JOURNAL OF BACTERIOLOGY, May 2005, p. 3391–3399 Vol. 187, No. 10
0021-9193/05/$08.00+0 doi:10.1128/JB.187.10.3391–3399.2005

Identification of *cptA*, a PmrA-Regulated Locus Required for Phosphoethanolamine Modification of the *Salmonella enterica* Serovar Typhimurium Lipopolysaccharide Core

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Received 15 December 2004/Accepted 8 February 2005

In response to the in vivo environment, the *Salmonella enterica* serovar Typhimurium lipopolysaccharide (LPS) is modified. These modifications are controlled in part by the two-component regulatory system PmrA-PmrB, with the addition of 4-aminoarabinose (Ara4N) to the lipid A and phosphoethanolamine (pEtN) to the lipid A and core. Here we demonstrate that the PmrA-regulated STM4118 (*cptA*) gene is necessary for the addition of pEtN to the LPS core. *pmrC*, a PmrA-regulated gene necessary for the addition of pEtN to lipid A, did not affect core pEtN addition. Although imparting a similar surface charge modification as Ara4N, which greatly affects polymyxin B resistance and murine virulence, neither *pmrC* nor *cptA* plays a dramatic role in antimicrobial peptide resistance in vitro or virulence in the mouse model. Therefore, factors other than surface charge/electrostatic interaction contribute to resistance to antimicrobial peptides such as polymyxin B.

In order to survive in the mammalian host, salmonellae have evolved means of evading killing by antimicrobial peptides (AP), central components of innate immunity. Such mechanisms have been demonstrated to be important for survival within hosts and host cells (9, 16, 27, 28). A common mode of increasing AP resistance involves alteration of the bacterial surface. In *Salmonella enterica* serovar Typhimurium, the two-component regulatory systems (TCRS) PhoP-PhoQ and PmrA-PmrB have been characterized as contributing to resistance to AP by mediating alterations to the outer membrane and lipopolysaccharide (LPS) (reviewed in reference 8). Both TCRS respond to specific cues from the host environment and have been shown to be activated within host cell vacuoles (1, 17). PhoP-PhoQ can be activated under conditions of low magnesium or low pH in vitro (3) and can activate PmrA-PmrB via induction of PmrD, whose product affects the phosphorylation state of PmrA (15, 19-21). PmrA can also be activated independent of PhoP in response to mild acid pH or high iron concentrations (11, 30). Such activation results in the induced transcription of *pmrE* and *pmrHFIJKLM* (also called *pbg* and *arn* elsewhere [12, 35]), which encode enzymes involved in biosynthesis of aminoarabinose (Ara4N) and its addition primarily to the 4′-phosphate of the lipid A portion of LPS (14).

PmrA is also predicted to control modification of LPS with phosphoethanolamine (pEtN), because PmrA mutants with increased polymyxin B (PM) resistance show enhanced pEtN substitutions to the lipid A and core oligosaccharide (OS) fractions of LPS (18). Recently, the PmrA-regulated *pmrC* gene product was shown to mediate the addition of pEtN to the 1-position of lipid A and affect resistance to PM (22). In *Neisseria meningitidis*, loci necessary for transferring pEtN to the heptose II (HepII) of the LPS core or to lipid A were also recently identified (5, 23, 38). Inactivation of *N. meningitidis lpt-3* resulted in the absence of pEtN from HepII of the core and bestowed increased resistance to bactericidal killing and decreased opsonophagocytosis by LPS-specific monoclonal antibody B5 (23). Similarly, inactivation of *lptA* led to loss of pEtN replacement of lipid A phosphates (5), and *lpt6* was required for the addition of pEtN-6 on HepII of the core. However, it is not known if these pEtN phosphotransferases play a role in neisserial AP resistance or animal virulence.

We recently identified a number of new *Salmonella* PmrA-regulated genes, including one locus with homology to both *lpt-3* and *lptA* of *N. meningitidis* (34). In this work, we describe the further characterization of this gene, its role in pEtN modification of LPS, and the role of pEtN modification of LPS in AP resistance and virulence of *Salmonella*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are described in Table 1. Salmonellae were grown in Luria-Bertani broth at 37°C with aeration. Antibiotics were used, when appropriate, at the following concentrations: chloramphenicol, 25 μg/ml; kanamycin, 45 μg/ml; tetracycline, 25 μg/ml.

Generation of mutations in *Salmonella* homologues to *lpt-3* of *N. meningitidis*. Initially, mutations were generated in PmrA^c (JSG435, *pmrA505 zjd*::Tn*10*d-cam) and PmrA-null (*pmrA*::Tn*10*d-tet) backgrounds using the lambda Red recombinase and FLP-mediated recombination techniques developed by Datsenko and Wanner (6). Briefly, the kanamycin resistance fragment was amplified from pKD4 using primers flanked with homology to *pmrC*, STM4118, STM3635, or STM0834 (Table 2). PCR fragments were transformed into PmrA^c and PmrA-null target strains in which lambda Red recombinase had been induced from pKD46. Correct integration of the kanamycin resistance gene insertion was verified by PCR. All strains and plasmids described herein are listed in Table 1.

