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Genetic and Functional Analysis of a PmrA-PmrB-Regulated Locus Necessary for Lipopolysaccharide Modification, Antimicrobial Peptide Resistance, and Oral Virulence of *Salmonella enterica* Serovar Typhimurium

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The two-component regulatory system PmrA-PmrB confers resistance of *Salmonella* spp. to cationic antimicrobial peptides (AP) such as polymyxin (PM), bactericidal/permeability-increasing protein, and azurocidin. This resistance occurs by transcriptional activation of two loci termed *pmrE* and *pmrHFIJKLM*. Both *pmrE* and *pmrHFIJKLM* produce products required for the biosynthesis of lipid A with 4-aminoarabinose (Ara4N). Ara4N addition creates a more positively charged lipopolysaccharide (LPS) and thus reduces cationic AP binding. Experiments were conducted to further analyze the regulation of the *pmrHFIJKLM* operon and the role of this operon and the surrounding genomic region in LPS modification and antimicrobial peptide resistance. The *pmrHFIJKLM* genes are cotranscribed and over 3,000-fold regulated by PmrA-PmrB. The *pmrHFIJKLM* promoter bound PmrA, as determined by gel shift analysis, as did a 40-bp region of the PmrA-PmrB-regulated *pmrCAB* promoter. Construction of nonpolar mutations in the *pmrHFIJKLM* genes showed that all except *pmrM* were necessary for the Ara4N addition to lipid A and PM resistance. The flanking genes of the operon (*pmrG* and *pmrD*) were not necessary for PM resistance, but *pmrD* was shown to be regulated by the PhoP-PhoQ regulatory system. BALB/c mice inoculated with *pmrA* and *pmrHFIJKLM* mutant strains demonstrated virulence attenuation when the strains were administered orally but not when they were administered intraperitoneally, indicating that Ara4N addition may be important for resistance to host innate defenses within intestinal tissues.

The key to success for many bacteria in causing infection is colonization of host tissues. Enteric bacteria, such as *Salmonella* spp., have to survive in harsh host microenvironments including the intestinal mucosa. At the intestinal mucosa, these bacteria encounter host defense mechanisms including antimicrobial peptides (AP), which are cationic, amphipathic molecules that kill bacteria by membrane permeabilization. Within the intestine, AP are secreted into the lumen by Paneth cells located in the base of intestinal crypts. AP are also found within phagocytic cells located in the intestinal submucosa. Following oral ingestion, typhoid fever-causing strains of *Salmonella* can transcytose through M cells and intestinal epithelial cells and are then taken up by and survive within resident phagocytes. The ability of salmonellae to survive within the host intestine and within professional phagocytes is likely to depend, at least in part, on mechanisms of resistance to AP.

Lipopolysaccharade (LPS) is the major surface component of gram-negative bacteria. Lipid A is the bioactive component of LPS that comprises the outer leaflet of the gram-negative bacterial outer membrane. Phosphate groups on lipid A and LPS core components result in the bacterial surface having a net negative charge. This charge plays a significant role in the electrostatic interaction of cationic AP with the bacterial surface. Gram-negative organisms can synthesize LPS with specific lipid A and core modifications in response to environmental conditions that include those found in host tissues. Lipid A modifications are best characterized for *Salmonella* spp. in which the addition of palmitate or 4-aminoarabinose (Ara4N) to lipid A is coordinately regulated by the two-component system PhoP-PhoQ (9–11), which is necessary for bacterial survival within macrophages and within the host (6, 12, 15). Ara4N-containing lipid A results in a less negatively charged bacterial surface, which reduces AP binding and promotes resistance to the cationic AP polymyxin B (PM), bactericidal/permeability-increasing protein, and azurocidin (17, 18).

PhoP-PhoQ-mediated Ara4N addition to lipid A occurs through transcriptional activation of the genes encoding another two-component system, PmrA-PmrB, which can itself be activated independent of PhoP-PhoQ by low-pH or high-iron conditions (9, 14, 20). Two loci, *pmrE* and *pmrHFIJKLM*, are regulated by PmrA-PmrB and are essential for both biosynthesis of lipid A containing Ara4N and for resistance to PM (1, 8). This study was conducted to examine the individual and collective roles of the genes of the *pmrHFIJKLM* operon, as well as flanking genes, in AP resistance, modification of lipid A, and *Salmonella enterica* serovar Typhimurium virulence.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and reagents. Bacterial strains used in this study are listed in Table 1. Cultures were grown on Luria-Bertani (LB) agar plates or broth at 37°C with aeration. Antibiotics were used at the following concentrations: chloramphenicol, 25 μg/ml; ampicillin, 50 μg/ml; kanamycin, 45 μg/ml; tetracycline, 15 μl/ml; streptomycin, 1,000 μg/ml. The chromogenic substrate for β-galactosidase, 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal), was used at a concentration of 40 μg/ml.

