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 \textit{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 in the PmrA-activated \textit{pbg} operon or \textit{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 \textit{pbgP} and \textit{ugd} mutants are not as sensitive to polymyxin B as a \textit{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 \textit{pmrC} gene encodes an inner membrane protein that is required for the incorporation of phosphoethanolamine into lipid A and for polymyxin B resistance. The \textit{pmrC} protein consists of an N-terminal region with five transmembrane domains followed by a large periplasmic region harboring the putative enzymatic domain. A \textit{pbgP pmrC} double mutant resembled a \textit{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 \textit{Paenibacillus polymyxa} (33). While the mechanism of killing of polymyxin B is not completely understood, the cationic polypeptide 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 \textit{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 aminoarabinosylated 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 \textit{ugd} gene and \textit{pbg operon} (designated \textit{pmrF} by Gunn et al. [13] and \textit{arn} by Trent et al. [43]) are necessary for both the biosynthesis and incorporation of aminoarabinosylated phosphoethanolamine into lipid A (13) and in polymyxin B resistance (12, 13). Yet, \textit{pbgP} and \textit{ugd} mutants are not as polymyxin sensitive as a \textit{pmrA} null mutant (24), indicating that an additional PmrA-regulated gene(s) is required for polymyxin B resistance. \textit{pmrA} null mutants produce lipid A species that lack aminoarabinosylated phosphoethanolamine, whereas strains with a block in the synthesis pathway for aminoarabinose due to mutations in the \textit{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 aminoarabinosylated 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 Fe$^{3+}$, which is sensed by the sensor protein PmrB (48), and by low levels of Mg$^{2+}$ 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), \textit{pmrA} null mutants are hypersusceptible to killing by Fe$^{3+}$ (2) and mildly attenuated for virulence in mice (15). The PmrA/PmrB system is encoded by the \textit{pmrCAB} operon and is apparently expressed from both a PmrA-activated promoter upstream of the \textit{pmrC} gene (47) and a constitutive promoter within the \textit{pmrC} coding region (14, 41).

In this paper, we demonstrate that the PmrA-activated \textit{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 \textit{pbgP} and \textit{pmrC} genes results in a strain that resembles a \textit{pmrA} mutant both in its susceptibility to polymyxin B and in its lipid A profile. Our results indicate that the PmrA-regulated incorporation of aminoarabinosylated phosphoethanolamine

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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, 38 mM glycerol, and 100 µg/ml kanamycin. Phage P22-mediated transductions were performed as described previously (7).

**Plasmids.** For construction of the single-copy plasmid pBAC108L, a 4.5-kb DNA fragment containing ampC, o r using plasmid pCP20 (3), was used as a host for plasmid pBAC108L.

**Construction of pmrC mutants.** For the construction of the *pmrA*::Kmr 41 strain, in which the *pmrC1* gene was disrupted, pKD3 was used as a marker. A DNA fragment containing a chloramphenicol resistance cassette was PCR-amplified with primers 2807 and 3179. A DNA fragment containing a chloramphenicol resistance cassette was PCR-amplified with primers 2807 and 3179.

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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; and grown for 4 h at 37°C with aeration to an optical density at 600 nm (OD600) of 0.3 to 0.4 and were then diluted 1:100 in LB broth. Fifty microliters of the diluted bacterial culture was mixed with 50 µl of polymyxin B dissolved in a phosphate-buffered saline (PBS) solution and was placed in a 96-well plate (Cell Culture Cluster; Costar). After 1 h of incubation at 37°C with aeration, cultures were serially diluted in PBS and plated onto LB agar plates to determine the number of CFU after an overnight incubation. The percent survival was calculated as follows: (CFU of polymyxin B-treated culture/CFU of PBS-treated culture) × 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 pmpR::CFLAG 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; and diluted 1:200 in 300 ml of N minimal medium, pH 7.7, with 10 µM 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 sucrose (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 (Molecular Research Center) for the amount of cells harvested from 30 ml of overnight culture grown in N minimal medium, pH 7.7, with 10 mM MgCl2. After 1 h of incubation at 37°C with aeration, cultures were serially diluted in PBS and plated onto LB agar plates to determine the number of CFU after an overnight incubation. The percent survival was calculated as follows: (CFU of polymyxin B-treated culture/CFU of PBS-treated culture) × 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).

