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The PmrA-Regulated pmrC Gene Mediates Phosphoethanolamine Modification of Lipid A and Polymyxin Resistance in Salmonella enterica

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The PmrA/PmrB regulatory system of Salmonella enterica controls the modification of lipid A with aminoarabinose and phosphoethanolamine. The aminoarabinose modification is required for resistance to the antibiotic polymyxin B, as mutations of the PmrA-activated pbg operon or ugd gene result in strains that lack aminoarabinose in their lipid A molecules and are more susceptible to polymyxin B. Additional PmrA-regulated genes appear to participate in polymyxin B resistance, as pbgP and ugd mutants are not as sensitive to polymyxin B as a pmrA mutant. Moreover, the role that the phosphoethanolamine modification of lipid A plays in the resistance to polymyxin B has remained unknown. Here we address both of these questions by establishing that the PmrA-activated pmrC gene encodes an inner membrane protein that is required for the incorporation of phosphoethanolamine into lipid A and for polymyxin B resistance. The PmrC protein consists of an N-terminal region with five transmembrane domains followed by a large periplasmic region harboring the putative enzymatic domain. A pbgP pmrC double mutant resembled a pmrA mutant both in its lipid A profile and in its susceptibility to polymyxin B, indicating that the PmrA-dependent modification of lipid A with aminoarabinose and phosphoethanolamine is responsible for PmrA-regulated polymyxin B resistance.

Polymyxin B is a cyclic antimicrobial lipopeptide produced by the soil bacterium Paenibacillus polymyxa (33). While the mechanism of killing of polymyxin B is not completely understood, the cationic polymyxin B is believed to bind initially to the anionic surfaces of gram-negative bacteria, in particular to the lipopolysaccharide (LPS) (46). This electrostatic interaction apparently allows polymyxin B to gain access to the bacterial inner membrane, which is its presumed target. Gram-negative bacteria that are resistant to polymyxin B possess mechanisms that modify the LPS by neutralizing its negative charge, which decreases the binding of polymyxin B (30, 37, 45). Strains that exhibit resistance to polymyxin B also display resistance to antimicrobial peptides and proteins from human neutrophils (36).

In Salmonella enterica serovar Typhimurium, polymyxin B resistance is controlled primarily by the PmrA/PmrB regulatory system (35, 44). A polymyxin B-resistant strain that expresses a constitutively active PmrA protein displays increased levels of aminoarabinose and phosphoethanolamine in the lipid A portion of the LPS (20), suggesting that these PmrA-controlled modifications are required for polymyxin B resistance. Consistent with this notion, the PmrA-activated ugd gene and pbg operon (designated pbgF) by Gunn et al. [13] and arm by Trent et al. [43] are necessary for both the biosynthesis and incorporation of aminoarabinose into lipid A (13) and for polymyxin B resistance (12, 13). Yet, pbgP and ugd mutants are not as polymyxin sensitive as a pmrA null mutant (24), indicating that an additional PmrA-regulated gene(s) is required for polymyxin B resistance. pmrA null mutants produce lipid A species that lack aminoarabinose and phosphoethanolamine, whereas strains with a block in the synthesis pathway for aminoarabinose due to mutations in the pbgP operon have increased levels of phosphoethanolamine-modified lipid A (52). While this indicates that the PmrA/PmrB system is absolutely needed for decorating lipid A with aminoarabinose and phosphoethanolamine, the PmrA-regulated determinant(s) responsible for the modification of lipid A with phosphoethanolamine and the role that such a modification plays in polymyxin resistance have remained unknown.

Transcription of PmrA-activated genes is promoted by Fe3+, which is sensed by the sensor protein PmrB (48), and by low levels of Mg2+ in a process that requires the PhoP/PhoQ regulatory system (41) and the PhoP-activated PmrD protein (24). In addition to the increased susceptibility towards polymyxin B (12), pmrA null mutants are hypersusceptible to killing by Fe3+ (2) and mildly attenuated for virulence in mice (15). The PmrA/PmrB system is encoded by the pmrCAB operon and is apparently expressed from both a PmrA-activated promoter upstream of the pmrC gene (47) and a constitutive promoter within the pmrC coding region (14, 41).