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference(s)
Strains		
CS019	*phoN2 zxx*::6251dTn*10*-cam	24
JSG210	Wild type, ATCC 14028s	24
JSG421	*pmrA*::Tn*10*d-tet	15
JSG435	*pmrA505 zjd*::Tn*10*d-cam	15
JSG485	*pmrA505 zjd*::Tn*10*d-cam *pmrF*::Tn*10*d-Tet	14
JSG1311	*rpsL phoN2 zxx*::6251dTn*10*-cam	This study
JSG1800	*pmrA505 zjd*::Tn*10*d-cam STM4118::Kan	This study
JSG1863	*pmrA505 zjd*::Tn*10*d-cam ΔSTM4118	This study
JSG1900	*pmrA505 zjd*::Tn*10*d-cam STM4118::*lacZ*	This study
JSG1901	*pmrA*::Tn*10*d-tet STM4118::*lacZ*	This study
JSG1960	14028s STM4118::Kan	This study
JSG2264	*pmrA505 zjd*::Tn*10*d-cam STM3635::Kan	This study
JSG2266	*pmrA505 zjd*::Tn*10*d-cam *pmrC*::Kan	This study
JSG2268	*pmrA505 zjd*::Tn*10*d-cam STM0834::Kan	This study
JSG2271	*pmrA505 zjd*::Tn*10*d-cam ΔSTM3635	This study
JSG2273	*pmrA505 zjd*::Tn*10*d-cam Δ*pmrC*	This study
JSG2275	*pmrA505 zjd*::Tn*10*d-cam ΔSTM0834	This study
JSG2278	*pmrA505 zjd*::Tn*10*d-cam STM3635::*lacZ*	This study
JSG2279	*pmrA*::Tn*10*d-tet STM3635::*lacZ*	This study
JSG2280	*pmrA505 zjd*::Tn*10*d-cam *pmrC*::*lacZ*	This study
JSG2281	*pmrA*::Tn*10*d-tet *pmrC*::*lacZ*	This study
JSG2282	*pmrA505 zjd*::Tn*10*d-cam STM0834::*lacZ*	This study
JSG2283	*pmrA*::Tn*10*d-tet STM0834::*lacZ*	This study
JSG2284	14028s STM3635::Kan	This study
JSG2285	14028s *pmrC*::Kan	This study
JSG2286	14028s STM0834::Kan	This study
JSG2339	*pmrA505 zjd*::Tn*10*d-cam ΔSTM4118 *pmrF*::Tn*10*d-Tet	This study
JSG2341	*pmrA505 zjd*::Tn*10*d-cam Δ*pmrC* *pmrF*::Tn*10*d-Tet	This study
JSG2435	*pmrA505 zjd*::Tn*10*d-cam Δ*pmrC* STM4118::Kan	This study
JSG2449	STM4118::Kan Δ*pmrC*::Cam	This study
JSG2512	*pmrA505 zjd*::Tn*10*d-cam Δ*pmrC* ΔSTM4118 *pmrF*::Tn*10*d-Tet	This study
Plasmids		
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS	6
pKD4	*bla* FRT *aph* FRT PS1 PS2 oriR6K	6
pCP20	*bla cat cl857* λP_R*flp*pSC101 oriTS	4, 6
pCE36	*aph* FRT *lacZY*$^+$ t_{his} oriR6K	7

TABLE 2. Primers used in this study

Primer	Sequence[a]
STM3635	5′ ACGCAACAGAAACTTAGTTTCTTGCTTGCG*TGTGTA*
	GGCTGGACGTGCTTC 3′
	5′ TATCGTATCGTATAGCTCCACATGACGACG*CATATG*
	AATATCCTCCTTA 3′
STM4293 (pmrC)	5′ CAGGTACTACAAGACCTACCGTTAAACTCG*TGTGTA*
	GGCTGGAGCTGCTTC 3′
	5′ CCGTCATTATCATTCCACAGTACGTTAATCC*CATAT*
	GAATATCCTCCTTA 3′
STM4118	5′ CGATTAAAGACAGGCTCTCATTATGCAATCC*TGTGT*
	AGGCTGGAGCTGCTTC 3′
	5′ GCGTTATCGGGCAACATACAGGCTATTGATT*CATAT*
	GAATATCCTCCTTA 3′
STM0834	5′ GCGTTTACCTGCCTGTTGCTGTTGCTCTGGTG*TGTA*
	GGCTGGAGCTGCTTC 3′
	5′ CTGCCCAGATGGTTATAGTCCACTTTCTGT*CATATG*
	AATATCCTCCTTA 3′

[a] Italicized sequences are complementary to sequence flanking the *aph* gene in pKD4 (6).

The insertion was eliminated using pCP20 as described previously, leaving a nonpolar deletion of the target gene and reintroducing a ribosome binding site (6). Transcriptional fusions (*lacZ*) were generated to each gene in both the PmrA^c and PmrA-null backgrounds by using site-specific recombination between the FLP-recognition target "scar site" generated in the deletion strains and that in pCE36 (7).

Mutations in STM4118, STM3635, STM0834, and *pmrC* were also introduced into a wild-type 14028s (JSG224) background using P22 phage-mediated transduction, generating JSG1960, JSG2284, JSG2285, and JSG2286.

pmrF double mutants in selected backgrounds were generated by transducing phage propagated on JSG485 (*pmrA505 zjd*::Tn*10*d-cam *pmrF*::Tn*10*d-tet) into JSG1863, JSG2271, JSG2273, and JSG2275. Tetracycline-, chloramphenicol-resistant colonies were selected and designated JSG2339 to -2342.

AP resistance assays. MIC assays were performed as described elsewhere (32). AP survival assays were performed by diluting logarithmic-phase bacteria to ~10^5 CFU/ml followed by the addition of an AP at a set concentration. At various times after the addition of the AP (as well as prior to the addition of AP), aliquots of the bacteria were washed, diluted, and plated onto solid medium to enumerate the surviving bacteria.

Virulence assays. Survival assays were performed as described previously (16). Wild-type 14028s, JSG1960 (14028s STM4118::kan), JSG2285 (14028s *pmrC*::kan), and JSG2449 (14028s ΔSTM4118::kan Δ*pmrC*::cat) were included in the assay. Briefly, each strain was grown to stationary phase (16 h) at 37°C. Approximately 5×10^6 stationary-phase bacteria (1 log unit above the 50% lethal dose) were washed and resuspended in 20 to 200 μl of phosphate-buffered saline, pH 7.4. Female BALB/c mice (weighing 16 to 18 g) were inoculated orally using the swallowing reflex or with a feeding needle. Five mice were used per strain. Dilutions of the stationary-phase cultures were plated to enumerate bacteria present in the inocula. Infected mice were observed for 21 days postinoculation. For competition assays, wild-type (JSG1311) and mutant (JSG2449) bacteria were inoculated into five BALB/c mice at a 1:1 ratio (1×10^6 of each in 200 μl total) and, at 4 days postinoculation, organs were harvested, macerated, and plated onto appropriate solid media to select for each of the competing strains.

