pmrA* mutations in strain LT2 because (i) LT2 provides a smooth, prototrophic background which is more amenable to genetic analysis and (ii) we wanted to avoid potential complications associated with previously described mutants which were isolated by chemical mutagenesis of whole cells (22). We used our *pmrA* mutants to map the *pmrA* locus precisely, to clone and sequence both the mutant and wild-type alleles of this gene, and to determine the molecular basis for the *pmrA*-encoded phenotype.

MATERIALS AND METHODS

Bacterial strains. The *S. typhimurium* strains used in this study are summarized in Table 1. *Escherichia coli* 71-18 (56) was the host for bacteriophage M13.

Media. LB broth (3), LB plates (3), E salts medium plus glucose (24), green plates (24), P22 broth (24), tryptone broth (pH 5.5) (3, 42), and Bochner plates (5) were prepared as previously described. Chloramphenicol (Cam) or tetracycline was included at 20 μg/ml where indicated. Spectinomycin was used at 50 μg/ml. LB-polymyxin E gradient plates (6) were prepared essentially as previously described, except that polymyxin E was included in the lower half of the plate

* Corresponding author.

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

Strain or plasmid	Genotype/plasmid	Source or reference
Strains		
LT2	*pmrA*$^+$	S. Maloy
JKS3	LT2 carrying *pmrA503*	This study
JKS5	LT2 carrying *pmrA505*	This study
JKS200	JKS5 carrying *zjd*::Tn*10*	This study
JKS203	JKS5 carrying *zjd*::Tn*10*	This study
JKS1169	LT2 carrying *zjd*::Tn*10d*-Cam	This study
JKS1170	LT2 carrying *pmrA505 zjd*::Tn*10d*-Cam	This study
JKS1184	LT2/pHSG576	This study
JKS1187	LT2/pKR100	This study
JKS1251	JKS5/pKR200	This study
JKS1252	JKS5/pKR201	This study
JKS1261	SH7426/pKR200	This study
JKS1271	SH7426/pKR201	This study
MS1868	*leu414 hsdL fels2*	T. Elliott
TN2409	*leuBCD485 oxd7*::MudJ	C. Miller
SH9178	*pmrA*$^+$ *rfaJ4041 metA22 metE551 trpB2 xyl-404 rpsL120 fla66 ilv-1178 thr-914 his-6116* (R471a)	M. Vaara
SH7426	SH9178 carrying *pmrA163*	M. Vaara
SR305	HfrA *hisD23 gal-50*	K. Sanderson
TT15275	MS1868 carrying *melAB396*::MudQ	4 (via K. Sanderson)
TT1662	*mel*::Tn*10*	C. Miller
TT10604	*proAB47*/F' 128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10d*-Cam	J. Roth
Plasmids		
pHSG576	pSC101 derivative, Cam^r	50
pGB2	pSC101 derivative, Spc^r	9
pKR100	pHSG576, *pmrA505*	This study
pKR101	pHSG576, *pmrA505*	This study
pKR102	pHSG576 + 3,579-bp *Cla*I fragment from pKR100	This study
pKR103	pHSG576 + 3,800-bp *Pst*I-*Eco*RI fragment from pKR100, *pmrA505*	This study
pKR104	pHSG576 + 4,000-bp *Sal*I-*Eco*RI fragment from pKR100, *pmrA505*	This study
pKR200	pGB2, Tn*10d*-Cam *pmrA*$^+$	This study
pKR201	pGB2, Tn*10d*-Cam	This study
pKR202	pUC18 + 4,200-bp *Sal*I fragment from pKR200, *pmrA*$^+$	This study
pKR207	Deletion of 614-bp *Mlu*I fragment from pKR202, *pmrA*$^+$	This study
pKR208	Deletion of 446-bp *Sac*II fragment from pKR202, *pmrA*$^+$	This study
pKR209	Deletion of ca. 200-bp *Pst*I-*Sal*I fragment from pKR202, *pmrA*$^+$	This study
pNK2881	pBR322 derivative, Amp^r *Ptac*::*ats1 ats2* transposase	19; N. Kleckner

instead of the upper half. We typically used a gradient of 0 to 50 μg of polymyxin E per ml in routine screening for the polymyxin resistance (PM^r) phenotype. We found this technique invaluable for rapid screening, as we noted that screening on plates containing a single concentration of polymyxin was difficult to interpret because of inoculum size effects.

DNA preparation and manipulations. Plasmid DNA was prepared by an alkaline lysis method (3). DNA used for cloning experiments was prepared from *E. coli* DH5α cells and further purified on CsCl gradients (3). Genomic DNA was prepared as previously described (3), except that the CsCl gradient step was omitted. The presence of inserts in plasmid DNA was assessed on agarose gels (3). Restriction enzymes were used in accordance with manufacturer recommendations. Transformation of *S. typhimurium* strains was done by electroporation (3). DNA used for dot blot experiments was radioactively labeled with a Pharmacia Oligolabeling Kit used in accordance with manufacturer recommendations. [^{32}P]dATP was purchased from Amersham. Preparation of phage DNA and dot blot analysis were done as previously described (3).

Isolation of *pmrA* mutants. Strain *S. typhimurium* LT2 was grown to the mid-exponential phase in LB broth. Cells were washed once in LB broth, and 0.1-ml portions of various serial dilutions were plated onto LB plates containing 0, 1, 3, 30, and 100 μg of polymyxin E per ml and incubated overnight at 37°C. Plates spread with less than 5×10^6 cells yielded no colonies, and plates spread with more than 5×10^7 cells yielded confluent growth. Plates spread with either 5×10^6 or 5×10^7 cells yielded a total of 26 isolated colonies. These were picked, restreaked, and tested for PM^r. Of these colonies, six (JKS1 to JKS5 and JKS7) were resistant to polymyxin E as judged from growth on gradient plates. The appearance of the other 20 colonies was presumably due to inoculum effects.

Genetic techniques. P22 HT105/1 *int-201* was used for production of transducing lysates as previously described (24). Transductants were routinely cleaned up on green plates and then restreaked back onto antibiotic-containing LB plates before further analysis, except in cases in which they were pooled or replica plated (see below). Strains JKS200 and JKS203 were constructed as follows A Tn*10* insertion library was prepared by infecting strain JKS5 with P22 *c2*ts::Tn*10* (20) and selecting for Tet^r colonies. Approximately 5,400 transductants were pooled, and a transducing lysate was prepared on the pooled transductants. This lysate was used to infect LT2 with selection for Tet^r. These

transductants were replica plated onto LB plates containing 8 and 16 μg of polymyxin E per ml. PM^r colonies were then isolated, and their phenotype was confirmed on gradient plates. Two isolates, strains JKS200 and JKS203, were chosen for further analysis. Linkage of the Tn*10* insertions to the PM^r phenotype was confirmed by preparing a transducing lysate for each strain and using the lysate to transduce LT2 to Tet^r. Transductants were then screened for PM^r on gradient plates.