Construction of luciferase fusions and transcription assays. *pmrD* was amplified from plasmid pKK01 (8) by PCR using primers JG177 (5′ GGG AAT TCT

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TABLE 1. Bacterial strains and plasmids and relevant properties

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Strains[a]		
SM10λpir	*thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA*::RP4-2-Tc::Mu	
JSG210	ATCC 14028s (WT)	ATCC
JSG430	*pho24 pmrA*::Tn*10d* (PhoP^c PmrA$^-$)	9
JSG437	*phoP*::Tn*10d-tet pmrA505 zjd*::Tn*10d-cam* (PhoP$^-$ PmrA^c)	9
JSG435	ATCC 14028s *pmrA 505 zjd*::Tn*10d-cam* (PmrA^c)	9
JSG421	*pmrA*::Tn*10d* (PmrA$^-$)	9
JSG485	JSG435 *pmrF1*::Tn*10d*	8
JM109	F′*traD36 lacI* Δ(*lacZ*)M15 *proA+B+*/e14$^-$ (*mcrA*) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* (r_K^- m_K^-) *relA1 supE44 recA1*	Promega
JSG224	*phoN2 zxx*::6251 Tn*10d-cam* (CS019)	15
JSG844	JSG435 *rpsL* (PmrA^c Sm^r)	This study
JSG1097	JSG844; Δ*pmrH*	This study
JSG911	JSG844; Δ*pmrF*	This study
JSG868	JSG844; Δ*pmrI*	This study
JSG1098	JSG844; Δ*pmrJ*	This study
JSG883	JSG844; Δ*pmrK*	This study
JSG884	JSG844; Δ*pmrL*	This study
JSG1046	JSG421 *pmrM*::*luc*	This study
JSG1047	JSG435 *pmrM*::*luc*	This study
JSG1048	JSG485 *pmrM*::*luc*	This study
JSG989	JSG224 *pmrD*::*luc*	This study
Plasmids		
pGPL01	Firefly luciferase recorder, suicide vector	9
pKAS32	*rpsL* suicide vector	19
pSR01	pKAS32; Δ*pmrH*	This study
pSR02	pKAS32; Δ*pmrI*	This study
pSR03	pKAS32; Δ*pmrK*	This study
pSR04	pKAS32; Δ*pmrL*	This study
pSR05	pKAS32; Δ*pmrF*	This study
pSR06	pKAS32; Δ*pmrJ*	This study
pLB02	Firefly luciferase recorder, suicide vector	7
pGPLM02	pGPL01 with *pmrM Eco*RI-*Kpn*I PCR fragment amplified with JG252 and JG253	This study
pMRD1	pGPL01 with *pmrD Eco*RI-*Kpn*I PCR fragment amplified with JG177 and JG178	This study
pLB2223	118-bp *pmrHFIJKLM* operon promoter-fragment (JG122 and JG123) in pLB02 (*Eco*RI-*Kpn*I)	This study
pQPA01	pQE30 containing the *pmrA* (WT) gene amplified with primers JG106 and JG107 (*Bam*HI-*Pst*I)	This study
pQP505	pQE30 containing the *pmrA*-constitutive (JSG435) gene amplified with primers JG106 and JG107 (*Bam*HI-*Pst*I)	This study
pQE30	Amino-terminal His tag vector	Qiagen

[a] Strain SMI0λpir is *E. coli*; the other strains are *S. enterica* serovar Typhimurium.

GCC ATG TTC TGG TGC TGT GC 3′) and JG178 (5′ GGG GTA CCG CGC GTC AAC CGC TGC CAT TC 3′). This fragment was digested with the restriction enzymes *Eco*RI and *Kpn*I and ligated into pGPL01, which is a Pir-dependent suicide vector containing a promoterless firefly luciferase gene downstream of the multiple cloning site (9). This clone, transformed into *Escherichia coli* SM10λPir, was mated with CS019, and a single recombinant was identified. P22HTint phage transduction was used to transduce the *pmrD*::*luc* fusion into various strains. Lucifierase activity was determined as previously described (9) after growth of strains to log phase (optical density at 600 nm [OD_{600}] ~0.6). A *pmrM* luciferase fusion was constructed in a manner identical to that for the *pmrD* fusion with primers JG252 (5′ GGAATTCGGACTGATAAGCGTTGCG 3′) and JG253 (5′ GGGGTACCTGATGCACGCTGTTATTCC 3′). The luciferase fusion to the *pmrHFIJKLM* promoter was also constructed similarly to the *pmrD* and *pmrM* fusions, except that the promoter fragment was cloned into pLB02 (7), which contains DNA homologous to a region downstream of *pagC*, where the vector is recombined. The primers used to amplify the *pmrHFIJKLM* promoter were JG122 (5′ GGGGTACCTGAAAGCCGCTTT TC 3′) and JG123 (5′ GGAATTCTTTTTACTTCACCT 3′).