In this paper, we demonstrate that the PmrA-activated pmrC gene encodes an inner membrane protein that is required for polymyxin resistance and for the incorporation of phosphoethanolamine into lipid A. We determined that the inactivation of both the pbgP and pmrC genes results in a strain that resembles a pmrA mutant both in its susceptibility to polymyxin B and in its lipid A profile. Our results indicate that the PmrA-regulated incorporation of aminoarabinose and phosphoethanolamine...
into lipid A is responsible for PmrA-mediated polymyxin B resistance in *S. enterica*.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used for this study are listed in Table 1. All *S. enterica* serovar Typhimurium strains used for this study were derived from the wild-type strain 14028s. Phage P22-mediated transductions were performed as described previously (7). Bacteria were grown at 37°C with aeration in Luria-Bertani (LB) broth or in N minimal medium, pH 7.7 or 5.8, supplemented with 0.1% Casamino Acids, 50 mM glycerol, and 10 mM MgCl₂ (39). When necessary, antibiotics were added to the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml. *Escherichia coli* DH5α (38) was used as a host for the preparation of plasmid DNA.

**Plasmid constructions.** For construction of the single-copy plasmid pBAC108L::pmrC (pmmrC), the pmrC coding region and its own promoter and a FLAG epitope sequence (5'-GGAATTCGGTACCATGGGATCCGCATATGG-3') and the DNA sequence encoding the FLAG epitope sequence (3'-GGATCCGTCGCGTTTGTGTATTGCACATATGAATATCCTCCTAG-3') and plasmid pKD13 (6) as the template, and was used to transform a derivative of pCP20 (3), was used as a host for the preparation of plasmid DNA.

**Construction of *pmrC* mutants.** For the generation of the *pmrC1* strain, which harbors a 1.096-bp deletion of the 1.641-bp pmrC gene, a DNA fragment containing a chloramphenicol resistance cassette was PCR amplified with primers 3109 and 3179 (5'-TCAGAACTCATTGTGCATCGTCGCTGAGCTGCTTCGACGGTGTGTCGCG-3') and 14028s genomic DNA as the template (the DNA sequence encoding the FLAG epitope sequence is underlined). The amplified PCR fragment was digested with BamHI and HindIII and cloned between the BamHI and HindIII sites of plasmid pBAC108L. DNA sequencing verified that the cloned segment had the expected pmrC sequence.

For construction of the *pmrC50* and *pmrC150* strains, a DNA fragment containing a kanamycin resistance cassette was PCR amplified with primers 2635 (5'-CCAGATCATAGTACCGTTAGTTACCAAGGCAGCTACTACACAGCCGTCGTCCTGTAAGCTTTCTCGTCTTCTCGACGGTGTGTCGCG-3') and 2636 (5'-TCTGCTGAGCTGCTTACCAAGGCAGCTACTACACAGCCGTCGTCCTGTAAGCTTTCTCGTCTTCTCGACGGTGTGTCGCG-3') and plasmid pKD3 (6) as the template, and was used to transform a derivative of strain 14028s as described previously (23). The *pmrC1* strain, in which the chloramphenicol resistance cassette was removed from the *pmrC* strain by using plasmid pCP20 (3), was used as a host for plasmid pBAC108L::pmrC, or pmmrCFLAG. Strains *pmrC50* and *pmrC150* exhibited the same lipid A profile and polymyxin B susceptibility.
overnight in N minimal medium, pH 7.7, with 10 mM MgCl₂. The next day, the
harvested, washed once with PBS, and resuspended in 4 ml of PBS containing
100 (11). The statistical significance of the polymyxin B susceptibility data was analyzed by a
two-tailed Student’s t test by using Excel software. The null hypothesis was zero for no mean difference comparisons, and P values are reported for this analysis (see
Fig. 3).