Strains JKS1169 and JKS1170 were constructed in several steps. First, plasmid pNK2881 (19), which contains the *ats-1 ats-2* transposase, was introduced into strain JKS5 by electroporation (3). One of the resulting transformants was infected with a P22 transducing lysate prepared on strain TT10604 (Table 1), and transductants were selected by plating onto LB-Cam plates. Approximately 5,000 transductants were pooled, and a P22 lysate was prepared on the pooled transductants. The resulting insertion library was used to transduce LT2 to Cam^r. The transductants were replica plated onto LB plates containing 20 μg of polymyxin E per ml. Three PM^r colonies were obtained, restreaked onto LB-Cam plates, and tested for PM^r on gradient plates. One isolate was used to prepare a transducing lysate. The lysate was used to transduce LT2 to Cam^r. Transductants were then screened for PM^r on gradient plates. One PM^s transductant and one PM^r transductant were designated JKS1169 and JKS1170, respectively. The transductional linkage between the Cam^r marker and *pmrA* allele was determined to be 50% in both strains.

Bactericidal assays. Bactericidal assays were done as previously described (33, 37, 42), except that plasmid-bearing strains were grown in the presence of an appropriate antibiotic. Briefly, mid-exponential-phase cells were harvested, diluted to a final concentration of 300/ml in tryptone-saline (pH 5.5) containing the indicated concentration of the test substance, and incubated in microtiter trays at 37°C for 1 h in a final volume of 200 μl. Then, 100 μl was plated onto LB plates which were incubated overnight at 37°C. Colonies were then counted, and percent survival was assessed. All assays were done in triplicate, and the data were plotted as the average of triplicate samples. The standard error between triplicate samples was less than 5%. Occasionally, growth in wells containing noninhibitory concentrations of the test antimicrobial agent was slightly greater than the growth in control wells, which resulted in values reported as >100% survival. For experiments with plasmid-bearing strains, several plates which contained greater than 100 colonies each were replica plated onto LB plus antibiotic to test for the presence of the plasmid. We found no drug-sensitive colonies, indicating that all of the plasmids used were stably maintained under the conditions used. All experiments were repeated at least twice with essentially identical results.

Preparation of CGE and Pep20-44. Crude granule extracts (CGE) from fresh human neutrophils were prepared as previously described (8, 41). Peptide Pep20-44 was prepared as previously described (33), and aliquots were stored in water at −20°C.

Construction of plasmids. Plasmids pKR100 and pKR101 were constructed as follows. Genomic DNA (3) was prepared from strain JKS5 and partially digested with *Sau*3AI. Fragments approximately 10,000 to 12,000 bp long were isolated by excising bands from agarose gels and purified by using the Millipore Ultrafree-MC purification system in accordance with manufacturer recommendations. Purified DNA was then ligated with *Bam*HI-cut pHSG576 (50) and used to transform *S. typhimurium* MS1868 with selection on LB-Cam. After overnight growth at 37°C, the Cam^r colonies were replica plated onto LB-Cam, incubated at 37°C for 4 h, replica plated onto LB plates containing 20 μg of polymyxin E per ml, and incubated overnight at 37°C. We found that replica plating of overnight colonies directly onto polymyxin E plates gave a large number of false positives, presumably because of inoculum size effects. When we plated first to LB-Cam and then to LB-polymyxin E, a greater proportion of the resulting colonies was confirmed to be PM^r. The 63 colonies we obtained were streaked onto LB-Cam plates and tested for PM^r on gradient plates. Plasmid DNA was isolated from confirmed PM^r isolates, and five independent pools were prepared and used to transform *S. typhimurium* LT2. Two nonidentical plasmids that conferred PM^r and were derived from two independent pools were selected and designated pKR100 and pKR101.

Plasmids pKR200 and pKR201 were constructed by isolating and purifying 12,000- to 14,000-bp *Sau*3AI fragments derived from strain JKS1169 chromosomal DNA as described above. The purified fragments were ligated to *Bam*HI-cut plasmid pGB2 (9), a pSC101-derived vector with a selectable Spc^r marker. Strain MS1868 was transformed with the ligation mixture with selection for Cam^r. Thirty-five transformants were obtained and pooled, and plasmid DNA was prepared. The pooled DNA was used to transform strain JKS5. Cam^r Spc^r transformants were screened for PM^s, and one isolate was found be less sensitive than strain JKS5. Plasmid DNA was isolated from this transformant and used to transform fresh JKS5 cells. All of the resulting transformants were PM^s. The plasmid that conferred PM^s on strain JKS5 was designated pKR200. Another plasmid from this experiment, which did not confer PM^s on strain JKS5, was chosen as a control and designated pKR201.

The derivatives of pKR100 and pKR200 described in the text were constructed by standard techniques (3).

Mapping of *pmrA*. The Tn*10* insertions in strains JKS200 and JKS203 were mapped by using the 54 mapping strains of Benson and Goldman as previously described (4). The lysate derived from strain TT15275 (Table 1) gave the highest number of Tet^s colonies.

Insert DNAs from plasmids pKR100 and pKR101 were mapped as follows. DNA was prepared from the 54 transducing lysates described above. A 100-μl aliquot was taken from each lysate, diluted 1:5 with water, and extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1). The extracted material was then precipitated with ethanol and air dried, and the pellet was resuspended in 100 μl of TE buffer (3). DNA was denatured by addition of NaOH to 0.3 M and heating to 60°C for 1 h. An equal volume of 6× SSC (3) was added, and samples of approximately 6 ng of DNA were spotted onto an Immobilon-N nylon membrane (Millipore). The membrane was then treated and probed with ^{32}P-labeled insert DNA from pKR100 (the ca. 10,000-bp *Hin*dIII-*Eco*RI fragment) in accordance with manufacturer recommendations.

Isolation of Tn*1000* (γ δ) insertions. Plasmid pKR100 was introduced into *E. coli* 71-18. One of the resulting transformants was mated with *E. coli* AB1157 (*rpsL31*), and exconjugants were selected by plating onto LB plates containing Cam and streptomycin. The exconjugants were pooled, and plasmid DNA was prepared and used to transform *S. typhimurium* LT2. Transformants were then screened for PM^r by streaking on gradient plates. The approximate locations of interesting insertions were determined by restriction enzyme analysis.

DNA sequence analysis. The *Pst*I-*Eco*RI insert DNA from pKR100 was ligated into *Pst*I-*Eco*RI-digested M13mp18 and M13mp19 (56) to obtain clones in both orientations. An ordered set of deletions from both strands was constructed by the T4 polymerase method (12) with the Cyclone I Biosystem kit purchased from International Biotechnologies, Inc. There were four gaps in the sequence data, due to failure to obtain a complete set of deletions, which were filled in with appropriate primers purchased from Operon Technologies, Inc. Sequencing of both strands was done with a Sequenase kit purchased from United States Biochemical. [α-^{35}S]dATP was purchased from Amersham. A similar approach was used to sequence the *Sac*II-*Mlu*I fragment from pKR200.

The locations of Tn*1000* insertions were determined by using primers GD1 and GD2 as previously described (48).

Sequence manipulations and data base searches. The DNA sequence was read manually and entered into the DNA Strider program for analysis. Location of open reading frames (ORFs), their subsequent translation, and production of hydropathy plots (21) based on the deduced amino acid sequences were done with Strider. Both the entire insert DNA sequence from pKR103 and the deduced amino acid sequences of the three ORFs were used to search the combined data bases of the National Center for Biotechnology Information by using the BLAST Network service on 23 March 1993 by the alignment algorithm of Altschul et al. (1).