Analysis of LPS modification. LPS was isolated by Mg^{2+}-ethanol precipitation as described by Darveau and Hancock (4), and lipid A was isolated by hydrolysis in 1% sodium dodecyl sulfate at pH 4.5 (3). Negative-ion matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry was performed as described previously (5). Lyophilized lipid A was dissolved with 5 μl of a 5-chloro-2-mercaptobenzothiazole (CMBT) MALDI matrix in chloroform-methanol (1:1 [vol/vol]) and then applied (1 μl) to the sample plate. All MALDI-TOF experiments were performed using a BiflexIII mass spectrometer (Bruker Daltonics, Inc., Billerica, Mass.). Mass spectroscopy tracings were analyzed for the presence or absence of Ara4N based on known peak locations at *m/z* 1,929 (hexa-acylated lipid A with Ara4N) and 2,167 (hepta-acylated lipid A with Ara4N).

Assays of DNA binding. PCR fragments of the *pmrHFIJKLM* and *pmrCAB* operons were digested with *Eco*RI (located at one end of each fragment) and labeled with [α-^{32}P]dATP (10 μCi/reaction) with the Klenow fragment of DNA polymerase I. Binding reactions were carried out by the incubation of the purified PmrA or PmrA-constitutive (PmrA^c) His-tagged proteins with binding buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA, 100 mM KCl, 0.1 mM dithiothreitol, 5% glycerol, 75 μg of sonicated salmon sperm DNA/ml, 10 mg of bovine serum albumin/ml for 5 min at room temperature. Labeled probe (1 to 5 ng) was added, and the reaction mixture was incubated for 10 min at room temperature. Samples were electophoresed at 4°C on a 5% acrylamide gel at 200 V. Gels were subsequently dried and autoradiographed.

Animal studies. Survival assays were accomplished as follows. Female BALB/c mice were inoculated orally with 20 μl of an overnight culture (washed and diluted to 10^6 organisms/20 μl in phosphate-buffered saline (PBS), which is 1 log unit above the 50% lethal dose [LD_{50}]) using the animal's swallowing reflex. Mice were prefed 20 μl of 10% sodium bicarbonate 30 min prior to bacterial inoculation. Alternatively, after dilution in PBS, animals were inoculated via the intraperitoneal route with various numbers of organisms in a 100-μl volume. Overnight cultures were plated to enumerate the numbers of organisms inoculated. The average days of survival and the numbers of surviving mice were recorded. In competition assays, female BALB/c mice were inoculated orally as described above. Mice were inoculated with a mixture of 10 μl of two different strains (each containing 10^6 organisms/10 μl). Mice were sacrificed when clearly moribund, and livers and spleens were removed. The organs were homogenized in 1 × PBS, diluted, and plated onto the appropriate antibiotic-containing agar plates, which selected for one of the two competing strains.

MIC assays of AP resistance. PM (U.S. Biochemicals; 8,040 U mg^{-1}) was used at concentrations of 0.0084 to 12 μg/ml in both plate and broth assays. Standard MIC testing of susceptibility to PM was accomplished as described by Steinberg et al. (21).

TABLE 2. Primers used for construction of each nonpolar deletion

Deleted gene	Primer	Primer sequence (5′ to 3′)
pmrH	JG234	CAT GCC ATG GCA TTG AAA GCC GCT TTT C
	JG235	GCT CTA GAC CCG TGC TGT CTG ACA GG
	JG236	CAT GCC ATG GGA TAG CAG GAC AAT AAG CAT G
	JG237	CGG AAT TCG TGA TGC ACC GGG ATC TC
pmrF	JG183	CAT GCC ATG GGT CAA ACA TGC TTA TTG TCC TGC TAT C
	JG181	GCT CTA GAC CCT TGA CCG TAA TCC CCG TG
	JG184	CAT GCC ATG GGC CGT TTA CTG AGG AAA GCC ACC AAT G
	JG202	CGG AAT TCG CCG TTA TAG CTG AAC GCG CC
pmrI	JG185	CAT GCC ATG GTG GTG GCT TTC CTC AGT AAA C
	JG187	GCT CTA GAC AGG ACA GCC TGT TTC GCA AAT CG
	JG186	CAT GCC ATG GCG ATA TCG CGG AAC GCG CAT C
	JG188	CGG AAT TCC CAG GTA TTG AAC GAC TGC GE
pmrJ	JG245	CAT GCC ATG GGC GTA AAC CGA CTT TCG TCA TGA TGC G
	JG246	GCT CTA GAC AGC GGT AAA CGT CGC ATT CG
	JG247	CAT GCC ATG GGG CAG CGA GCG CCT CAT GAT G
	JG248	CGG AAT TCG AAA GAA CAT AGG TGG GCA GTT TC
pmrK	JG191	CAT GCC ATG GGC GTA TCG ATT TCA TCA TGA G
	JG193	GCT CTA GAC ACC ATG CGT GGC AGA CTC AC
	JG192	CAT GCC ATG GGG TGT TAA TTC AGT ATC GGC C
	JG196	CGG AAT TCG CAA GCC ATC ATG ATC TGG CG
pmrL	JG194	CAT GCC ATG GCA GAA CGA CGC CGA TCA TTT AGG C
	JG193	GCT CTA GAC ACC ATG CGT GGC AGA CTC AC
	JG195	CAT GCC ATG GGG CAT AAT GGG CGT AAT GTG G
	JG196	CGG AAT TCG CAA GCC ATC ATG ATC TGG CG