Construction of genes encoding chimeric PmrC::LacZ and PmrC::PhoA proteins. 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'-GATCCCTAAGGCGCCTGTTTACAAAGCT CGTCAG-3') and 4141 (5'-CCGGAACGTGTTATTTTGCAACAGCACA ACTG-3'), introducing AvrII (CTTGAAG) and HindIII (AAGCTT) restriction sites, respectively, and 4142 (5'-GATCCCTAAGGCGCCTGTTTACAAAGCT CGTCAG-3') and 4141, introducing Nhel (GTCAG) and HindIII (AAGCTT) restriction sites, respectively. (Restriction sites in the primers are underlined.) PhoA genes were fused in frame to pmrC right after the sequence encoding the fourth predicted transmembrane domain. In plasmids pPmrC181-lacZ, pPmrC181-phoA, pPmrC295-lacZ, and pPmrC295-phoA, the lacZ' and phoA' genes were fused in frame in pmrC right after the sequence encoding the sixth 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 pKD4 as the template and with primers 2935 (5'-GGTTACAATGTTTTATTTTGCAACCAGG GAGGTTTGTTGTGATCAACTGCTCTTGGGAAAC 3') and 2936 (5'-GGTTGTTTGATCAACTGCTCTTGGGAAAC 3') by a modification of published methods (27). The fragments digested with NheI and HindIII were verified by Southern blot hybridization and/or PCR analysis.

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 LB broth. The next day, the cells were harvested; washed three times with 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; and diluted 1:100 in 200 to 300 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 one time with PBS, and resuspended in 4 ml of PBS containing sucrose (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.cmdr.ucb.ca/bobh/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 for 24 h at 40,000 rpm for 24 h. Bands between the 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 integrity. 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).

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 N minimal medium, pH 7.7, with 10 µM 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 Tri-Reagent (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 experiment was performed twice. The combined upper phases were dried in a speed-vac apparatus (model RC10/22, Jouan, Winchester, Va.) and dissolved in 500 µl of hydrolys buffer, pH 4.5, containing 12.5 mM sodium acetate and 1% SDS. For the release of lipid A from the LPS, samples were boiled for 1 h at 100°C, dried in a speed-vac, and resuspended in a mixture of 100 µl of water and 500 µl of acidic ethanol (made by mixing 100 µl of 4 M HCl with 20 ml of 95% ethanol). The pellet was harvested by centrifugation at 2,000 × 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) and was dried under nitrogen.

Lipid A samples were then dissolved in 100 µl of water and analyzed by ESI-MS, including matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. MALDI-TOF mass spectrometry analyses of lipid A were performed with the negative-ion mode of a Voyager DE STR mass spectrometer (PerSeptive Biosystems, Framingham, Mass.) equipped with a 337-nm nitrogen laser with delayed laser. Analyses were carried out in the reflector mode at a mass range of m/z 1,500 to 3,000, with an accelerating voltage of 20 kV and a delay of 100 ns. The reflector mass instrument was operated with a delay time of 40 ns, an accelerating voltage of 20 kV, and a pulse duration of 30 ps. A laser power of 100 mW was used to avoid saturation of the detector. 2,5-Dihydroxybenzoic acid at 10 µg/ml in 70% acetonitrile–0.1% trifluoroacetic acid was used as a matrix.
TABLE 2. Salmonella open reading frames exhibiting sequence similarity to the lpt-3 gene product of N. meningitidis MC58

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene name</th>
<th>% Amino acid identity (no. with identity/total)</th>
<th>% Amino acid similarity (no. with similarity total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STM3635</td>
<td>yhiW</td>
<td>24 (137/562)</td>
<td>41 (236/562)</td>
</tr>
<tr>
<td>STM4293</td>
<td>pmrC</td>
<td>25 (71/279)</td>
<td>43 (122/279)</td>
</tr>
<tr>
<td>STM4118</td>
<td>yijP</td>
<td>23 (64/272)</td>
<td>38 (104/272)</td>
</tr>
<tr>
<td>STM0834</td>
<td>yhiP</td>
<td>26 (61/260)</td>
<td>41 (107/260)</td>
</tr>
</tbody>
</table>