Chemicals and reagents. Polymyxin B (7,730 U/mg), polymyxin E (colistin methanesulfate; 13,700 U/mg), and all of the other antibiotics used were obtained from Sigma. Restriction enzymes were purchased from New England BioLabs. Bacterial growth medium was obtained from Difco.

Nucleotide sequence accession number. The entire DNA sequence (wild type) reported here has been submitted to GenBank and assigned accession number L13395.

RESULTS

Isolation of *pmrA* mutants of *S. typhimurium* LT2. Since preparation of the large amounts of CAP required to screen directly for CAP^r mutants would be cost prohibitive, we used the finding that *pmrA* strains are cross-resistant to CAP (40, 41) as a basis for isolation of CAP^r mutants of smooth strain LT2. Dilutions of exponential-phase cells were plated onto LB plates containing various concentrations of polymyxin E (see Materials and Methods). Twenty-six colonies were obtained on the polymyxin E plates, and six of these were found to maintain the increased polymyxin resistance phenotype stably. Preliminary experiments indicated that all six isolates were at least two- to fourfold more resistant to CGE. Notably, all six isolates remained sensitive to phage P22, indicating that they had retained the smooth phenotype, and they remained prototrophs, as judged by growth on E salts medium plus glucose. Isolates JKS3 and JKS5 were chosen for more detailed analysis.

We had previously designated the mutation in JKS5 as *gapA*, for the granule antimicrobial protein resistance gene, to distinguish it from the *pmrA* mutations previously isolated by chemical mutagenesis and to emphasize our interest in the CAP^r phenotype (45). However, since the results presented below are consistent with results we (40, 41, 46) and others (22) have previously obtained with rough *pmrA* mutant strains, we designate these mutants *pmrA* mutants.

Characterization of polymyxin resistance in *pmrA* mutant strains. Selected *pmrA* strains were tested for resistance to polymyxin B in a killing assay (37, 42) in which exponential-phase cells were incubated with various concentrations of polymyxin B for 1 h at 37°C and the number of surviving cells was determined. As summarized in Fig. 1A, killing was dose dependent and both JKS3 and JKS5 were greater than 1,000-fold more resistant than parent strain LT2. Strains JKS1169 and JKS1170 have a Tn*10d*-Cam element (14) 50% linked to *pmrA*$^+$ and *pmrA505*, respectively (Table 1; see Materials and Methods). The level of polymyxin resistance of strain JKS1170 is identical to that of strain JKS5, indicating that just one region of the chromosome is responsible for the entire 1,000-fold effect. Control strain JKS1169 was as sensitive as LT2, which shows that the Tn*10d*-Cam element did not influence polymyxin resistance.

To confirm that these *pmrA* mutants are cross-resistant to human CAP, we tested a crude granular extract prepared from human neutrophil granules (8, 40). Comparison of strains LT2 and JKS5 (Fig. 1B) showed that killing was dose dependent and that the *pmrA* mutation increased resistance to crude CAP approximately three- to fourfold. This increase in resistance is similar to the results we obtained by comparing isogenic rough *pmrA*$^+$ and *pmrA* strains SH9178 and SH7426, respectively (40). Comparison of strains JKS1169 and JKS1170 yielded similar results (Fig. 1B).

Pereira et al. (33) have previously shown that a synthetic peptide corresponding to amino acids 20 to 44 of CAP37 retains many of the properties of CAP37 related to its bactericidal action, including the ability to bind to LPS and the ability to kill the same range of gram-negative bacteria. When we tested the action of this peptide on strains LT2, JKS3, and JKS5 (Fig. 1C), we found that killing was dose dependent and strains JKS3 and JKS5 were twofold more resistant than strain LT2. Strains JKS1169 and JKS1170 gave similar results (data not shown). These data are consistent with our findings with whole CAP37 tested against strains SH9178 and SH7426 (41).

Mapping of *pmrA*. The *pmrA* mutation was mapped to approximately 91 to 93 min on the standard *S. typhimurium* genetic map (39) by the method of Benson and Goldman (4) (data not shown). We then used strain JKS203, which has Tn*10* linked to *pmrA505*, as the donor for transduction of strain TT15275 (*melAB*::Mu*d*Q) to Tet^r. The results from this experiment are summarized in Table 2. One hundred and twelve Tet^r transductants were screened for PM^r and Cam^r (the Mu*d*Q element carries a Cam^r marker). We found that 34% of the Tet^r transductants had also been transduced to Cam^s, indicating that the two markers are linked. Additionally, when the transductants were screened for PM^r we found that 13% of the transductants were both Cam^s and PM^r. Since Mu*d*Q and Tn*10* are large elements (ca. 36.4 and 9.3 kb, respectively), this result probably reflects an underestimation of their true transductional linkage. This appears to be the case, because when strain TT1662 (*melAB*::Tn*10*) was used as the donor in a transductional cross with strain JKS5, there was 32% cotransductional linkage between *melAB*::Tn*10* and *pmrA* (Table 2). These results confirm that *pmrA505* maps near *melAB*.

Strauch et al. (49) have previously reported that the *oxdB* locus is linked to *melAB*. To determine whether *pmrA* was linked to *oxdB*, we used strain TN2409 (*oxdB7*::Mu*d*J) as the donor in a transductional cross with strain JKS5. There was 13% cotransductional linkage between *oxdB* and *pmrA* (Table 2). These data (Table 2), combined with the transductional data of Strauch et al. (49), suggest the gene order *pmrA-melAB-oxdB*, although further experiments are required to confirm this conclusion.