Deletion analysis of the *pmrHFIJKLM* operon. Flanking regions of the genes of the operon were amplified using PCR and digested with the restriction enzyme *Nco*I. Primers that were used are listed in Table 2. The flanking regions were then ligated together and digested with the restriction enzymes *Eco*RI (3′) and *Xba*I (5′). This fragment was then ligated into plasmid pKAS32, a suicide vector containing a dominant streptomycin sensitivity allele (19). Deletion constructs in SM10λPir were mated with JSG844, and single recombinants were selected by plasmid-encoded resistance to ampicillin and chromosome-encoded resistance to chloramphenicol and sensitivity to streptomycin. Losses of the plasmid (secondary recombination events) were selected following overnight incubation by growth on 1,000-μg/ml streptomycin-containing agar. Colonies were further screened by PCR to confirm the incorporation of the deletion. Mutation of the *pmrM* gene was accomplished not by the construction of a nonpolar deletion but by the construction of the *pmrM*::*luc* fusion described above, which essentially inserts plasmid pGPL01 into the *pmrM* coding sequence.

Confirmation of in-frame deletions and complementation experiments. PCR fragments overlapping the deletion sites were sequenced to confirm correct incorporation of the in-frame deletion. Deletions in *pmrH* and *pmrF* were complemented with plasmid pKK013 (carrying only the intact *pmrH* and *pmrF* genes). The *pmrJ, pmrK, pmrL*, and *pmrM* genes were complemented by pKK012-1 (carrying only the intact *pmrJKL* and *pmrM* genes). To further confirm nonpolarity, the *pmrM*::*luc* fusion was mated into each deletion strain and luciferase activity was monitored.

Isolation of the PmrA protein. The *pmrA* gene or the mutant, constitutive allele of this gene (*pmrA505*) was amplified by PCR with primers JG106 (5′ GCGGATCCAAGATACTGATTGTTGAAG 3′) and JG107 (5′ AACTGCAG TTAGCTTTCCTCAGTGGC 3′) from strains ATCC 14028s (wild type [WT]) and JSG435, respectively. These fragments were cloned into the N-terminal His tag-encoding vector pQE30 and transformed into strain XL1-Blue-MRF′. Transcription of the genes was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) upon movement of the plasmids into strain M15/pREP4, and the His-tagged proteins were purified as previously described (7).

RESULTS

Definition of the *pmrHFIJKLM* transcriptional unit and promoter. The *pmrHFIJKLM* genes are unidirectionally transcribed, and the individual open reading frames are separated by no more than 5 bp, as shown in the diagram of this chromosomal region (Fig. 1). This arrangement suggested that these genes formed a typical prokaryotic operon. To demonstrate that these genes were indeed cotranscribed, a luciferase fusion to *pmrM*, the last gene of the operon, was constructed in single copy on the chromosome. Upon the movement of this fusion into PmrA-null (PmrA$^-$), PmrA^c, and PmrA^c *pmrF*::Tn*10d* backgrounds by P22-mediated transduction, luciferase activity was monitored. As shown in Fig. 2, this fusion was highly regulated by PmrA-PmrB, and the reporter activity in a PmrA$^-$ strain was similar to that in a strain containing the *pmrF*::Tn*10d* insertion, demonstrating that the insertion in *pmrF* had a polar effect on downstream gene transcription. Therefore, these data strongly suggest that the *pmrHFIJKLM* genes are cotranscribed and that the promoter is upstream of this operon.