LPS and lipid A isolation and gel analysis. LPS was purified from stationary-phase cells using the hot phenol-water method as previously described (2). Deoxycholine-polyacrylamide gel electrophoresis (DOC-PAGE) was performed with an 18% polyacrylamide gel. The LPS samples were dissolved at a concentration of 4 μg/μl, and 1 μl was loaded in each well. The samples were electrophoresed at 30 mA constant current mode and were developed by silver staining (Bio-Rad).

Size exclusion chromatography. For the LPS samples, chromatography was performed with a Sephacryl S-200 (HR) column (120 cm by 1 cm). The samples were eluted with Tris-EDTA buffer (pH 9.2), and the eluted fractions were monitored by an on-line refractive index detector and assayed by DOC-PAGE. Samples were pooled according to the molecular size, dialyzed extensively to remove the DOC, and lyophilized. This fractionated LPS was used for further analysis.

Isolation and purification of the oligosaccharide. The LPS samples were delipidated with 1% acetic acid at 100°C for 2 h. The lipid precipitate was removed by centrifugation at 10,000 rpm for 10 min. The supernatant was fractionated with a Bio-Gel P10 column, using water as eluent. There was a single major fraction of each oligosaccharide that was lyophilized and used for further analysis.

NMR analysis. Proton-nuclear magnetic resonance (NMR) of each oligosaccharide (2 mg) was performed after exchanging three times with 99.8% D_2O (Aldrich) and dissolving in 0.5 ml D_2O (100%) (Cambridge Isotopes). All of the NMR experiments were accomplished with a 600-MHz Varian-Inova instrument at 30°C. Two-dimensional NMR (2D-NMR) was performed using standard parameters as provided by Varian. The gCOSY (gradient sensitive ^{1}H-^{1}H correla-

tion spectroscopy) spectra were measured over a spectral width of 2.5 kHz using a data set of ($t_1 \times t_2$) of 256 by 2,048 points, and 16 scans were acquired. For g-HSQC (gradient-sensitive ^1H-^{13}C heteronuclear single quantum coherence spectroscopy), the spectral widths in proton and carbon dimensions were selected to be 2.5 and 13.9 kHz, respectively, and 96 scans were acquired.

MALDI mass spectrometry. The oligosaccharide samples were dissolved in Milli-Q water at a concentration of 1 μg/μl and mixed with 2,5-dihydroxy azobenzoic acid matrix at a 1:1 ratio. The samples were spotted on a 100-well stainless steel matrix-assisted laser desorption ionization (MALDI) plate and air dried. A 337-nm N_2 laser was used to desorb the molecules, and spectra were collected in delayed, linear, and negative mode using an acceleration voltage of 20,000.

RESULTS

Identification of *Salmonella* homologues to *N. meningitidis* phosphoethanolamine phosphotransferases and their regulation by PmrA. The predicted protein sequences of *lpt-3* (NMB2010), *lptA* (NMB1638), and *lpt6* (NMA0408) were compared to the *S. enterica* serovar Typhimurium LT-2 genome sequence using the NCBI database (BLASTX and BLASTP). Similar searches and results that were restricted to the use of *lpt-3* have been previously reported (22). The products of four *S. enterica* serovar Typhimurium loci showed significant similarity (*E* value $< 4e^{-7}$) to Lpt-3: STM3635 (*yhjW*; 24% identity, 41% similarity), STM4293 (*pmrC yjdB*; 24% identity, 43% similarity), STM4118 (*yijP*; 23% identity, 38% similarity), and STM0834 (*ybiP*; 23% identity, 41% similarity). LptA showed similar levels of identity and similarity to the same *S. enterica* serovar Typhimurium loci but demonstrated a greater degree of conservation to STM4293 (43% identity, 62% similarity). Lpt6 showed no significant similarity to *Salmonella* proteins. STM3635 is predicted to encode a membrane-associated, metal-dependent hydrolase. STM4118, *pmrC*, and STM0834 encode putative integral membrane proteins, consistent with the predicted location of an LPS phosphoethanolamine phosphotransferase, and PmrC has been demonstrated to be necessary for the transfer of pEtN to the 1-phosphate of lipid A (22).

Two of these loci, *pmrC* and *yijP* (STM4118, hereafter called *cptA*), have been previously shown to be regulated by PmrA (15, 31, 34), while the role of PmrA-PmrB in the regulation of STM0834 and STM3635 was unknown. Therefore, to determine whether PmrA activated transcription of these genes, expression of *lacZ* transcriptional fusions to STM3635 and STM0834 in PmrA^c and PmrA-null backgrounds was compared. This analysis demonstrated that neither STM0834 nor STM3635 was regulated by PmrA (data not shown). Because the transferase that catalyzes addition of pEtN to the *Salmonella* LPS core is predicted to be regulated by PmrA (18, 40), STM0834 and STM3635 were not further characterized.

Structural analysis of LPS demonstrates that *cptA*, but not *pmrC*, is required for pEtN addition to the LPS core. To further examine the role of *pmrC* and *cptA* in LPS modification, deletions in each gene were created in a JSG435 (PmrA-constitutive, PmrA^c) background. The PmrA^c strain possesses high-level PM resistance and high levels of Ara4N- and pEtN-modified LPS (18). Following the isolation of LPS from these mutants, the DOC-PAGE profile showed the characteristic ladder pattern, indicating molecular heterogeneity in the LPS. No obvious differences were detected in the patterns of the LPS between the *cptA* and *pmrC* mutants, or in comparison to the controls (PmrA-null or PmrA^c). After separation by size exclusion chromatography, LPS fractions were identified that were enriched in LPS molecules bearing a low number of O-antigenic repeats (Fig. 1). Compositional analysis showed that higher-molecular-weight material contained more mannose and rhamnose sugars compared to the low-molecular-weight materials (data not shown). Appropriate low-molecular-weight fractions were chosen, and the OS was prepared from the core-enriched LPS molecule by mild acid hydrolysis.