Our mapping data do not agree with those of Makela et al.,

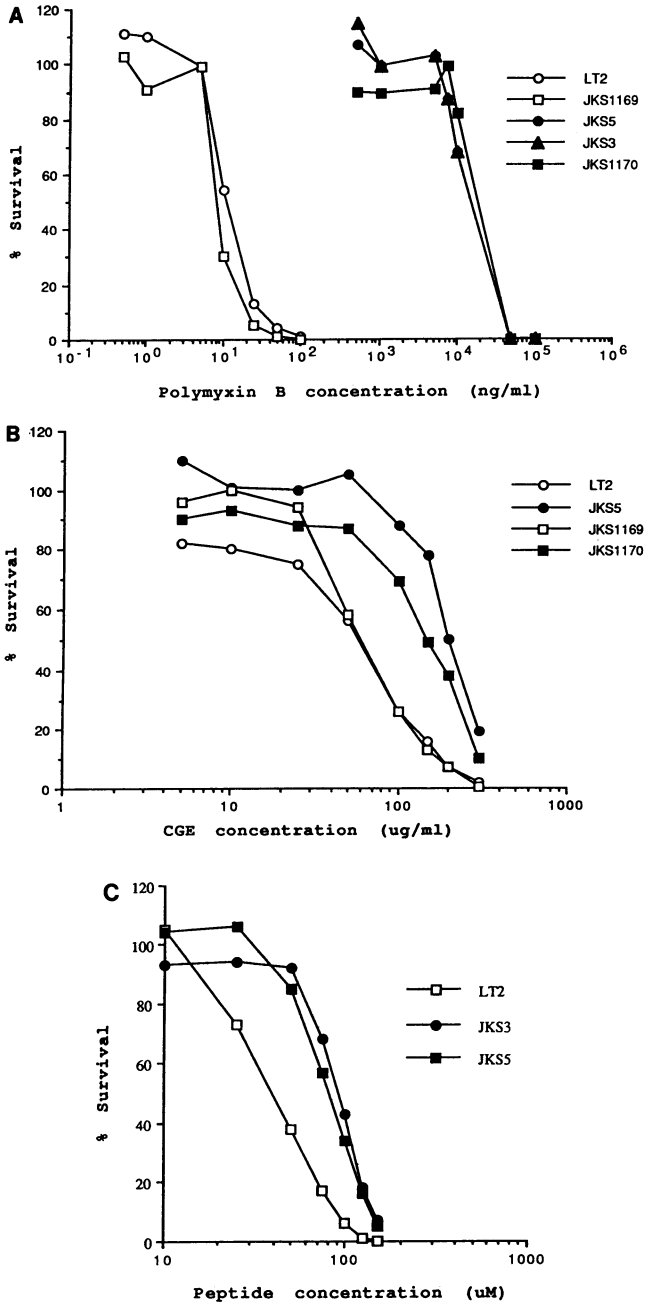

FIG. 1. Effect of *pmrA* on resistance to polymyxin B, CGE, and Pep20-44. Open symbols are used for *pmrA*$^+$ strains, and closed symbols are used for *pmrA* strains. (A) Resistance to polymyxin B. The 50% lethal doses for strains LT2 (*pmrA*$^+$), JKS1169 (Tn*10d*-Cam *pmrA*$^+$), JKS5 (*pmrA505*), JKS1170 (Tn*10d*-Cam *pmrA505*), and JKS3 (*pmrA503*) were 11, 8, 20,000, 20,000, and 15,000 ng/ml, respectively. (B) Resistance to a CGE derived from human neutrophils. The 50% lethal doses for strains LT2 (*pmrA*$^+$), JKS1169 (Tn*10d*-Cam *pmrA*$^+$), JKS5 (*pmrA505*), and JKS1170 (Tn*10d*-Cam *pmrA505*) were 56, 56, 200, and 160 μg/ml, respectively. (C) Resistance to synthetic peptide Pep20-44. The 50% lethal doses for strains LT2 (*pmrA*$^+$), JKS5 (*pmrA505*), and JKS3 (*pmrA503*) were 41, 82, and 93 μM, respectively.

who reported the map position for *pmrA* to be between 94 and 100 U (22). We believe not that the discrepancy is due to the fact that our mutations are at a different locus but rather that the transductional methods used here are more accurate than the conjugational methods used previously. Genetic evidence presented below indicates that our isolates and those of Makela et al. (22) harbor mutations in the same loci.

Strain JKS1169, which has a Tn*10d*-Cam element linked to *pmrA*$^+$, was used as the donor strain to transduce all six of our *pmrA* isolates to Cam^r. All six strains showed approximately 50% linkage between the Cam^r marker and PM^r (data not shown), indicating that all of our PM^r isolates contain mutations that map to the same region of the chromosome.

Cloning of *pmrA505*. JKS5 chromosomal DNA was isolated, and random *Sau*3AI fragments ca. 10,000 to 12,000 bp long were ligated to medium-copy-number plasmid pHSG576 (50). We used this plasmid because we suspected that the *pmrA505* gene might be involved in outer membrane synthesis and therefore its expression from a higher-copy-number vector might be lethal. The ligation mixture was transformed into *S. typhimurium* MS1868 ($r^- m^+$) by electroporation, and the resulting transformation mixture was plated onto LB plates containing Cam. Approximately 40,000 Cam^r transformants were then replica plated onto LB plates containing 20 μg of polymyxin E per ml. We obtained 64 PM^r colonies which were tested for polymyxin resistance on polymyxin E gradient plates. Forty-two of the isolates showed either an intermediate level of resistance or no resistance and were not examined further. The other 22 plasmids were isolated, combined into six pools, retransformed into *S. typhimurium* MS1868, and screened for PM^r on gradient plates. Two of the plasmids, pKR100 and pKR101, were chosen for further analysis, and a partial restriction map was determined (Fig. 2A). Note that while pKR100 and pKR101 have overlapping inserts, their orientations are opposite with respect to the vector, indicating that expression of *pmrA505* in these constructions is orientation independent.

The location of the insert from pKR100 was mapped by a dot blot method in which phage lysate DNA derived from the mapping strains of Benson and Goldman (4) was spotted onto a nylon membrane and probed with ^{32}P-labeled DNA from pKR100 (see Materials and Methods). We found that the probe hybridized to a lysate derived from strain TT15275 (Table 1), indicating that the insert DNA maps to the same region of the chromosome as *pmrA505*. Insert DNA from pKR101 was also included on the blot and cross-hybridized with the pKR100 probe. We conclude, on the basis of these mapping data and the fact that the plasmids were isolated on the basis of conferral of PM^r to *S. typhimurium*, that the DNAs in plasmids pKR100 and pKR101 contain the *pmrA505* allele.

We introduced pKR100 into strain LT2, and the resulting strain, JKS1187, was tested for PM^r by the 1-h killing assay. The plasmid conferred an eightfold increase in resistance (Fig. 3) over LT2. This is intermediate with respect to the level of resistance seen in strain JKS5 (Fig. 3). One explanation for this observed difference is the fact that strain JKS1187 contains both the *pmrA505* and *pmrA*$^+$ alleles, while strain JKS5 contains only *pmrA505*. Therefore, in strain JKS1187, the PmrA505 and PmrA$^+$ products may interfere with each other. Following this line of reasoning, the fact that *pmrA505* is present in multiple copy number and yet the increase in resistance is only 1% of the increase due to a single copy on the chromosome suggests that the wild-type gene is partially dominant over the mutant version.

TABLE 2. Mapping of *pmrA* by transduction[a]

Donor	Recipient	Selected marker	Unselected marker(s)	% Linkage[b]
JKS203 (*zjd*::Tn*10 pmrA505*)	TT15273 (*melAB*::Mud*Q*)	Tet^r	PM^r	60 (67/112)
JKS203 (*zjd*::Tn*10 pmrA505*)	TT15273 (*melAB*::Mud*Q*)	Tet^r	Cam^s	34 (38/112)
JKS203 (*zjd*::Tn*10 pmrA505*)	TT15273 (*melAB*::Mud*Q*)	Tet^r	PM^r Cam^s	13 (15/112)
TT1662 (*mel*::Tn*10*)	JKS5 (*pmrA505*)	Tet^r	PM^s	32 (18/56)
TN2409 (*oxd7*::Mud*J*)	JKS5 (*pmrA505*)	Kan^r	PM^s	13 (7/56)

[a] Element Tn*10* confers Tet^r, Mud*Q* confers Cam^r, and Mud*J* confers Kan^r.
[b] In parentheses is the number of colonies displaying the unselected marker over the total number of transductants screened.

Alternatively, expression of *pmrA505* may be altered in this plasmid construction. A combination of these effects is also possible. The presence of the pHSG576 vector alone did not influence the level of PM^r in either strain LT2 or JKS5 (data not shown). Plasmid pKR100 did not confer a detectable increase in CGE resistance on strain LT2 (data not shown). This result was not unexpected, since the relative increase in resistance to polymyxin B was so small.