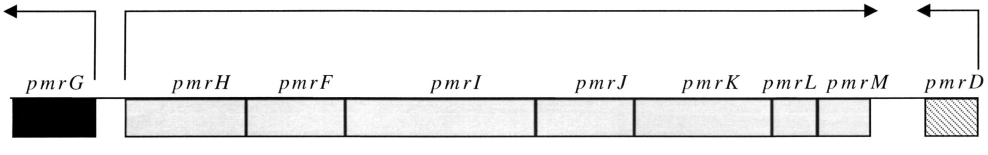

FIG. 1. Diagram of the chromosomal region containing the *pmrG, pmrD*, and *pmrHFIJKLM* genes. Arrows, direction of transcription.

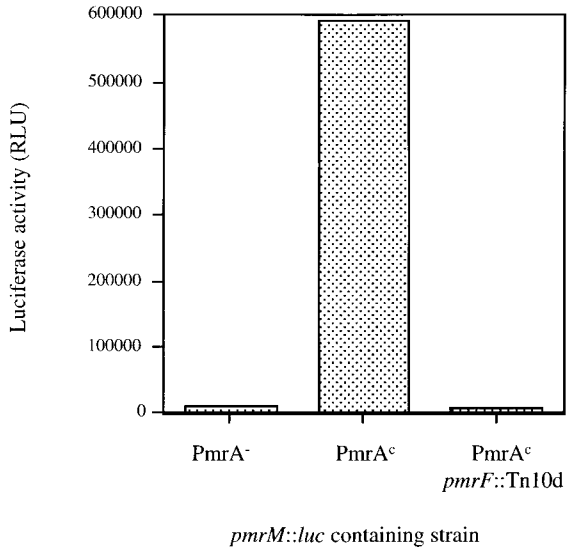

FIG. 2. The *pmrHFIJKLM* genes are expressed as an operon. PmrA$^-$, PmrA^c, and PmrA^c *pmrF*::Tn*10d* strains were examined. Each strain contained a single-copy *pmrM*::*luc* fusion and was assayed in logarithmic phase (OD_{600}, 0.6) for firefly luciferase-activity (expressed as relative light units [RLU]). The data are from a single experiment of three independent assays that gave similar results.

To define the DNA region of the *pmrHFIJKLM* operon necessary for PmrA-mediated activation, a 118-bp region of the predicted promoter was examined for regulation in a single-copy luciferase assay system and also by gel mobility shift experiments for DNA binding. To examine promoter regulation, the fragment was cloned into the luciferase fusion/suicide vector pLB02 (7) and subsequently recombined (into an innocuous site downstream of *pagC*) onto the chromosomes of PmrA$^-$ and PmrA^c strains. As shown in Fig. 3, analysis of luciferase activity showed this promoter to be 3,442-fold activated by PmrA. Furthermore, little activity of the *pmrHFIJKLM* promoter-luciferase gene fusion was observed in a PmrA$^-$ or PhoP^c PmrA$^-$ strain. This demonstrates that there is little transcription of the *pmrHFIJKLM* genes in the absence of PmrA and that direct activation of the promoter is mediated by PmrA and not PhoP.

To demonstrate PmrA binding to this promoter fragment, gel mobility shift experiments were conducted with the purified PmrA-His protein, as well as a mutant version of this protein (17) previously shown to result in constitutive activation of PmrA-PmrB regulated genes (PmrA^c-His). As shown in Fig. 4, the *pmrHFIJKLM* promoter fragment bound to both PmrA-His and PmrA^c-His. Densitometry studies suggest that the PmrA^c-His protein resulted in a threefold increase in the amount of shifted promoter fragment, suggesting that constitutive activation of PmrA-regulated genes by this protein may be due to increased affinity for promoter binding.

To extend the results discussed above to the definition of a consensus DNA binding site for PmrA, we performed gel shift analysis on promoter fragments of another PmrA-regulated locus, *pmrCAB*. The *pmrCAB* operon is positively autoregulated by PmrA (9, 20). PCR fragments extending 75, 100, 125, and 200 bp upstream from the ATG start codon of the *pmrC* gene were examined by gel mobility shift experiments, and all fragments were shown to bind PmrA (Fig. 4; data not shown for the 100- and 125-bp fragments). Based on these studies, a 40-bp fragment corresponding to a region directly upstream of the predicted −35 sequence was amplified by PCR and analyzed in the gel shift assay. As shown in Fig. 5, this fragment also bound PmrA. In addition, the binding of PmrA to the *pmrCAB* promoter was shown to be specific. Binding could be completely abolished with 50 ng of unlabeled promoter fragment DNA; however, 100 ng of unlabeled, noncompetitive (pUC19) DNA did not produce any reduction of PmrA-*pmrCAB* promoter binding. Therefore, the PmrA binding site is contained within this 40-bp sequence.