Subcellular localization of PmrC protein. Inner and outer membranes were prepared as follows. A pmrC strain harboring the pppmCFLAG plasmid, which carries the pmrC gene with its own promoter and a sequence encoding a FLAG epitope at the 3′ end immediately upstream of the stop codon, was grown overnight in N minimal medium, pH 7.7, with 10 mM MgCl₂. The next day, the cells were harvested; washed three times with N minimal medium, pH 7.0, without MgCl₂; diluted 1:100 in 200 ml of N minimal medium, pH 7.7, with 10 mM MgCl₂; and grown for 4 h at 37°C with aeration. The cells were then harvested, washed once with PBS, and resuspended in 4 ml of PBS containing
100 (20) and lysozyme (100 μg/ml). After being incubated on ice for 30 min, the cells were opened by sonication. A sucrose gradient ultracentrifugation procedure (32, 49) was used, with modifications (www.cmed.ncbi.nlm.nih.gov/ methodsall.html), to isolate the inner and outer membranes. Cell debris was removed by centrifugation at 4,000 × g for 15 min, and the whole-cell lysate was loaded on top of a sucrose gradient made with 4 ml each of 60 and 70% sucrose in a Beckman Ultra-Clear centrifuge tube followed by centrifugation in an SW41 rotor at 100,000 rpm for 20 h at 10°C. Bands between 60% (upper, reddish band) and between 60 and 70% (lower, white band) sucrose corresponding to the inner and outer membranes, respectively, were collected and dialyzed overnight against PBS. Protein concentrations were determined by a modified Lowry method (1), with bovine serum albumin used as a standard protein. NADH oxidase activity, which was measured as described previously (32), was used as a marker for inner membrane purity. Inner and outer membrane preparations (20 μg of protein each) were run in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and developed by using an anti-FLAG M2 monoclonal antibody (Sigma), an anti-mouse immunoglobulin G horseradish peroxidase-linked antibody, and the ECL detection system (Amersham Biosciences).

Construction of genes encoding chimeric PmrC-LacZ and PmrC-PhoA pro-
teins. A lacZ DNA fragment missing nine codons at the 5′ end (lacZ′) (26) was PCR amplified by using E. coli MG1655 genomic DNA as a template and the following primer pairs: 4140 (5′-GGATCCTAGGCGCCTTCGTTATACAGCGGTAC-3′) and 4141 (5′-CCGGCAAGCTTTCATTCGATGATCTTCTTTACTCAATAAATTATTTTTGTCGTCATATGAATATCCTGGACGGAACGTTTAC-3′) for PmrC (CTGACG-3′) and 4144, introducing NheI (GTCGAC) and HindIII (AAGCTT) restriction sites, respectively. (Restriction sites in the primers are underlined.)

DNA fragments encoding the truncated PmrC proteins PmrC1-150, PmrC1-181, and PmrC1-295 were PCR amplified by using 14026 genomic DNA as a template and primers 2935 (5′-GGATCCTAGGCGCCTTCGTTATACAGCGGTAC-3′) and 4145 (5′-GATCCTGGACGCGGCTTCGGCTTTATTTTTGTCGTCATATGAATATCCTGGACGGAACGTTTAC-3′) for PmrC1-150, 3109 and 4147 (5′-GATCCTGGACGCGGCTTCGGCTTTATTTTTGTCGTCATATGAATATCCTGGACGGAACGTTTAC-3′) for PmrC1-181, and 3109 and 4148 (5′-GATCCTGGACGCGGCTTCGGCTTTATTTTTGTCGTCATATGAATATCCTGGACGGAACGTTTAC-3′) for PmrC1-295. The PCR-amplified DNA fragments were first digested with BamHI and PstI and cloned between the BamHI and PstI sites of plasmid pC1920 (27) to generate plasmids pPmrC150, pPmrC181, and pPmrC295. The fragments harboring the lacZ′ and phoA′ genes digested with AvrII and HindIII were cloned between the BamHI and HindIII sites of plasmids pPmrC150 and pPmrC295 to generate plasmids pPmrC150phoA′, pPmrC181phoA′, and pPmrC295phoA′. The fragments digested with NheI and HindIII were cloned between the NheI and HindIII sites of plasmid pPmrC150 to generate plasmids pPmrC150-lacZ′ and pPmrC181-lacZ′. In plasmids pPmrC150-lacZ′ and pPmrC181-lacZ′, the lacZ′ and phoA′ genes were fused in frame to pmrC right after the sequence encoding the fourth predicted transmembrane domain. In plasmids pPmrC150-lacZ′ and pPmrC181-lacZ′, the lacZ′ and phoA′ genes were fused in frame to pmrC right after the sequence encoding the fifth predicted transmembrane domain. These plasmids were transformed into a Salmonella strain with a deletion of the phoB gene, which was constructed as described previously (6), with plasmid pKD3 as the template and with primers 2945 (5′-GGATCAACTGTTTACATTTAGGTATGCTGACAGGAGGAGCTTCGCTTGTTGATCGCTAATAGCTTACAGCGGTAC-3′) and 2946 (5′-GGATCCTAGGCGCCTTCGTTATACAGCGGTAC-3′) for PmrC (CTGACG-3′). The production of PmrC-PhoA proteins was determined on LB agar plates containing 5-bromo-4-chloro-3-indolylphosphate (XP, 40 μg/ml). The production of β-galactosidase by strains expressing PmrC-LacZ proteins was determined on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 40 μg/ml).