Cloning of *pmrA*$^+$. We reasoned that if indeed *pmrA*$^+$ is dominant over *pmrA505*, we might be able to exploit that fact to aid in the cloning of *pmrA*$^+$. The rationale we used for this cloning was first to clone large fragments containing the Tn*10d*-Cam element from strain JKS1169 (Table 1) and then to test those clones in strain JKS5 for the ability to reduce the level of PM^r in that strain. By using this approach, we identified one plasmid (pKR200) which reduced the PM^r of strain JKS5 as judged by growth on gradient plates. We then compared the resistance of strain JKS1251 (JKS5/pKR200) with that of strain JKS5 in the 1-h killing assay and found that the presence of plasmid pKR200 completely reversed the effects of the *pmrA505* mutation on resistance to both polymyxin B (Fig. 4A) and CGE (Fig. 4B). As an additional control, we included strain JKS1252 (JKS5/pKR201), which is a transformant of strain JKS5 that carries another Cam^r isolate from the above-described cloning experiment but did not appear to affect the PM^r of strain JKS5 on gradient plates and had no effect on the PM^r of strain JKS5 in our more quantitative assay (Fig. 4). When these plasmids were introduced into strain LT2, they caused no change in the level of resistance to polymyxin B or CGE (Fig. 4), indicating that these plasmids do not have some nonspecific effect that results in an overall increase in membrane permeability to these agents.

We determined a partial restriction map of plasmid pKR200 (Fig. 2B) and found that there is a large region (ca. 9 kb) of overlapping sequences among pKR200, pKR100, and pKR101, as judged by the identical locations of restriction sites. The restriction map of plasmid pKR201 did not significantly overlap with that of plasmid pKR200, although the Tn*10d*-Cam element was present (data not shown). This result, along with our complementation data, supports the conclusion that we cloned *pmrA*$^+$.

If plasmid pKR200 does, in fact, carry *pmrA*$^+$, then it may complement other alleles. To test this, we introduced plasmids pKR200 and pKR201 into strain SH7426 (*rfaJ pmrA163*) and tested the resulting transformants for resistance to polymyxin B and CGE. The effect of these plasmids is nearly identical to that observed in strain JKS5 (Fig. 4). Strain SH9178, the isogenic *pmrA*$^+$ parent of SH7426, was included in the experiment summarized in Fig. 4B, and the data show that plasmid pKR200 reduces the level of CGE resistance in strain SH7426 to the same level as its *pmrA*$^+$ parent. Smooth LT2 derivatives are 100-fold more resistant to CGE than are *rfaJ* mutant strains, as has been noted previously (15, 37, 42, 46), while the presence of O antigen offers no protection to polymyxin B (Fig. 4A). It is also apparent from these results that the increase in resistance to both CAP and polymyxin B conferred by *pmrA* is not dependent on the presence or absence or O antigen.

Insertion analysis, subcloning, and DNA sequence analysis of pKR100. We isolated five Tn*1000* ($\gamma\ \delta$) insertion mutations in pKR100 that no longer conferred PM^r on strain LT2. Restriction analysis showed that they all mapped toward one end of the plasmid and within a 3,800-bp *Pst*I-*Eco*RI fragment (Fig. 2A). Subcloning experiments showed that this *Pst*I-*Eco*RI fragment defined the minimal region required to confer PM^r on strain LT2. A smaller, 3,600-bp *Cla*I fragment did not (Fig. 2A). Therefore, the 3,800-bp *Pst*I-*Eco*RI fragment was used as the source of DNA for sequencing.

Both strands of the *Pst*I-*Eco*RI fragment were sequenced as outlined in Materials and Methods. The results are presented in Fig. 5. Three ORFs are present in the fragment and are accompanied by potential ribosome-binding sites upstream of the initiation codon of each ORF (Fig. 5). The organization of the ORFs suggests that they constitute an operon. The stop and start codons at the junction of ORF1 and ORF2 overlap by one nucleotide, and there are only nine bases between the end of ORF2 and the start of ORF3. We could not identify an obvious consensus σ^{70} promoter sequence upstream of ORF1, although it is likely that there is a promoter in that region, since the 3,600-bp *Cla*I fragment, which does not contain the sequence upstream from ORF1 (Fig. 5), does not confer PM^r (Fig. 2). There is also a region that encodes a potential RNA secondary structure downstream of ORF3 that may serve as a factor-independent transcriptional terminator.

The deduced amino acid sequences of the three ORFs indicate that ORF1, ORF2, and ORF3 encode 547-, 222-, and 356-amino-acid proteins with corresponding molecular masses of 61,579, 25,019, and 40,237 daltons, respectively.

The precise locations of the Tn*1000* insertions were determined by DNA sequence analysis. Three of the insertions lie in ORF1, and two lie in ORF2 (Fig. 5). If one assumes that the insertions in ORF1 are polar, then it is likely that either ORF2 or ORF3 is the *pmrA* gene. We present data below that confirm that ORF2 is *pmrA*.

Deletion analysis of a *pmrA*$^+$ clone. We subcloned a 4,200-bp *Sal*I fragment from pKR200, which overlaps the *Pst*I-*Eco*RI fragment of pKR103 (Fig. 2), in both orientations into plasmids pUC18 and pHSG576. All four constructions complemented JKS5 to wild-type PM^s, as judged by growth on PM gradient plates. One of the pUC18 isolates, pKR202 (Fig. 2B), was used for further analysis. We took advantage of fortuitous restriction sites to construct the deletions shown in Fig. 2B. All three deletions retained the ability to complement JKS5 to PM^s. These results suggested the