Regulation and role in PM resistance of the flanking genes of the operon. It has been shown that the gene upstream of and divergently transcribed from the *pmrHFIJKLM* operon is the PmrA-PmrB-regulated gene, *pmrG* (8). The gene directly downstream and divergently transcribed from the *pmrHFIJKLM* operon is *pmrD*, which imparts increased PM resistance to *S. enterica* serovar Typhimurium when expressed from a multicopy plasmid (16). To further the studies of this chromosomal region, experiments were designed to determine the potential regulation of *pmrD* and the role of *pmrD* and *pmrG* in resistance to PM.

To examine both the regulation and role in AP resistance of the *pmrD* gene, a fragment internal to the gene was cloned into the luciferase fusion/suicide vector pGPL01, and this fusion was recombined onto the chromosome. This resulted in both a gene disruption and a transcriptional fusion of *pmrD* to the *luc* gene. Regulation of this locus was examined by moving this region into PhoP$^-$, PhoP^c, PmrA$^-$, and PmrA^c backgrounds by P22-mediated transduction, followed by assay of firefly luciferase activity. We chose to examine regulation by PhoP and PmrA as these two regulators are directly or indirectly involved in the regulation of *pmrG* and the *pmrHFIJKLM* operon. As shown in Fig. 6, this locus is activated 66-fold by PhoP-PhoQ but is not regulated by PmrA-PmrB. This *pmrD* mutant (in a high-level-PM-resistant strain background [PmrA^c]) was also examined on PM-containing plates and by MIC analysis for an effect on resistance to PM. Both plate and MIC assays showed that the (single-copy) loss of *pmrD* had no effect on resistance to PM under the growth conditions employed (data not shown).

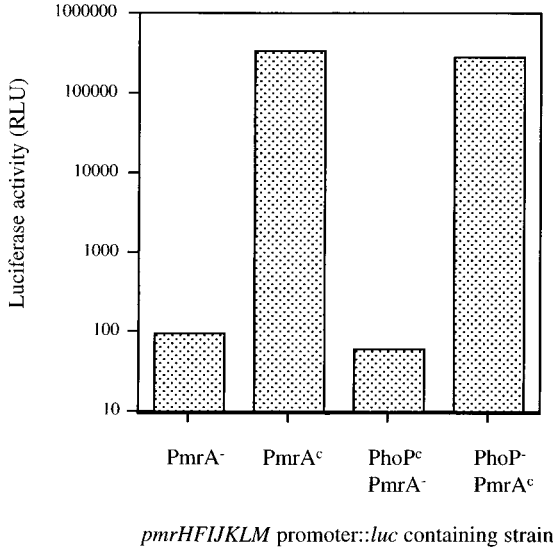

FIG. 3. The *pmrHFIJKLM* promoter is highly PmrA-PmrB activated and tightly regulated. PmrA$^-$, PmrA^c, PhoP^c PmrA$^-$, and PhoP$^-$ PmrA^c strains containing a single-copy *pmrHFIJKLM* promoter::*luc* fusion were assayed in logarithmic phase (OD_{600}, 0.6) for firefly luciferase activity (expressed as relative light units [RLU]). Note that the *y*-axis scale is logarithmic. The data are from a single experiment of three independent assays that gave similar results.

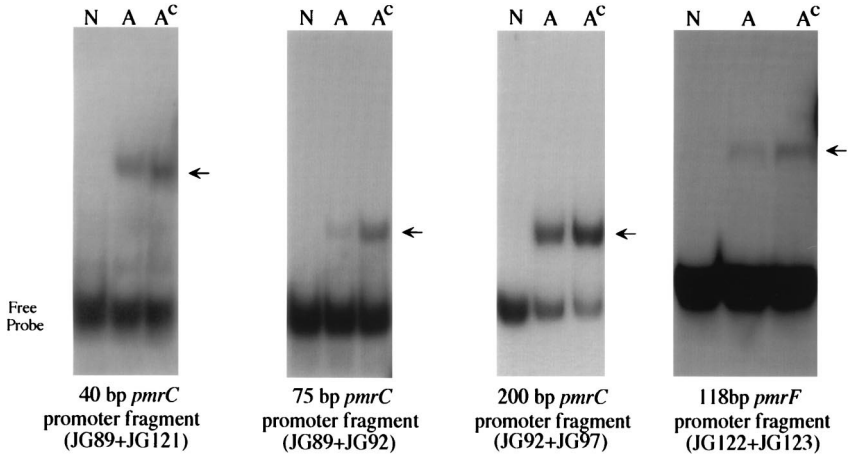

FIG. 4. Binding of the PmrA-His and PmrA^c-His proteins to the *pmrHFIJKLM* promoter (called *pmrF*) and the *pmrCAB* promoter (called *pmrC*). The sizes of the fragments used and the primers used to amplify the fragments are noted. Arrows, shifted fragments. N, no protein added; A, PmrA-His added; A^c, PmrA^c-His added.