Preparation of lipid A samples for MALDI-TOF mass spectrometry analysis. Lipid A samples were prepared as described previously (50), with a slight modification: cells were harvested from overnight cultures grown in N minimal medium, pH 7.7, with 10 mM MgCl₂; washed three times with N minimal medium, pH 7.0, without MgCl₂; and diluted 1:100 in 200 ml of N minimal medium, pH 7.7, with 10 mM MgCl₂. After growth for 4 h at 37°C with aeration, the cells were harvested, washed once with PBS, and resuspended in 300 μl of Tris-Regent (Molecular Research Center) for the amount of cells harvested from 30 ml of culture at an OD₆₀₀ of ~0.4. After an incubation for 20 min at room temperature, 30 μl of chloroform was added, and the samples were vortexed vigorously and incubated for 15 min at room temperature. The phases were separated by centrifugation at 12,000 × g for 10 min, and the upper phase was transferred to a new tube. One hundred microliters of water was added to the lower phase, vortexed, incubated for 15 min, and centrifuged at 12,000 × g for 10 min. The upper phase was combined with the upper phase recovered as described above. This extraction was performed twice. The combined upper phases were dried in a speed-vac apparatus (model RC10.22; Jouan, Winchester, Va.) and dissolved in 100 μl of water and 500 μl of 1 M trifluoroacetic acid (made by mixing 500 μl of 4 M HCl with 20 μl of 95% ethanol). The pellet was harvested by centrifugation at 2,060 × g for 10 min, washed with 500 μl of 95% ethanol, and centrifuged again at 2,060 × g for 10 min. The washing steps were repeated to completely remove SDS. The pellet was dried at room temperature for 5 min, and lipid A was dissolved by the addition of 100 μl of chloroform and methanol (1:1). Then, 25 μl of chloroform and 10 μl of 1% (w/v) of 2,5-dihydroxybenzoic acid were added to the 100 μl of lipid A sample. The 100 μl was removed. The 25 μl of 2,5-dihydroxybenzoic acid at 10 μg/ml in 70% acetonitrile–0.1% trifluoroacetic acid was used as a matrix.
The final mass spectra were obtained from an average of 5 to 10 spectra, and each spectrum was a collection from 200 laser shots.

### RESULTS

The *Salmonella* PmrC protein exhibits sequence identity with *Neisseria* proteins implicated in the incorporation of phosphoethanolamine into LPS. To identify *Salmonella* genes responsible for the phosphoethanolamine modification of lipid A, we conducted a BLAST search of the *Salmonella* genome by using as the query the amino acid sequence of the *Neisseria meningitidis* Lpt-3 protein, which had been implicated in the phosphoethanolamine modification of the heptose residue in the core oligosaccharide portion of the LPS (28). We recovered four open reading frames (PmrC, YbiP, YhjW, and YijP) (Table 2) and decided to focus on the PmrC protein because it is encoded in the PmrA-dependent *pmrCAB* operon (41) and because we were interested in phosphoethanolamine modifications that are regulated by PmrA. We then used the amino acid sequence of the PmrC protein to query the *Neisseria* genome and obtained three genes: the expected NMB2010 gene (*pmrC*), NMB0415, which appears to be a pseudogene, *lptA* (5), which has been shown to be required for the incorporation of phosphoethanolamine into the lipid A moiety of the LPS (5). The *Salmonella* PmrC protein exhibited the highest identity with the NMB1638 gene product (42% identity and 60% similarity). However, the regions of sequence identity and similarity were not evenly distributed: these proteins were 48% identical (65% similar) in the C-terminal 340 amino acids. This analysis suggested that the PmrA-regulated *pmrC* gene might be involved in the phosphoethanolamine modification of the LPS.