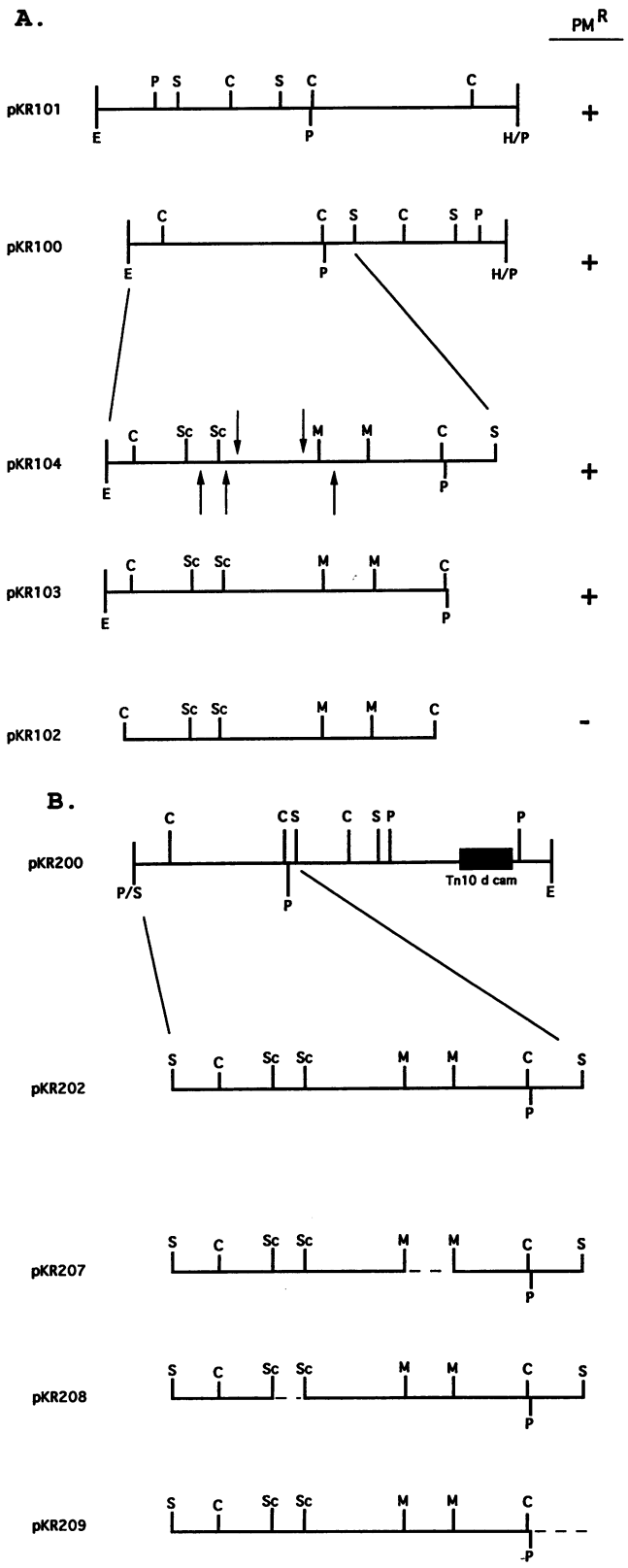

FIG. 2. Restriction map of plasmids carrying cloned *pmrA505* (A) and *pmrA*$^+$ (B) alleles. Plasmid designations are shown on the left. Restriction site abbreviations: E, *Eco*RI; P, *Pst*I; S, *Sal*I; C, *Cla*I; H, *Hin*dIII; Sc, *Sac*II; M, *Mlu*I. Restriction sites shown as lines that extend above and below the horizontal line are derived solely from the vector. (A) All plasmids are derivatives of pHSG576. Note that pKR100 and pKR101 contain overlapping inserts in opposite orientations with respect to the vector sequences. The ability to confer polymyxin resistance on *S. typhimurium* LT2, as judged by growth on gradient plates, is marked by a plus sign. A minus sign denotes wild-type sensitivity. The ca. 4,000-bp *Eco*RI-*Sal*I fragment from pKR100 is shown in an expanded view and is the insert in pKR104. The arrows indicate the locations of Tn*1000* insertions present in pKR100 that eliminate the ability to confer PM^r on strain LT2. (B) All plasmids are derivatives of pUC18, except for pKR200, which is a pGB2 derivative. The Tn*10d*-Cam element is shown as a black box. The 4,200-bp *Sal*I fragment from pKR200 is shown in an expanded view and constitutes the insert DNA in pKR202. Derivatives of pKR202 were constructed by deleting the restriction fragment indicated by the broken line.

possibility that one of the three restriction fragments present in all three constructs contained the *pmrA*$^+$ gene. We subcloned the *Sal*I-*Sac*II, *Sac*II-*Mlu*I, and *Mlu*I-*Pst*I fragments into pUC18 and screened for complementing ability. Only plasmids carrying the 1,320-bp *Sac*II-*Mlu*I insert were capable of restoring JKS5 to PM^s. This fragment contains the last 285 codons of ORF1 and the first 156 codons of ORF2. Since ORF3 is not present in this construct, it cannot encode the *pmrA* gene. The entire fragment was sequenced, and a single G-A transition was found (Fig. 5) which would result in the substitution of an Arg residue for His at position 81 of ORF2. We also sequenced the rest of ORF2 and found no other differences between the *pmrA505* and *pmrA*$^+$ DNAs. From these results, combined with the data obtained from analysis of the Tn*1000* insertions (see above), we conclude that ORF2 is the *pmrA* gene. We also designate ORF3 *pmrB* on the basis of its proximity to *pmrA* and the data presented below.

The *pmrA* locus encodes a two-component regulatory system. A data base search for the DNA sequence resulted in the finding of 78% identity between 2,678 bp of our clone (bp 1146 to 3824) and the *basRS* genes from *E. coli* (30), indicating that *pmrAB* comprises the *S. typhimurium* version of these genes. We noted that there were two insertions in our sequence, relative to *basRS*, which occurred near the end of the two sequences. Only one influenced a coding region. Because the *basRS* sequence lacked an 11-bp segment that contains the stop codon for the *pmrB* gene (bp 3598 to 3608), the BasS protein contains eight more amino acids than PmrB. The second insertion is a single C-G base pair at position 3740 of the *pmr* sequence that is not present in *basRS*. The *basRS* sequence also contained an insertion of 42 bp relative to *pmr* at position 3771. This insertion is discussed below.

We also found that the final 213 nucleotides of our sequence showed 73% identity with the coding strand sequence downstream of the *E. coli proP* gene (11), not including a gap of 42 bases that are present in *proP* (and *basRS*; see above) but are not present in our sequence. Although the *proP* gene from *S. typhimurium* has not been sequenced, we infer from this result that the *pmrA* locus is located within 300 bp of the *S. typhimurium proP* gene. This conclusion is supported by the fact that *proP* has been mapped to 93 U on the *S. typhimurium* chromosome (39). There are several interesting features of the overlap sequence. First, the sequence identity is between the noncoding strand of *pmr* and the coding strand of *proP*, indicating that *pmr* and *proP* are transcribed convergently. Second, the 42 bp missing in our sequence corresponds to one of two

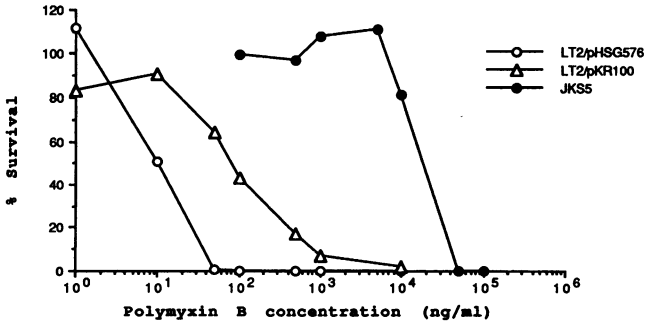

FIG. 3. Effect of pKR100 (*pmrA505*) on polymyxin B resistance in *S. typhimurium* LT2 (*pmrA*$^+$). The 50% lethal doses for LT2/pHSG576, LT2/pKR100, and JKS5 were 11, 80, and 19,000 ng/ml, respectively.

potential secondary structure regions identified by Culham et al. (11) and proposed to serve as transcriptional terminators. However, the second potential secondary structure region they identified is present our sequence (Fig. 5). The region shows 100% identity with our sequence, except for seven of the eight bases that would constitute the loop of the proposed hairpin. The fact that this structure has been conserved to the nucleotide indicates that it serves a specific function, perhaps as a bidirectional transcriptional termina-