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 phosphoethanolamine modification of 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, YhiW, 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 (lpt-3), NMB0415, which appears to be a pseudogene, and NMB1638 (lptA), 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 residues but only 30% identical (53% similar) in the N-terminal 176 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 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 appears directly the constitutive expression of the downstream pmrA and pmrB genes (13, 41) (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 pgbP 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 Mg²⁺ (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 pgbP 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

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 pgbP gene that were grown logarithmically in N-minimal medium, pH 5.8, with 10 μM MgCl₂. Transcription was investigated in wild-type (14028s), ΔpmrC1 (EG13927), and ΔpmrC2 (EG13633) genetic backgrounds. Data correspond to mean values from three independent sets of experiments performed in duplicate. Transcription of the PmrA-activated pgbP gene was similar in the wild-type and ΔpmrC1 strains, but it was decreased in the ΔpmrC2 mutant. (C) Western blot analysis of cell extracts prepared from the ΔpmrC1.1 mutant (EG14592) containing the pmrCFLAG 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) MgCl₂. The total protein from equal amounts of bacterial cells, as adjusted by the OD₆₀₀, 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 Mg²⁺ concentration but is not detected when bacteria are grown in a high Mg²⁺ concentration.
DeltapmrC1.1 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, DeltapmrC1.1, and pmrC4 strains and strains grown at a low pH and with a low level of Mg$^{2+}$, 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 aminoarabinose, 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 1,4,1,944, a hydroxylated form of palmitoylated form of aminoarabinose (132 amu) at the 1 or 4 position (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 position 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 aminoarabinose are as follows: m/z 1,928, a diphosphorylated lipid A (m/z 1,796) bearing an aminoarabinosyl (132 amu) at the 1 or 4 position 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-Mg$^{2+}$ conditions used to grow the organisms (39), and were used as internal controls for our lipid A analyses.

The DeltapmrC1.1 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 aminoarabinosyl 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 DeltapmrC1.1 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 DeltapmrC1.1 strain harboring a plasmid with a wild-type copy of the pmrC gene (Fig. 2D), but not in a DeltapmrC1.1 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 DeltapmrC1.1 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 DeltapmrC1.1 mutant (Fig. 3A). Moreover, the DeltapmrC1.1 mutation decreased polymyxin B resistance even in the polymyxin-resistant pmaA505 strain (Fig. 3C), which expresses PmrA-regulated genes even under noninducing conditions (24). Because the DeltapmrC1.1 strain lacked phosphoethanolamine but retained aminoarabinosyl 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 *pbgP* and *pmrC* genes has the same lipid A profile and susceptibility to polymyxin B as a *pmrA* null mutant. When grown under low-Mg$^{2+}$ and mildly acidic conditions, mutants defective in the *pbgP* or *pmrC* genes are more sensitive to polymyxin B than the wild-type strain but are not quite as sensitive as a *pmrA* null mutant (Fig. 3B) (24). On the other hand, a *pbgP* Δ*pmrC*1 double mutant displayed the same level of polymyxin B susceptibility as a *pmrA* null mutant (Fig. 3B). Consistent with this result, the inactivation of both the *pmrC* and *pbgP* genes in the polymyxin B-resistant *pmrA*505 genetic background reduced polymyxin B resistance to the levels of the *pmrA* null mutant (Fig. 3C). These results indicate that the *pbg* operon and the *pmrC* genes are solely responsible for PmrA-regulated polymyxin B resistance. (This is in addition to the *ugd* gene, which exhibits a similar susceptibility phenotype as the *pbgP* mutant, consistent with these loci encoding proteins mediating the biosynthesis of aminoaabinose.)

To further explore the association between polymyxin B resistance and lipid A modifications, we examined the lipid A profiles of *pmrA*, *pbgP*, Δ*pmrC*, *pmrA*505, and *pmrA*505 *pbgP* Δ*pmrC*1.1 strains. The lipid A from the *pmrA* mutant lacked molecular ions ([M−H]$^-$) corresponding to those modified with either phosphoethanolamine (peaks at m/z 1,919, 1,935, 2,153, and 2,173) or aminoaabinose (peaks at 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 *pbgP* and *pmrC* genes in either a *pmrA*505 (Fig. 4C) or *pmrA*505 (Fig. 4B) background resulted in a strain with the same lipid A profile as that exhibited by the *pmrA* null mutant (Fig. 4D), which缺乏 the modifications displayed by the *pmrA*505 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 aminoaabinose and phosphoethanolamine modifications of lipid A.