**Construction of a nonpolar pmrC mutant.** To examine the function of the *pmrC* gene, we constructed a nonpolar *pmrC* mutant that lacked 1,096 of the 1,641 bp of the *pmrC* gene (Fig. 1A). This mutant retained 185 bp at the 5' end of the *pmrC* coding region as well as the last 360 bp of the *pmrC* open reading frame. The latter region contains a putative promoter that apparently directs the constitutive expression of the downstream *pmrA* and *pmrB* genes (13) (Fig. 1A). The generated mutation (designated ΔpmrC1) was not polar on the *pmrA* and *pmrB* genes because the same levels of transcription of the PmrA-activated *pbgP* gene were displayed by isogenic wild-type and ΔpmrC1 strains (Fig. 1B). Moreover, a PmrC-FLAG protein that was expressed from the *pmrC* promoter carried by a single-copy-number plasmid exhibited normal regulation in the ΔpmrC1.1 mutant: the protein was produced when bacteria were grown in low, but not high, levels of Mg\(^{2+}\) (Fig. 1C). The behavior of the ΔpmrC1 mutant contrasted with that exhibited by a strain with a deletion of the complete *pmrC* coding region (ΔpmrC2) (Fig. 1A), which showed levels of *pbgP* transcription that were 10 times lower than those displayed by the wild-type strain (Fig. 1B). These results support the notion that there is a promoter within the *pmrC* coding region that promotes the transcription of the downstream *pmrA* and *pmrB* genes. Furthermore, they indicate that the generated ΔpmrC1 and

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### FIG. 1

(A) Schematic representation of the *pmrCAB* operon in wild-type *Salmonella* and in mutants with a partial (ΔpmrC1 and ΔpmrC1.1) or complete (ΔpmrC2) deletion of the *pmrC* open reading frame. (B) β-Galactosidase activity (in Miller units) expressed by strains harboring a chromosomal lac transcriptional fusion to the PmrA-activated *pbgP* gene that were grown logarithmically in N-minimal medium, pH 5.8, with 10 μM MgCl\(_2\). Transcription was investigated in wild-type (14028Δ, ΔpmrC1 (EG13927), and ΔpmrC2 (EG13633) genetic backgrounds. Data correspond to mean values from three independent sets of experiments performed in duplicate. (C) Western blot analysis of cell extracts prepared from the ΔpmrC1.1 mutant (EG14592) containing the pmmrCFLAG plasmid, which expresses the pmrCFLAG gene from its own promoter, after logarithmic growth in N-minimal medium, pH 7.7, with 10 μM (L) or 10 mM (H) MgCl\(_2\). The total protein from equal amounts of bacterial cells, as adjusted by the OD\(_{600}\), was run in an SDS-10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and developed by using anti-FLAG antibodies. The ΔpmrC1.1 mutant displays normal PmrA regulation, as the PmrC-FLAG protein is produced by bacteria grown in a low Mg\(^{2+}\) concentration but is not detected when bacteria are grown in a high Mg\(^{2+}\) concentration.
ΔpmrC1.1 mutations do not affect the expression of the pmrA and pmrB genes, and this allowed us to examine the phenotypes resulting from the absence of a functional pmrC gene.

Mutation of the pmrC gene results in lipid A that lacks phosphoethanolamine. To examine whether the pmrC gene is necessary for the incorporation of phosphoethanolamine into lipid A, we used negative-ion-mode MALDI-TOF mass spectrometry to analyze the lipid A species from wild-type pbgP, ΔpmrC1.1, and pmrC strains and strains grown at a low pH and with a low level of Mg2+, which are conditions that promote the transcription of PmrA-activated genes (41). Because the chemical structures and m/z values for most of the lipid A species in S. enterica had been previously assigned (16, 51-53), we focused on the differences in lipid A profiles between wild-type and mutant strains, putting particular emphasis on the representative molecular ions ([M − H]−) of lipid A species modified with phosphoethanolamine or aminoorabinose, which are governed by the PmrA/PmrB system.