To examine the effect of the loss of the PmrA-PmrB-regulated *pmrG* gene on PM resistance, *pmrG*::Tn*phoA* was transduced into a PmrA^c background and the resulting strains were examined for resistance to PM on plates and by MIC analysis. Like the loss of *pmrD*, the loss of *pmrG* had no effect on resistance to PM under standard growth conditions. Therefore, this chromosomal region contains three regulated loci, one by PhoP-PhoQ (*pmrD*) and two by PmrA-PmrB (*pmrG* and *pmrHFIJKLM*), while of these three loci only mutations in *pmrHFIJKLM* affect PM resistance when strains are grown on standard laboratory media.

Deletion analysis of the *pmrHFIJKLM* operon: roles of individual loci in LPS modification and PM resistance. To examine the roles of individual genes of the *pmrHFIJKLM* operon in resistance to PM and in Ara4N modification of lipid A, nonpolar deletions were constructed in *pmrH*, *pmrF*, *pmrI*, *pmrJ*, *pmrK*, and *pmrL*. An insertion mutation was created in *pmrM* by recombination of a suicide plasmid within this locus. The chromosomally incorporated deletions were confirmed to be nonpolar by sequencing the deletion junctions and by demonstrating the lack of an effect on luciferase activity of a *pmrM*::*luc* fusion mated into each deletion background. These nonpolar deletion or mutant strains (constructed in a PmrA^c background) were then examined for resistance to PM. All mutations except that in *pmrM* had a major effect on resistance to PM (MIC of PmrA^c and Δ*pmrM* strains, 4 μg/ml; MIC of strains with *pmrHFIJKL* deletions, 0.1 μg/ml). To determine if the loss in resistance to PM was due to the loss of Ara4N in lipid A, MALDI-TOF mass spectroscopy was performed on purified lipid A. This analysis confirmed the absence of Ara4N in deletion strains with reduced resistance to PM (Fig. 7). This can be seen by the absence of peaks at *m*/*z* 1,929 and 2,167, which correspond to hexa- and hepta-acyl lipid A with Ara4N, respectively. Loss of the peaks at *m*/*z* 1,949 and 2,189 is also evident in the mutants, as these peaks correspond to hexa- and hepta-acyl lipid A, respectively, with Ara4N and the PhoP-PhoQ-mediated hydroxyl addition to myristate. The profile of the *pmrM* mutant was identical to that of the PmrA^c strain (the parental strain of all mutants examined [8]), further demonstrating that this gene plays no role in Ara4N addition to lipid A or PM resistance.

LPS modification affects oral virulence. It has been shown previously that a strain containing a *pmrF*::Tn*10d* insertion was unable to add Ara4N to lipid A, which resulted in a significant decrease in resistance to PM (8). To determine the effect on *S. enterica* serovar Typhimurium virulence of the lipid A modification with Ara4N, the *pmrF*::Tn*10d* mutant was examined in the mouse model of typhoid fever.

Mice inoculated orally with the *pmrF*::Tn*10d* mutant at a dose approximately 1 log unit above the LD_{50} had a sevenfold increase in survival versus mice inoculated with the WT strain (62 versus 9%) (Fig. 8A). Furthermore, of those mice inoculated with the *pmrF*::Tn*10d* mutant, the time to death of some of the mice was greater than 20 days, while the mice inoculated with the WT consistently averaged about 12 days (Fig. 8B). To confirm the observed virulence defect, competition assays were performed with the *pmrF*::Tn*10d* mutant and the WT strain. These data show that the *pmrF*::Tn*10d* mutant was dramatically impaired (10- to 1,000-fold) in its ability to compete with the WT strain (Fig. 8C). In vitro competition assays demonstrated that the defect was not due to a growth deficiency (Fig. 8C). A PmrA$^-$ strain was also examined in all of the above-mentioned virulence assays. Although it was expected that a PmrA$^-$ strain should show the same, if not a greater, defect than the *pmrF*::Tn*10d* mutant, the virulence defect that was observed was not as dramatic as that seen with the *pmrF*::Tn*10d* mutant (Fig. 8).

Intraperitoneal inoculation of JSG485 (*pmrF*::Tn*10d*) and PmrA$^-$ strains into mice had no effect on the LD_{50} (<20 organisms) or the survival time of the mice compared to in-

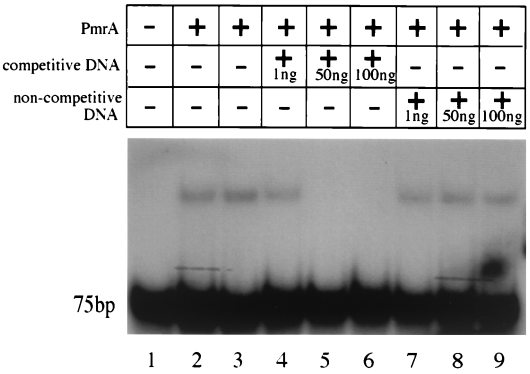

FIG. 5. PmrA-His protein binding to the *pmrCAB* promoter is specific. The presence or absence of the PmrA-His protein, competitive DNA, and noncompetitive DNA is noted at the top. The numbers at the bottom are the lane numbers.