The *pmrC* gene is dispensable for resistance to Fe$^{3+}$. The *pmrC* mutant exhibits hypersusceptibility to killing by Fe$^{3+}$, but the targets of PmrA regulation that are responsible for Fe$^{3+}$ resistance have remained unknown (48). Thus, we tested the Δ*pmrC* and *pbgP* Δ*pmrC* mutants for Fe$^{3+}$ sensitivity and found that they retained wild-type levels of resistance to Fe$^{3+}$ (data not shown), suggesting that the *pmrC* gene is not required for this property.

*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 of 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).

The PmrC protein exhibits sequence identity with two *Neisseria* proteins that are implicated in the incorporation of phosphoethanolamine into lipid A and the core region of the LPS (5, 28). There is a higher degree of sequence identity between the *Salmonella* PmrC and *Neisseria* LptA proteins in the C-terminal region, possibly reflecting the fact that both of these proteins are necessary for the modification of lipid A with phosphoethanolamine (Fig. 2) (5). A search of the conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), using the C-terminal region (amino acids 177 to 547) of PmrC as a query, retrieved the catalytic domains of the phosphoglycerol transferase and sulfatase families. This makes sense because phosphoglycerol transferase uses phosphatidylglycerol as a donor of phosphoglycerol in *E. coli* (22) and because phosphatidylglycerol and phosphatidylethanolamine are structurally similar. Moreover, a sulfatase catalyzes the hydrolysis of a sulfate group, which is similar in size to a phosphate group (31P versus 32S). Interestingly, the sulfatase is closely related to the sulfotransferases of mycobacteria in terms of substrate binding, i.e., binding of a sulfate group (31), which incorporate a sulfate group into the glycopeptidolipid (4), the equivalent of the LPS in gram-negative bacteria. Cumulatively, our results suggest that PmrC is a phosphoethanol-
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 at which in-frame fusions were generated to the PhoA and LacZ proteins. The right panel shows alkaline phosphatase and β-galactosidase activities displayed by the phoN strain (EG14286) harboring plasmids pPmrC150- lacZ', pPmrC150-phoA', pPmrC181- lacZ', pPmrC181-phoA', pPmrC295- lacZ', and pPmrC295-phoA' when streaked onto LB agar plates containing either XP (40 μg/ml) or X-Gal (40 μg/ml). These data suggest that the C-terminal region (amino acids 177 to 547) of PmrC localizes to the periplasm.

FIG. 5. (A) Western blot analysis of inner and outer membranes prepared from the ΔpmrC1.1 strain containing either the pBAC108L vector (EG14656) or the pppmrCFLAG plasmid (EG14592), which carries a pmrC gene directed by its own promoter and expresses a PmrC protein tagged with a FLAG epitope at its C terminus. Bacteria were grown to the logarithmic phase in N-minimal medium, pH 7.7, with 10 μM MgCl₂. Inner and outer membranes were prepared by sucrose density gradient centrifugation. Twenty micrograms of protein from the inner and outer membranes was boiled for 10 min, run in an SDS–10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and developed by using anti-FLAG antibodies. To examine the purity of the membrane preparations, we determined the NADH oxidase activity by measuring the oxidation of NADH at 340 nm, and these values are expressed as follows: 100 × μmol of substrate oxidized/min/mg of protein. The analysis demonstrates that the PmrC protein localizes to the inner membrane. (B) Kyte-Doolittle hydropathy plot (25) of the PmrC protein generated by DNA Strider 1.3 software. (C) The left panel shows the predicted topology of the PmrC protein. The numbers correspond to the positions in the PmrC protein at which in-frame fusions were generated to the PhoA and LacZ proteins. The right panel shows alkaline phosphatase and β-galactosidase activities displayed by the phoN strain (EG14286) harboring plasmids pPmrC150- lacZ', pPmrC150-phoA', pPmrC181- lacZ', pPmrC181-phoA', pPmrC295- lacZ', and pPmrC295-phoA' when streaked onto LB agar plates containing either XP (40 μg/ml) or X-Gal (40 μg/ml). These data suggest that the C-terminal region (amino acids 177 to 547) of PmrC localizes to the periplasm.
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 pmrAS505 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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