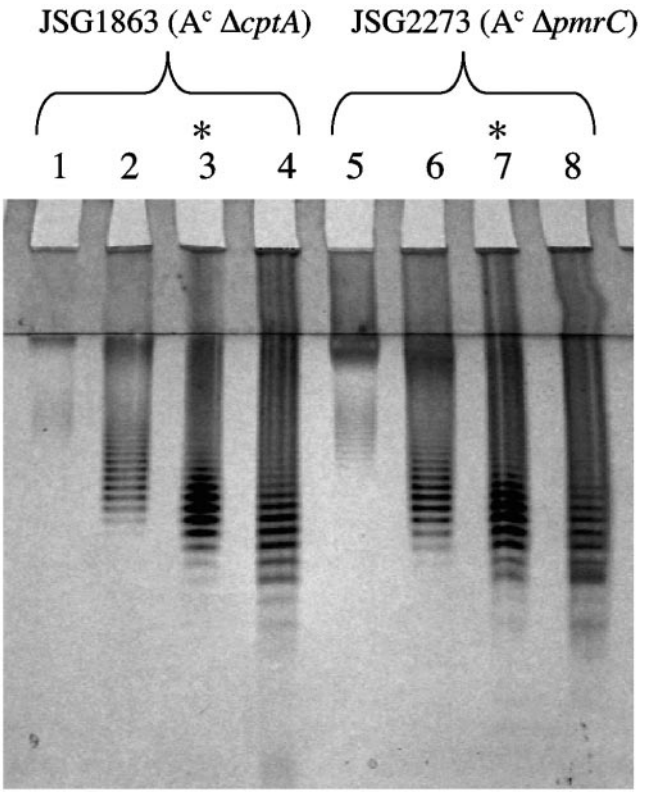

FIG. 1. DOC-PAGE analysis of LPS fractions. Sephacryl S-200 fractions of whole LPS from strains JSG1863 and JSG2273 were collected and electrophoresed. Four lanes corresponding to different fractions are shown for each strain (lanes 1 to 4, JSG1863; lanes 5 to 8, JSG2273). Similar profiles of strains JSG421 and JSG435 were performed but are not shown. Asterisks mark the lanes with the polysaccharide chosen for further analysis.

The NMR data indicated a mixture of molecules, as expected from LPS heterogeneity. The anomeric region indicated that most of the sugars are α-linked and lie between 5.8 and 4.8 ppm; however, there was one β-linked sugar at 4.5 ppm, as was evident from 2D-NMR spectroscopy. The other reporting group signals were 6-deoxy methyl doublets from rhamnose at 1.0 to 1.35 ppm and *N*-acetyl methyl singlets at 2.1 ppm. The H-3 methylene protons of the Kdo residue resonate at 1.9 and 2.2 ppm. The other nonanomeric signals from ring protons overlap between 4.4 and 3.5 ppm. For the controls, the characteristic chemical shift for the distal methylene (-CH_2-) protons from the phosphorylated ethanolamine group (pEtN/PEA) was found in both JSG435 (PmrA^c) and also in JSG421 (PmrA-null) at 3.3 ppm, which was evident from the NMR spectra (Fig. 2). However, the ratio of the integral area for the methylene to anomeric proton at 5.21 ppm was 2:1 for JSG435