A molecular ion ([M − H]−) at m/z 1,796 was considered to be the prototype lipid A, a hexa-acylated lipid A 1,4-bisphosphate (i.e., diphosphorylated lipid A) (Fig. 2A). The m/z values corresponding to phosphoethanolamine-modified lipid A molecular ions ([M − H]−) are as follows: m/z 1,919, a diphosphorylated lipid A (m/z 1,796) bearing a phosphoethanolamine of 124 average mass units (amu) at the 1 or 4 hydroxyl phosphate of lipid A; m/z 1,935, a hydroxylated form of m/z 1,919; m/z 2,157, a palmitoylated form of m/z 1,919; and m/z 2,173, a palmitoylated form of m/z 1,935. The molecular ions ([M − H]−) of lipid A species modified with aminoorabinose are as follows: m/z 1,928, a diphosphorylated lipid A (m/z 1,796) bearing an aminoorabinose (132 amu) at the 1 or 4 hydroxyl phosphate of lipid A; m/z 1,944, a hydroxylated form of m/z 1,928; m/z 2,166, a palmitoylated form of m/z 1,928; and m/z 2,182, a hydroxylated form of m/z 2,166. m/z 1,812 represents a hydroxylated form of the prototype lipid A (m/z 1,797), and m/z 2,035 and 2,051 represent a diphosphorylated lipid A molecular ion ([M − H]−) bearing a palmitate group and a hydroxyl group, respectively (Fig. 2A). The latter modifications are known to be regulated by the PhoP/PhoQ system (10, 16, 17), which is activated under the low-Mg2+ conditions used to grow the organisms (39), and were used as internal controls for our lipid A analyses.

The ΔpmrC1.1 mutant lacked peaks at m/z 1,919, 1,935, 2,157, and 2,173, which correspond to phosphoethanolamine-modified lipid A species (Fig. 2C). On the other hand, this mutant retained molecular ions corresponding to lipid A species modified with aminoorabinose at m/z 1,928, 1,944, 2,166, and 2,182 (Fig. 2C), which, as expected (14), were absent from the pbgP mutant (Fig. 2B). The lipid A profile of the ΔpmrC1.1 mutant was solely due to the absence of the pmrC gene function, as the phosphoethanolamine-modified lipid A molecular ions (peaks at m/z 1,919, 1,935, 2,157, and 1,935) were present in the lipid A species of a ΔpmrC1.1 strain harboring a plasmid with a wild-type copy of the pmrC gene (Fig. 2D), but not in a ΔpmrC1.1 strain with a vector control (Fig. 2E). These results demonstrate that the pmrC gene is required for the incorporation of phosphoethanolamine into lipid A.

The pmrC gene is required for resistance to polymyxin B. We determined that the ΔpmrC1.1 mutant was three- to five-fold more sensitive to polymyxin B than was the wild-type strain (Fig. 3A). This phenotype was due to the lack of the pmrC gene function, as a plasmid carrying a wild-type copy of the pmrC gene restored wild-type levels of polymyxin B resistance to the ΔpmrC1.1 mutant (Fig. 3A). Moreover, the ΔpmrC1.1 mutation decreased polymyxin B resistance even in the polymyxin-resistant pmrA505 strain (Fig. 3C), which expresses PmrA-regulated genes even under noninducing conditions (24). Because the ΔpmrC1.1 strain lacked phosphoethanolamine but retained aminoorabinose in lipid A (Fig. 2C), these results demonstrate that the ability to modify lipid A with phosphoethanolamine is necessary for polymyxin B resistance.
A mutant defective in both the 

\[ \text{pmgP} \] and \[ \text{pmrC} \] genes has the same lipid A profile and susceptibility to polymyxin B as a \[ \text{pmrA null mutant} \]. When grown under low-\( \text{Mg}^{2+} \) and mildly acidic conditions, mutants defective in the \[ \text{pmgP} \] or \[ \text{pmrC} \] genes are more sensitive to polymyxin B than the wild-type strain but are not quite as sensitive as a \[ \text{pmrA null mutant} \] (Fig. 3B) (24). On the other hand, a \[ \text{pmgP} \Delta \text{pmrC1} \] double mutant displayed the same level of polymyxin B susceptibility as a \[ \text{pmrA null mutant} \] (Fig. 3B). Consistent with this result, the inactivation of both the \[ \text{pmrC} \] and \[ \text{pmgP} \] genes in the polymyxin B-resistant \[ \text{pmrA505} \] genetic background reduced polymyxin B resistance to the levels of the \[ \text{pmrA null mutant} \] (Fig. 3C). These results indicate that the \[ \text{pmgP} \] operon and the \[ \text{pmrC} \] genes are solely responsible for PmrA-regulated polymyxin B resistance. (This is in addition to the \[ \text{ugd} \] gene, which exhibits a similar susceptibility phenotype as the \[ \text{pmgP} \] mutant, consistent with these loci encoding proteins mediating the biosynthesis of aminoarabinose.)