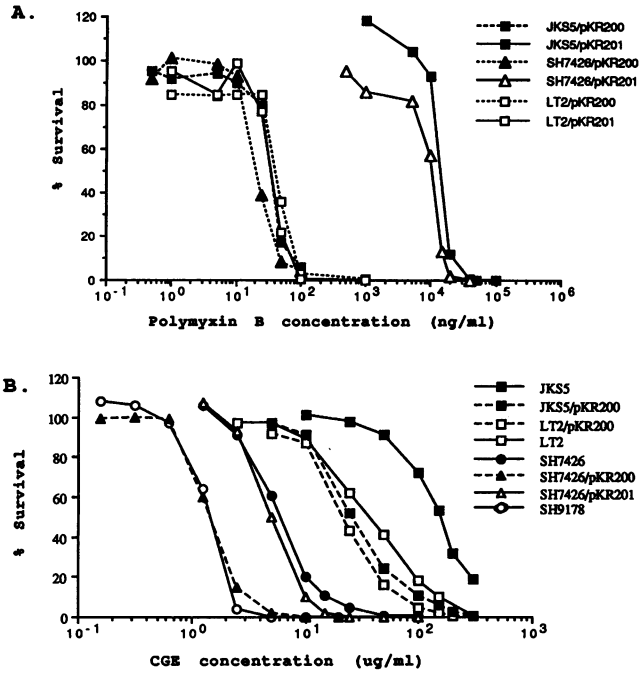

FIG. 4. Effect of a plasmid-borne copy of *pmrA*$^+$ in strains LT2 (*pmrA*$^+$), JKS5 (*pmrA505*), and SH7426 (*rfaJ pmrA163*). (A) Resistance to polymyxin B. The 50% lethal doses for strains JKS5/pKR200, JKS5/pKR201, SH7426/pKR200, SH7426/pKR201, LT2/pKR200, and LT2/pKR201 were 36, 15,000, 20, 11,000, 20, and 16 ng/ml, respectively. (B) Resistance to CGE. The 50% lethal doses for smooth strains JKS5, JKS5/pKR200, LT2/pKR200, and LT2 were 160, 27, 21, and 36 μg of CGE per ml, respectively. The 50% lethal doses for *rfaJ* strains SH9178 (*pmrA*$^+$), SH7426/pKR200, SH7426/pKR201, and SH7426 were 1.5, 1.4, 6.3, and 6.3 μg/ml, respectively.

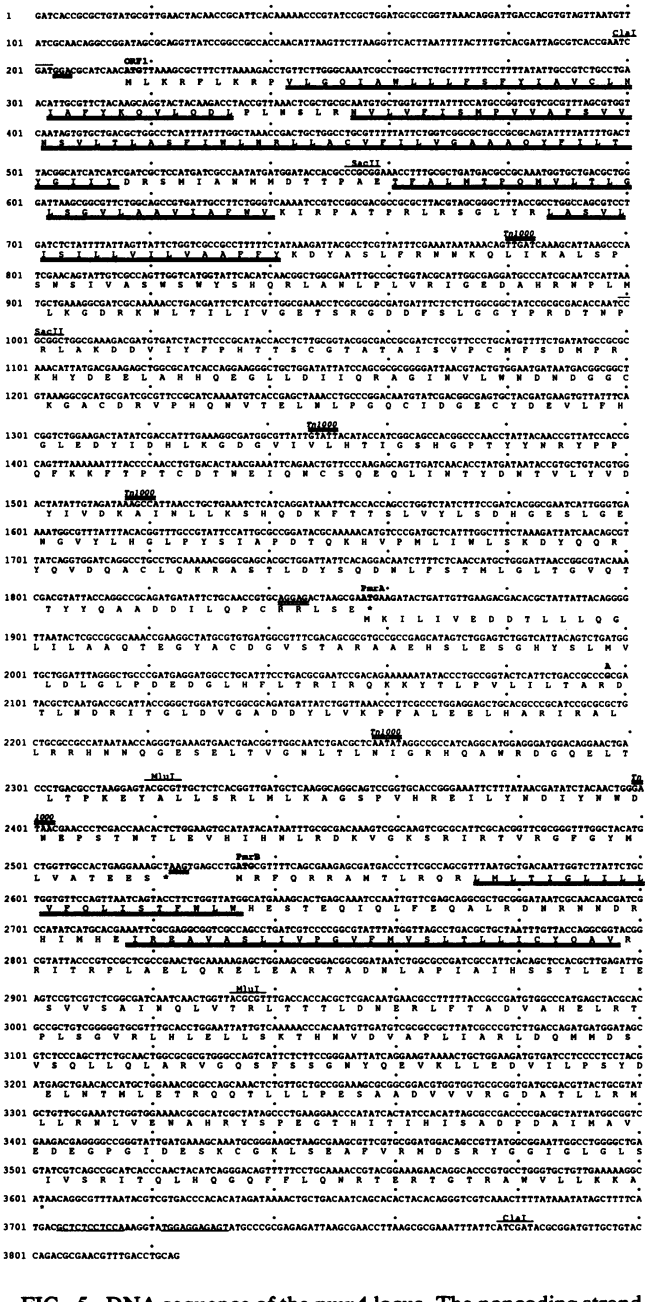

FIG. 5. DNA sequence of the *pmrA* locus. The noncoding strand of the wild-type *pmrA* gene is shown. The locations of *Cla*I, *Sac*II, and *Mlu*I restriction sites are indicated with single overlines. Double overlines indicate the locations of Tn*1000* insertions that abolish the PM^r phenotype conferred by pKR100. A double-underlined DNA sequence denotes the putative ribosome-binding site before each ORF. Putative initiation codons are shown in boldface. Hydrophobic regions that may constitute membrane-spanning domains are shown as double-underlined amino acid sequences. The G-A transition that is the basis of the *pmrA505* mutation is shown as a boldface A above the corresponding G at base 2097 of the sequence. The region of dyad symmetry conserved between the *S. typhimurium pmrA* locus and *E. coli proP* is denoted by an underline.

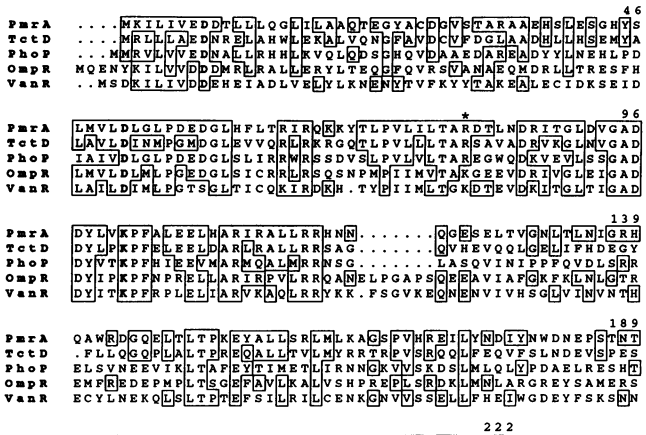

FIG. 6. Amino acid sequence homology between PmrA and regulatory proteins TctD (55), PhoP (17, 28), OmpR (10), and VanR (2). Regions of identity are boxed. The three amino acid residues that are conserved among all response regulators are in boldface (47). Gaps have been introduced to maximize the homology with PmrA. The numbers refer to amino acids of the PmrA sequence. The location of the R81H mutation in *pmrA505* is marked with an asterisk.

tor. The specific sequence of the hairpin loop is apparently not important.