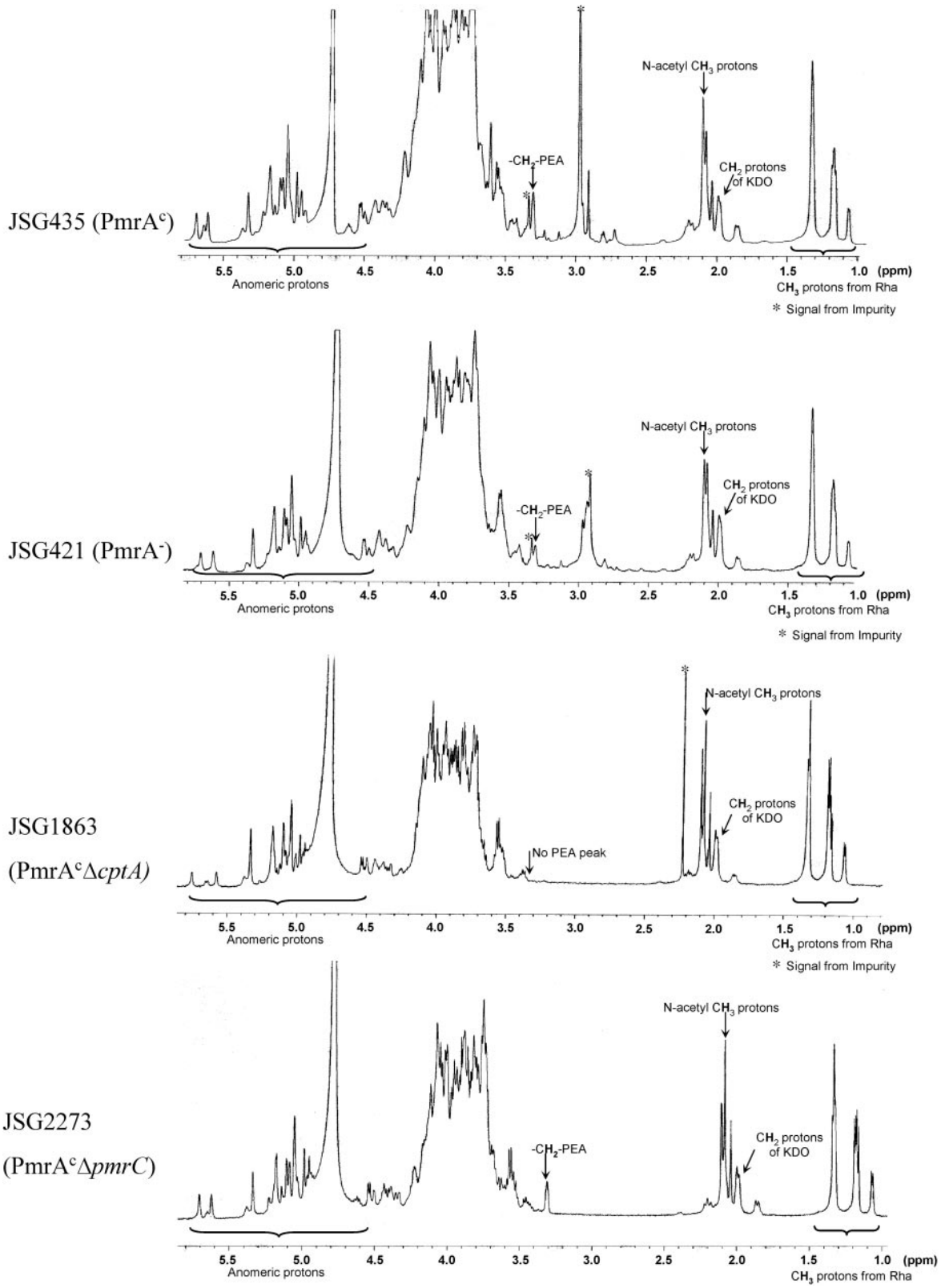

FIG. 2. Proton NMR spectra of LPS core-enriched polysaccharide preparations. The proton signals are as indicated in the figure. Strain JSG1863 (PmrA^c Δ*cptA*) is missing the signal at 3.3 ppm corresponding to the chemical shift for the distal methylene (-CH_2-) protons of the pEtN (also called -PEA). All other samples, including that of JSG2273 (PmrA^c Δ*pmrC*) and JSG421 (*pmrA*::Tn*10*d-tet), still possess a signal at 3.3 ppm (although the signal is less intense in JSG421). The signals at about 2.9 ppm (marked with an asterisk) are due to an unidentified contaminant.

and 1.5:1 for JSG421, indicating that the latter strain contained less pEtN. 2D-NMR further confirmed the presence of the pEtN group on both OS preparations from JSG435 and JSG421 in that there was a distinct COSY correlation between the distal and proximal -CH_2- protons at 3.3 and 4.2 ppm, respectively (data not shown). Further, the presence of pEtN was confirmed by C-H one-bond heteronuclear g-HSQC analysis, which showed that the distal -CH_2 protons at 3.3 ppm were correlated to the nitrogen-bearing carbon of the pEtN group, which resonated at 40.0 ppm (data not shown).

MALDI was also performed on the controls. The MALDI mass spectrum showed the presence of more than one molecular species. The 123 mass unit difference between *m*/*z* at 2426 and 2549 in the JSG435 OS indicates the presence of pEtN in this mutant (Fig. 3). There also was a molecular distribution of oligosaccharides with a larger number of repeating units (OS1) that have the pEtN-bearing oligosaccharide. We also observed similar ions in JSG421 samples, with a 123 mass difference indicating the presence of pEtN distribution on that oligosaccharide (Fig. 3). However, again, the intensity of pEtN-bearing signals was less in the JSG421 OS than in JSG435 OS.

Examination of JSG1863 (PmrA^c Δ*cptA*) and JSG2273 (PmrA^c Δ*pmrC*) by NMR and MALDI was also performed. As with the controls, the distal (with respect to phosphate) methylene protons (-CH_2-) of the pEtN group had a characteristic chemical shift near 3.3 ppm. The 1D-^{1}H-NMR of the OS isolated from JSG1863 and JSG2273 showed the presence of the distal -CH_2 signal of pEtN at 3.3 ppm in the latter, while it was completely absent on the former (Fig. 2). 2D-NMR further detected the presence of the pEtN group on the OS from JSG2273, where there is a distinct COSY correlation between the distal and proximal -CH_2- at 3.3 and 4.2 ppm, respectively. The presence of the pEtN signal was confirmed by the C-H one-bond heteronuclear g-HSQC spectrum, where the distal -CH_2 protons at 3.3 ppm correlated with a nitrogen-bearing carbon of the pEtN group that resonated at 40.0 ppm (data not shown). In the MALDI mass spectrum, the presence of the pEtN signal in JSG2273 was observed (the −123 mass unit difference at *m*/*z* at 2417.3 and 2540.3 in the JSG2273 OS) (Fig. 3). However, there was no indication of −123 mass differences in the JSG1863 mutant, demonstrating the lack of pEtN in the core of this mutant. Other minor differences were noted between JSG1863 and controls, but they did not correlate with pEtN modification. Therefore, the core of the LPS in JSG2273 (PmrA^c Δ*pmrC*) still possesses pEtN, whereas the LPS core of JSG1863 (PmrA^c Δ*cptA*) does not.

***pmrC* and *cptA* have a modest effect on resistance to PM.** Strains with various combinations of deletions in *pmrC* and *cptA* (in a PmrA^c background; JSG1863 and JSG2273) were tested for increased susceptibility to PM using standard MIC assays. Strains JSG1863 and JSG2273 each showed a twofold decrease in PM resistance (4 μg/ml PM) compared to the parent PmrA^c strain (8 μg/ml PM). Because the loss of Ara4N has been shown to have a dramatic effect on PM resistance in *S. enterica* serotypes, mutants containing defined *pmrHFIJKLM* operon insertions, or those deficient in this operon plus either *pmrC* or *cptA*, were examined in MIC assays. Such double mutants were shown to be no more susceptible to PM than mutation of *pmrF* alone (0.03 μg/ml PM). While these data suggested that elimination of pEtN phosphotransferase genes does not amplify PM susceptibility beyond that conferred by abolishing Ara4N modification of lipid A, this analysis was furthered with additional PM sensitivity assays. Survival assays using 0.5 μg/ml polymyxin B and strains containing *pmrC*, *cptA*, or *pmrC cptA* double mutations in a PmrA^c background were performed. As shown in Fig. 4A, these mutants possessed no statistically significant differences in their levels of survival in comparison to the PmrA^c parental strain, further demonstrating that pEtN modification of LPS plays a modest role in regard to PM resistance. When these mutations (*pmrC*, *cptA*, or *pmrC cptA*) were examined in a PmrA^c *pmrF*::Tn*10*d background, both *cptA* and *pmrC* resulted in a statistically significant reduction in survival compared to the PmrA^c *pmrF*::Tn*10*d strain (Fig. 4B). However, while the *cptA* mutation resulted in a ~2-fold drop in survival, the PmrA^c *pmrF*::Tn*10*d *pmrC* strain (as well as the PmrA^c *pmrF*::Tn*10*d *pmrC cptA* strain) demonstrated a 94-fold drop in survival, which obtained a level similar to the survival of a PmrA-null strain. Thus, *cptA* appears to play a minor role in PM resistance, which can be primarily observed in the absence of Ara4N modification of lipid A. The *pmrC* gene appears to play a larger role in PM resistance, but it also primarily plays a role only in the absence of Ara4N lipid A modification.