To further explore the association between polymyxin B resistance and lipid A modifications, we examined the lipid A profiles of \[ \text{pmrC} \], \[ \text{pmgP} \Delta \text{pmrC1} \], \[ \text{pmrA505} \], and \[ \text{pmrA505} \text{pmgP} \Delta \text{pmrC1} \] strains. The lipid A from the \[ \text{pmrA} \] mutant lacked molecular ions (\( \text{[M} - \text{H}^- \) ) corresponding to those modified with either phosphoethanolamine (peaks at \( \text{m/z} 1,919 \), 1,935, 2,153, and 2,173) or aminoarabinose (peaks at \( \text{m/z} 1,928 \), 1,944, 2,166, and 2,182) (Fig. 4D), which was consistent with previous reports (52). Likewise, inactivation of both the \[ \text{pmgP} \] and \[ \text{pmrC} \] genes in either a \[ \text{pmrA}^{-} \] (Fig. 4C) or \[ \text{pmrA505} \] (Fig. 4B) background resulted in a strain with the same lipid A profile as that exhibited by the \[ \text{pmrA null mutant} \] (Fig. 4D), which lacks the modifications displayed by the \[ \text{pmrA505} \] strain (Fig. 4A). Taken together with the results of the polymyxin susceptibility assays (Fig. 3), this analysis indicates that PmrA-controlled polymyxin B resistance is mediated by the aminoarabinose and phosphoethanolamine modifications of lipid A.

The \[ \text{pmrC} \] gene is dispensable for resistance to \( \text{Fe}^{3+} \). The \[ \text{pmrA} \] mutant exhibits hypersusceptibility to killing by \( \text{Fe}^{3+} \), but the targets of PmrA regulation that are resistant to \( \text{Fe}^{3+} \) resistance have remained unknown (48). Thus, we tested the \[ \text{pmrC1} \] and \[ \text{pmgP} \Delta \text{pmrC1} \] mutants for \( \text{Fe}^{3+} \) sensitivity and found that they retained wild-type levels of resistance to \( \text{Fe}^{3+} \) (data not shown), suggesting that the \[ \text{pmrC} \] gene is not required for this property.

\[ \text{PmrC} \] is an inner membrane protein with a large periplasmic domain. The PSORT-B subcellular localization program
that amino acids 1 to 291 mediate membrane association and that the remaining C-terminal region of the PmrC protein is in the cytoplasm.

To investigate the topology of the PmrC protein, we evaluated the β-galactosidase and alkaline phosphatase activities of a Salmonella strain with a deletion of the phoN gene and harboring plasmids with in-frame lacZ or phoA fusions to the 3' end of the pmrC gene truncated at different positions. These fusions were predicted to generate chimeric proteins with LacZ or PhoA immediately after the predicted fourth, fifth, and sixth transmembrane domains of PmrC (Fig. 5C). (The use a phoN mutant facilitated the detection of alkaline phosphatase activity, which can be obscured by phoN-encoded nonspecific acid phosphatase.) We detected alkaline phosphatase activity in the strains expressing the PhoA chimera harboring the N-terminal 181 and 295 residues of PmrC but not in that expressing a chimera harboring the N-terminal 150 residues (Fig. 5C). Consistent with these results, the strains expressing the LacZ chimera harboring the N-terminal 181 and 295 residues of PmrC produced no β-galactosidase activity, whereas the strain with LacZ fused to the N-terminal 150 residues did (Fig. 5C). These results suggest that the PmrC protein harbors five transmembrane domains that are followed by a large periplasmic region.

**DISCUSSION**

The PmrA/PmrB two-component regulatory system has been implicated in the modification of the 1 and 4' positions of lipid A with aminoarabinose and phosphoethanolamine (52). The synthesis of aminoarabinose is mediated by the PmrA-activated ugd gene and pbp operon (43), which are necessary for resistance to polymyxin B. We have now established that the PmrA-activated pmrC gene is necessary for the phosphoethanolamine modification of lipid A (Fig. 2) and for resistance to polymyxin B (Fig. 3).

An analysis of the PmrC protein by the TMPred program (www.ch.embnet.org/software/TMPRED_form.html) suggested the presence of five to six transmembrane domains in the N-terminal region that could mediate membrane association (Fig. 5B) and of a C-terminal region that could be responsible for the predicted phosphoethanolamine transferase activity. The five-transmembrane-domain model predicts that amino acids 1 to 176 mediate membrane association and that the C-terminal 371 amino acids are located in the periplasm. On the other hand, the six-transmembrane-domain model predicts that amino acids 1 to 291 mediate membrane association and that the remaining C-terminal region of the PmrC protein is in the cytoplasm.