Data base searches of amino acid sequences deduced from the three ORFs revealed that PmrA and PmrB show 90 and 86% identity with the amino acid sequences deduced from the *basR* and *basS* genes, respectively. The *E. coli basRS* genes were cloned in a screen that was designed to identify new two-component regulatory systems and was dependent on the ability of the cloned DNA to encode a phosphotransferase activity (30). PmrA and PmrB also show strong homology with other members of the OmpR-EnvZ family of two-component regulatory systems. The PmrA sequence shows homology with a number of response regulator proteins (Fig. 6), including TctD (55), PhoP (17, 28), OmpR (10), and VanR (2). PmrB was found to have homology with sensor-kinase components of the two-component system (Fig. 7), including PhoR (23) and EnvZ (10). The homology of each protein with its respective response regulator and sensor-kinase counterpart includes all of the conserved residues described by Stock et al. (47). A hydropathy plot (21) of the PmrB sequence indicated that like many sensor-kinase proteins, it has potential membrane-spanning domains near its amino-terminal end (Fig. 5). On the basis of these results, we conclude that the *pmrA* locus encodes a two-component regulatory system.

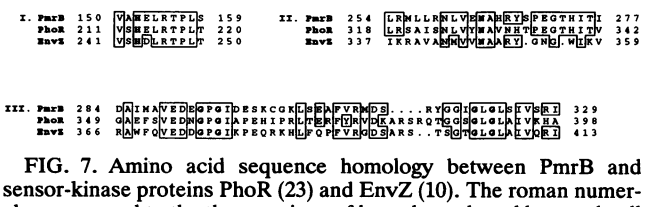

FIG. 7. Amino acid sequence homology between PmrB and sensor-kinase proteins PhoR (23) and EnvZ (10). The roman numerals correspond to the three regions of homology shared by nearly all of the sensor-kinase proteins identified by Stock et al. (47). Amino acids conserved among all sensor-kinases are in boldface. Regions of identity are boxed.

A data base search of the ORF1-encoded protein was not as informative. We found no compelling homology with any known protein. A hydropathy plot (21) of the ORF1-encoded amino acid sequence indicated that there are at least four membrane-spanning domains (Fig. 5). The best matches from data base searches were between these putative membrane domains and the membrane-spanning domains of a number of eucaryotic and viral proteins, most notably with one of the membrane domains of the band 3 anion-exchange protein (32). We infer from these results that ORF1 encodes an integral membrane protein, but the similarities were not strong enough to allow any inferences as to function.

DISCUSSION

Previously, the LPSs of several *pmrA* mutants were examined and shown to have increased levels of both 4-aminoarabinose and phosphorylethanolamine substitution in the lipid A moiety (53). One would expect different enzymes to be responsible for the addition of each of these compounds. Either two mutations, one in each of the structural genes, or a single mutation, in a locus that regulates the expression of both structural genes, would be required to facilitate this result. The genetic data presented here support the latter hypothesis, since only a single locus is involved (Table 2 and Fig. 4). DNA sequence analysis confirmed that *pmrA505* is a missense mutation in what appears to be the response regulator component of a two-component regulatory system (Fig. 5). Therefore, we suspect that the *pmrA* locus plays a role in regulating at least two genes involved in the modification of lipid A. It is also clear from our mapping data that this gene is not associated with other known genes that influence LPS structure (39).

The PhoP^c protein acts to increase the levels of acid phosphatase in rich media (18), bypassing the requirement for an environmental signal. The fact that *pmrA505* is a missense mutation in a response regulator, combined with our genetic analysis, suggests that it confers a constitutive phenotype, analogous to PhoP^c (18). The lines of evidence for this are as follows. First, the mutation does not confer a complete loss-of-function phenotype because (i) the mutant allele can confer PM^r in the presence of a wild-type allele (Fig. 3), (ii) Tn*1000* insertions in the cloned *pmrA505* allele, which should result in loss of function, abolished its ability to confer PM^r to strain LT2, and (iii) when we screened a library of 25,000 Tn*10d*-Cam insertions in strain LT2, we failed to find any insertions that resulted in PM^r (38). Second, by analogy with OmpR (51), it is likely that the carboxy terminus of PmrA contains the DNA-binding domain. Plasmid pKR207 is a derivative of pKR202 in which the 446-bp *Sac*II fragment is deleted (Fig. 2). This deletion results in a carboxy-terminal 66-amino-acid truncation of the wild-type PmrA protein, deleting at least part of the putative DNA-binding domain. The fact that this truncated protein can still complement JKS5 suggests that complementation does not require wild-type DNA-binding ability. Rather, it may be due to the formation of inactive dimers or higher-order structures. Conversely, a Tn*1000* insertion within the *Sac*II fragment of pKR100 (Fig. 2 and 5), the same fragment deleted in pKR207, abolishes the associated PM^r phenotype, suggesting that the DNA-binding domain is required for the mutant phenotype. Finally, the *pmrA* mutation correlates with an increase in the substitution of lipid A phosphates, which indicates an increase in the activity of at least two

enzymes, as described above. The simplest interpretation of this result is that the genes are up-regulated. Although inactivation of repressor function could account for this finding, it is inconsistent with the first two lines of evidence.

A number of *pmrA* mutants have been isolated (22, 52, 53). Although it has not been shown that they are allelic, they all map to the same region of the chromosome and we expect that the associated PM^r phenotype is caused by mutations in the *pmrA* gene. However, it is possible that some of them resulted from a mutation in the *pmrB* gene, by analogy with known EnvZ mutations that result in a hyperactive kinase or decreased phosphatase activity (discussed in reference 47). In any case, it appears that the PM^r phenotype requires altered expression of several genes because all of the single-step mutants isolated to date have mapped to the *pmrA* regulatory locus (22, 53). If a single gene or enzymatic activity were sufficient, then one would expect at least some of the mutations to map to a distinct structural gene.

A growing number of two-component regulatory systems have been identified (47). The common theme among them is that they sense changes in the environment and alter the expression of various genes in response to these changes. Many of these adaptive systems are directly involved in the virulence of a wide variety of organisms, including the VirG protein of *Agrobacterium tumefaciens* (26), the VanRS proteins of *Enterococcus faecium* (2), and the PhoPQ proteins of *S. typhimurium* (17, 28). In this work, we identified a mutation in what appears to be a response regulator gene that confers resistance to polymyxin B, to a CGE from human neutrophils, and to a 25-amino-acid peptide derived from the sequence of human neutrophil protein CAP37 (Fig. 1). In addition, Stinavage et al. (46) have shown that strain SH7426 (Table 1 and Fig. 4) survives longer in human neutrophils than does its parent strain, SH9178 (Table 1 and Fig. 4). Recent evidence that splenic neutrophils constitute a "safe site" for *S. typhimurium* in a murine host (13) supports the hypothesis that survival in neutrophils is an important step in infection. On the basis of these findings, we suggest that the PmrAB system plays a role in the adaptation of salmonellae to the host environment, in particular to neutrophils, and therefore it plays a role in virulence as well.

PM^r mutants of *E. coli* have been reported (27). None of the details of the genetics have been presented, but a study of the biochemistry of their lipid A suggests that the basis of their resistance is similar to *pmrA*-mediated resistance (35). Although it is not known whether an *E. coli* version of *pmrA* is responsible for this phenotype, we have found that plasmid pKR100 confers a 1,000-fold increase in PM^r to several strains of *E. coli* (38), indicating that the *S. typhimurium pmrA* gene is functional in those strains. Therefore, it is at least feasible that a *pmrA* mutation is involved, presumably in the *basR* or *basS* gene.

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