Strains lacking both *cptA* and *pmrC*, but not those lacking either gene alone, show a modest virulence defect in the mouse model of typhoid fever. Because the loss of pEtN plays a modest role in resistance to AP and AP resistance has been shown to play a role in virulence, pEtN mutants were examined for virulence defects in the mouse model. Mice infected by the intraperitoneal or oral routes with strains containing *cptA*, *pmrC*, or *cptA pmrC* double deletions in a wild-type background showed no differences in the 50% lethal dose compared to controls (data not shown).

To further examine the in vivo relevance of pEtN LPS modification on virulence, competition infection experiments were performed. While single mutations in *cptA* or *pmrC* had no defect in competition experiments when evaluated against the wild-type strain (data not shown), a *cptA pmrC* double mutant showed a consistent, but less than 10-fold decrease in survival in all tissues examined (Fig. 5). Therefore, the inability to modify LPS in vivo with pEtN on both core and lipid A regions can be shown to play a minor role in virulence.

DISCUSSION

The expression of mechanisms for resistance to AP is an important determinant of pathogenicity, and susceptibility of bacteria to AP is associated with attenuation of virulence (9, 10, 13, 28). The TCRS PmrA-PmrB of *S. enterica* serovar Typhimurium has been shown to be activated within host cell vacuoles (1, 17) and has been characterized as contributing to AP resistance by mediating alterations to the outer membrane and LPS (reviewed in reference 8). PmrA controls the expression of *pmrE* and *pmrHFIJKLM*, which encode enzymes involved in producing Ara4N substitutions on lipid A (16). In addition, PM-sensitive strains have been reported to lack the ability to produce pEtN substitutions in the lipid A and core oligosaccharide fractions of LPS (18, 40).

Four *Salmonella* open reading frames (STM3635, STM4293 [*pmrC*], STM4118 [*cptA*], and STM0834) were shown to pos-