FIG. 4. Lipid A species profiles for the pmrA505 (EG9492) (A), pmrA505 pbp ΔpmrA1.1 (EG14369) (B), pbp ΔpmrA1 (EG14372) (C), and pmrA (EG7139) (D) strains grown to logarithmic phase in N-minimal medium, pH 5.8, with 10 μM MgCl₂, and analyzed by negative-ion-mode MALDI-TOF mass spectrometry. These profiles show that the pmrC promoter. The PmrC protein localized to the inner membrane (Fig. 5A), which makes physiological sense because that is where the largest pool of phosphatidylethanolamine in the bacterial cell is located, and phosphatidylethanolamine is the donor of phosphoethanolamine in E. coli (19) and Salmonella (Yixin Shi and Eduardo A. Groisman, unpublished results).

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lamine transferase that uses phosphatidylethanolamine as a donor of phosphoethanolamine.

The PmrC protein localizes to the inner membrane (Fig. 5A) and appears to have two distinct domains. The N-terminal 176-amino-acid domain harbors several stretches of hydrophobic amino acids that may constitute transmembrane segments (Fig. 5B) and likely mediates the membrane association of the PmrC protein. The alkaline phosphatase and β-galactosidase activities displayed by strains expressing different chimeric PmrC proteins suggest that the C-terminal 370-amino-acid domain is present in the periplasmic side of the inner membrane (Fig. 5C). The location and topology of the PmrC protein would allow it to catalyze the incorporation of phosphoethanolamine into lipid A by using phosphatidylethanolamine (19), the most abundant phospholipid in E. coli (34) and Salmonella (Shi and Groisman, unpublished results), as a substrate.

Mutants in the regulatory protein PmrA that are resistant to polymyxin B exhibit increased levels of aminoarabinose and phosphoethanolamine in lipid A (20). We have now established that both of these PmrA-controlled modifications are
required for polymyxin B resistance, as a pbgP ΔpmrC1 double mutant is as susceptible to polymyxin B as a pmrA null mutant (Fig. 3B) and has a lipid A profile that is identical to that of a pmrA null mutant, lacking both aminoarabinose and phosphoethanolamine (Fig. 4). This is true even when the pbgP and pmrC genes are mutated in the hyperactive pmrA505 genetic background (Fig. 3C and 4). While the pmrA null mutant is ~10,000-fold more susceptible to polymyxin B than the wild-type strain, we were surprised to find that this is more than the sum of the susceptibilities displayed by mutants defective in pbgP or pmrC (Fig. 3B). This suggests that when Salmonella lacks the ability to perform a particular type of lipid A modification, a different type of modification may be enhanced. Indeed, phosphoethanolamine-modified lipid A accumulates to higher levels in a pbgP (pmrF) mutant of E. coli than in the wild-type strain (52). Taken together, these results establish that the PmrA-controlled phosphoethanolamine modification of lipid A is essential for full resistance to polymyxin B.

It has been hypothesized that two promoters mediate the transcription of the pmrA and pmrB genes: a PmrA-activated promoter located upstream of the pmrC gene in the pmrCAB operon and a constitutive promoter located within the pmrC open reading frame. Whereas the PmrA-regulated promoter has been defined by S1 mapping experiments (47), evidence for the constitutive promoter is based on the ability of a 346-bp fragment from the pmrC coding region to promote transcription from a plasmid-linked promoterless reporter gene (14) and the fact that pmrC-lac fusions generated with the MudJ transposon near the 3′ end, but within the pmrC coding region, exhibit normal PmrA-dependent transcription (41). We have now provided genetic evidence for the presence of a promoter within the pmrC gene by establishing that the deletion of the complete pmrC open reading frame abolished PmrA-mediated transcription, whereas a strain retaining 360 bp at the 3′ of the pmrC gene exhibited normal PmrA-controlled transcription (Fig. 1B). As described for the PhoP/PhoQ two-component regulatory system (42), this constitutive promoter may provide the basal levels of PmrA and PmrB proteins that are required in order to respond to environmental changes.

Finally, the availability of strains that are specifically defective in the phosphoethanolamine modification of lipid A makes it possible to examine the role that this modification plays in resistance to other antimicrobial peptides and in potential interference with signaling by host cells.

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