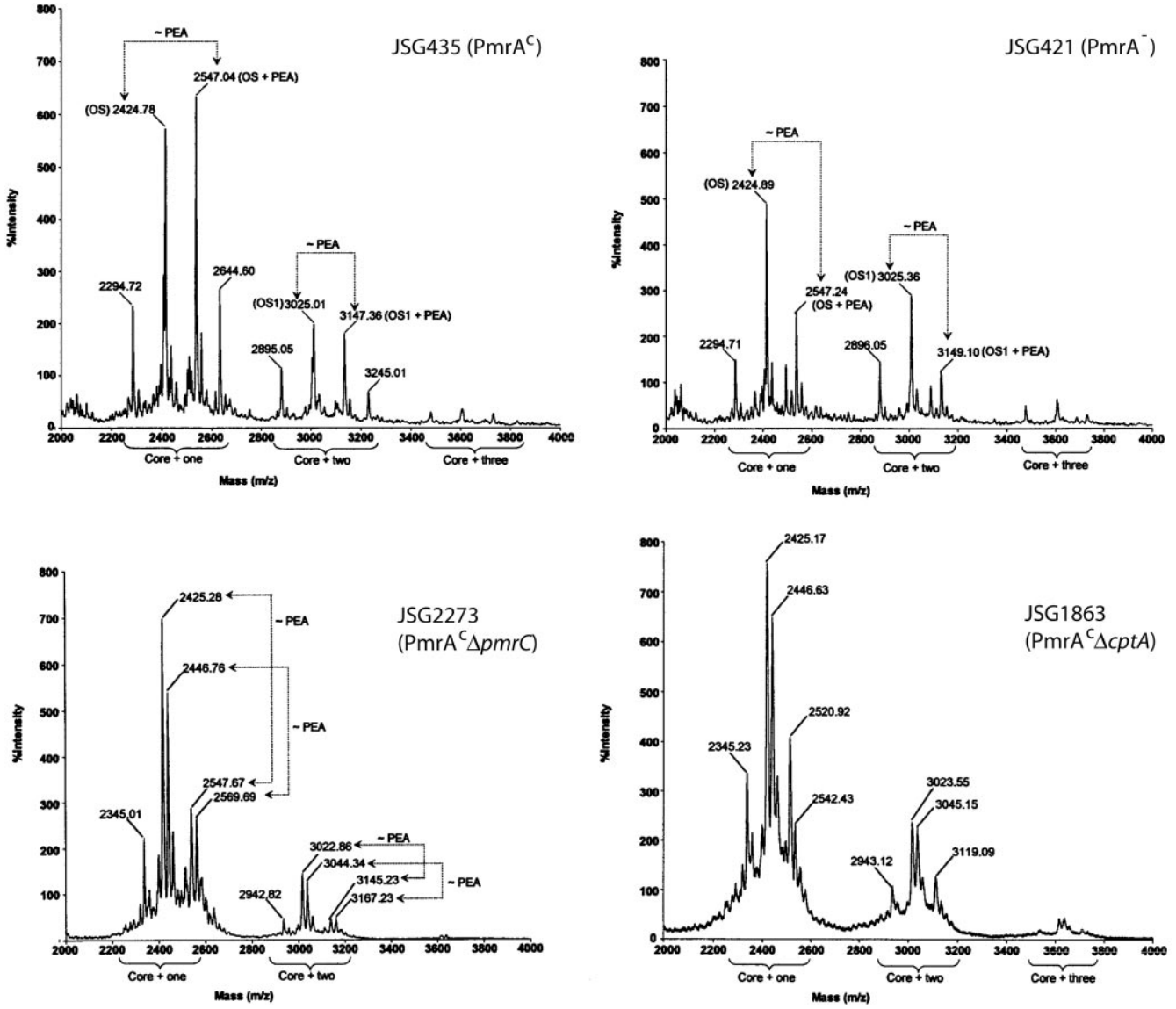

FIG. 3. The MALDI-TOF mass spectrometry spectrum of LPS core-enriched polysaccharide preparations. As indicated in the figure, the clusters of signals are due to polysaccharides comprised of the core oligosaccharide plus one O-chain repeat unit, core plus two repeat units, etc. The core (after mild acid hydrolysis) is defined as that published by Olsthoorn et al. (25). The structures corresponding to *m*/*z* 2644.6 and *m*/*z* 3245.0, 220 mass units larger than *m*/*z* 2424.8 and *m*/*z* 3025.0, respectively, are likely due to structures that still contain the second Kdo residue of the core oligosaccharide. Ion peaks at 2446.76 and 3044.34 in JSG2273 (2446.63 and 3045.15 in JSG1863) are due to sodiated (+22 mass units) forms of other apparent ions. These spectra clearly reveal the presence of structures with an added pEtN (PEA) component in JSG345, JSG421, and JSG2273, i.e., ions that are increased 123 mass units, as indicated in the figure. The lack of a 123-mass unit increase in any observed peak confirms proton NMR data concerning the lack of pEtN in the LPS core of strain JSG1863 (PmrA^c Δ*cptA*).

sess significant similarity to the *N. meningitidis* genes (*lpt-3* and *lptA*) that encode pEtN phosphotransferases (5, 23). Two of these genes, STM4293 (*pmrC*) and STM4118 (*cptA*), were previously shown to be PmrA regulated (34). STM3635 and STM0834 are not PmrA regulated and their function remains unknown, although by homology they appear to be phosphoethanolamine phosphotransferases. *pmrC*, the first open reading frame in the *pmrCAB* operon, has been shown to be necessary for the transfer of pEtN to lipid A (22). However, it was not shown in this work whether PmrC could add pEtN to other positions in the LPS, such as the core. We hypothesized that PmrC may be a phosphoethanolamine phosphotransferase specific for lipid A and *cptA* may encode a phosphoethanolamine phosphotransferase specific for the LPS core.

NMR and mass spectrometry of PmrA^c *pmrC* and PmrA^c *cptA* mutants demonstrated that *cptA* but not *pmrC* is necessary for adding pEtN to the LPS core. Therefore, there exist distinct PmrA-regulated phosphoethanolamine phosphotransferases of the LPS core and lipid A (as it is likely that CptA does not add pEtN to lipid A). It is highly probable that the pEtN group observed in this work is that added to heptose I of the core. A second PmrA-regulated modification of the inner

core Kdo with pEtN has been reported (18) but not confirmed. Such a modification could not have been observed in our NMR or mass spectroscopy studies due to the biochemical processing of the oligosaccharide prior to analysis, but it will be the subject of future studies with additional PmrA-regulated gene mutants. Also of interest in these biophysical studies was the identification of pEtN in the LPS core of the PmrA-null strain. This was surprising, because PmrA regulates the expression of *cptA*, which has a clear pEtN-deficient phenotype. This may suggest that core pEtN modification and/or *cptA* expression is not completely dependent upon PmrA.

Increased substitution of lipid A with Ara4N results in increased resistance to AP, including PM, neutrophil granule AP azurocidin (CAP-37), and bactericidal permeability-increasing protein (CAP-57) (18, 29, 33, 36). It is surmised that substitution of the anionic phosphates of LPS with moieties such as Ara4N reduces the electrostatic attraction and binding between the LPS and cationic AP. LPS from PM-resistant *Salmonella* binds PM poorly in vitro (37). Because pEtN addition to lipid A and core phosphate moieties would also result in decreased anionic charge of the LPS, it was expected that pEtN modification of LPS should play a similar role in AP resistance. This reasoning is also in part due to the results of Yethon et al., which demonstrated that the loss of *waaP*, which catalyzes the phosphorylation of the HepI of the *Salmonella* LPS core and is required for further pEtN modification of this Hep residue, affected both PM resistance and virulence (39). Here, we show that the lack of PmrC or CptA activity reduced the MIC of PM 2-fold, whereas elimination of Ara4N modification reduced resistance to PM 100-fold (14, 16). Similar major effects of Ara4N and minor effects of pEtN were observed in PM survival assays. Therefore, while both modifications decrease the anionic charge of LPS, only Ara4N substitution dramatically affects the ability of PM to associate with LPS. These results suggest that LPS charge is only partially responsible for interactions between PM and LPS and that the structure and/or location of the Ara4N modification to lipid A has an inhibitory effect on PM binding. With respect to *pmrC*, these results are somewhat contradictory to the results of Lee et al. (22). In that work, a strain with a mutation in *pmrC* had a significant effect on PM resistance but, similar to our findings, the role of *pmrC*-mediated LPS modification was inferior to that of Ara4N LPS modification with regard to PM resistance. As a possible explanation of these contradictory results, variations in experimental conditions may have led to differential *pmrC* expression and/or PM killing that influenced in vitro bacterial survival.

The PmrA-PmrB TCRS, and also the dependent AP resistance, has been demonstrated to affect virulence of *S. enterica* serovar Typhimurium in the mammalian host (16). A null mutation in *pmrA*, *pmrE*, or *pmrHFIJKLM* decreases AP resistance and results in 10- to 1,000-fold decreased survival of the bacteria in the murine model of infection (16). Therefore, it was of interest to determine whether the PmrA-mediated pEtN substitution of core and lipid A contributed to AP resistance and/or survival in the host. Mutation of *pmrC* or *cptA* did not result in decreased virulence as individual mutations but, when combined, resulted in a consistent but less than 10-fold decrease in the competitive index compared to a wild-type strain. This decrease in competitive index was even observed in the intestinal lumen, an early point after infection. This may suggest that antimicrobial factors in the lumen (possibly including AP) substantially contribute to the observed reduction in the competitive index. However, the decreased AP resistance evinced by loss of pEtN modifications was not sufficient to be dramatically detrimental to survival of *Salmonella* in the murine model. The previously mentioned *waaP* mutation had a dramatic affect on virulence, which might have been due to the increased susceptibility to AP in this mutant (39). However, since both this *waaP* and the *cptA* mutant are missing pEtN on the core but do not have equal virulence defects, it is

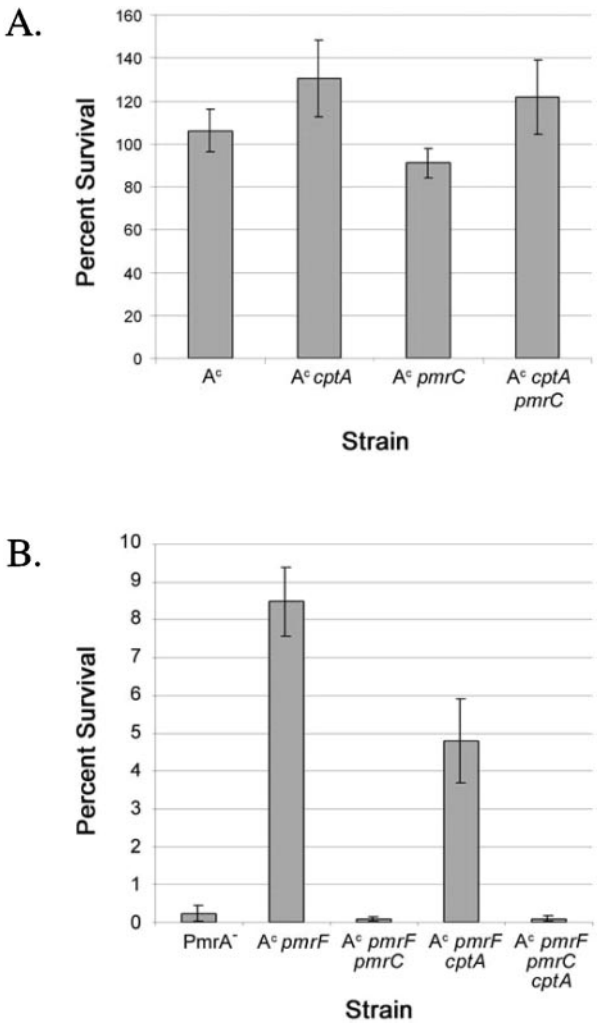

FIG. 4. PM resistance assays of strains deficient in LPS modifications. Strains were incubated in the presence of 0.5 μg/ml PM for 1 h at room temperature followed by plating to determine the percent survival. (A) Strains with mutations in *cptA*, *pmrC*, or both genes were examined for PM sensitivity in a PmrA-constitutive (PmrA^c) background. Neither mutation had a significant effect on PM resistance in this background compared to the PmrA^c parent strain (*t* test or analysis of variance [ANOVA], $P > 0.05$). (B) Strains with mutations in *cptA*, *pmrC*, or both genes were examined for PM sensitivity in a PmrA^c *pmrF* (aminoarabinose-deficient) background. Strains containing *cptA*, *pmrC*, or both mutations in the PmrA^c *pmrF* background demonstrated a level of sensitivity to PM that was significantly different from the PmrA^c *pmrF* parent strain (*t* test or ANOVA, $P < 0.05$). Error bars indicate the standard errors.

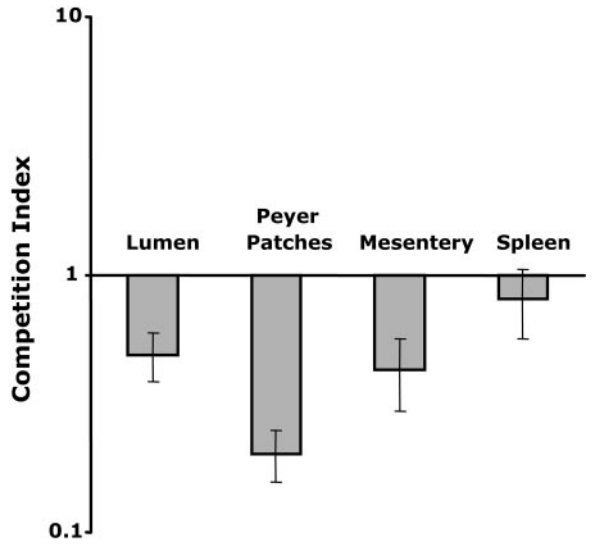

FIG. 5. Competition infection experiments of the wild type (JSG1311) and double mutant (*cptA pmrC*; JSG2449). At 4 days postinfection, selected organs and tissues were harvested and the contents were examined for surviving bacteria by culture on selective, solid medium. Error bars indicate the standard errors.

likely that the virulence attenuation of the *waaP* mutant can be attributed to its other known membrane effects, such as an increase in outer membrane phospholipids.

Activation of *pmrC* and *cptA* by PmrA suggests that these loci, and the consequent pEtN modifications, are up-regulated during infection of the mouse (17), but the function of pEtN LPS modifications remain unclear. Evidence in *N. meningitidis* indicates that LOS is a receptor for complement component C4b and that substitution of core with pEtN affects binding by C4b (26). Specifically, pEtN substitutions on HepII form amide bonds with C4b and augment complement-mediated killing in serum bactericidal assays. Bacteria with pEtN additions at position 6 of HepII were more efficiently eliminated than those with substitutions on position 3, and strains with pEtN at position 3 of HepII were more likely to be found in clinical isolates (26). *Salmonella* has been shown to add pEtN to Kdo and HepI, but not HepII; however, pEtN substitution of the core may likewise affect complement killing in *Salmonella*. If so, AP resistance attributed to pEtN substitution may be negated by increased recognition and elimination by complement. Further studies of the effect of pEtN substitution of LPS on resistance to AP other than PM and on immune recognition are required in order to understand the physiological role of pEtN modification in vivo.

ACKNOWLEDGMENTS

This work was funded by grants from the National Institutes of Health to J.S.G. (AI43521 and AI43521-S1) and a CCRC center grant from the DOE to R.C. (DE-FG-02-93ER20097).

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