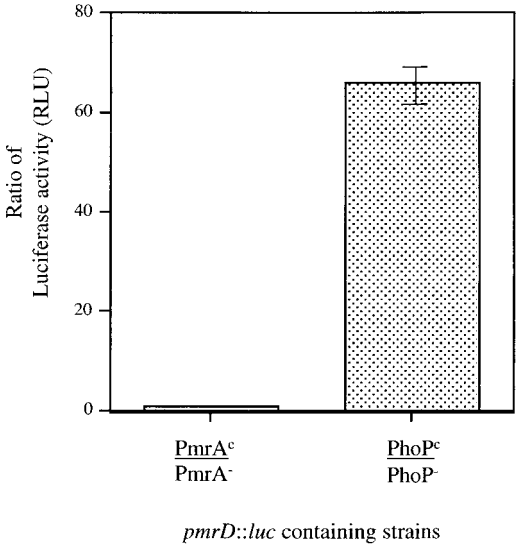

FIG. 6. PhoP-PhoQ but not PmrA-PmrB regulates the *pmrD* gene. PmrA$^-$, PmrA^c, PhoP^c, and Phop$^-$ strains containing a single-copy *pmrD*::*luc* fusion were assayed in logarithmic phase (OD_{600}, 0.6) for firefly luciferase activity (expressed as relative light units [RLU]). Results are expressed as ratios of firefly luciferase activity in PmrA^c versus PmrA$^-$ backgrounds and in PhoP^c versus PhoP$^-$ backgrounds.

oculation with the WT (data not shown). Therefore, loss of Ara4N affects *S. enterica* serovar Typhimurium virulence during early events in a natural infection but not at later stages of macrophage survival and growth within the liver and spleen.

DISCUSSION

Activation of the PmrA-PmrB two-component regulatory system by environmental signals or by mutations within *pmrA* that result in a constitutive phenotype activates the transcription of genes whose products covalently modify LPS. These modifications (Ara4N and phosphoethanolamine) result in increased resistance to AP, which are membrane-active pore formers present at mucosal surfaces and within macrophages and neutrophils. Increased resistance to these peptides provides the bacterium with a survival advantage within the host. In this work, we presented a detailed characterization of an island of PmrA-PmrB- and PhoP-PhoQ-regulated genes. Experiments were conducted to examine the roles of the individual genes in this region in resistance to AP and what effect the loss of genes in this region has on *S. enterica* serovar Typhimurium virulence.

The *pmrHFIJKLM* locus is regulated by PmrA-PmrB, and a *pmrF*::Tn*10d* insertion was shown to eliminate the ability to modify lipid A with Ara4N and markedly reduced resistance to PM (8). These genes were shown to be cotranscribed, as a *pmrM*::*luc* fusion was activated by PmrA-PmrB and luciferase activity was dramatically reduced when the reporter fusion was recombined downstream of the *pmrF*::Tn*10d* insertion. Fusion of the promoter of this operon to the gene encoding firefly luciferase showed it to be over 3,000-fold activated by PmrA-PmrB. It is intriguing to propose that this highly expressed in vivo-activated promoter may be useful in a live-attenuated *Salmonella* vaccine for the expression of heterologous antigens. This approach has several advantages including high-level expression in host antigen-presenting cells and the ability to construct stable, single-copy chromosomal constructs. In fact, this approach has been successfully tested using *phoP*-regulated gene promoters (2, 13).

Studies of the regulators of two-component systems have shown that they bind to consensus sequences in the promoters of regulated genes. Therefore, experiments were designed to examine the interaction of PmrA with *pmr* promoters. The PmrA protein was purified (both WT and constitutive forms) and used in promoter binding studies. A 118-bp region of the *pmrHFIJKLM* promoter and a 40-bp region of the *pmrCAB* operon promoter were found to shift with each protein. The 40-bp region of the *pmrCAB* promoter is located just upstream of the −35 region, which places the putative binding site in a position comparable to those of other transcriptional activators of the OmpR family. Recently, Wøsten and Groisman (22) demonstrated similar binding of the PmrA protein to the promoters of activated genes and defined a consensus binding site of 5′ TTAAKTTCTTAAKGTT 3′ for PmrA. This site is located within both the *pmrCAB* and *pmrHFIJKLM* operon promoter fragments that bound PmrA (16 of 16 match in each). The PmrA^c protein bound approximately threefold more pro-

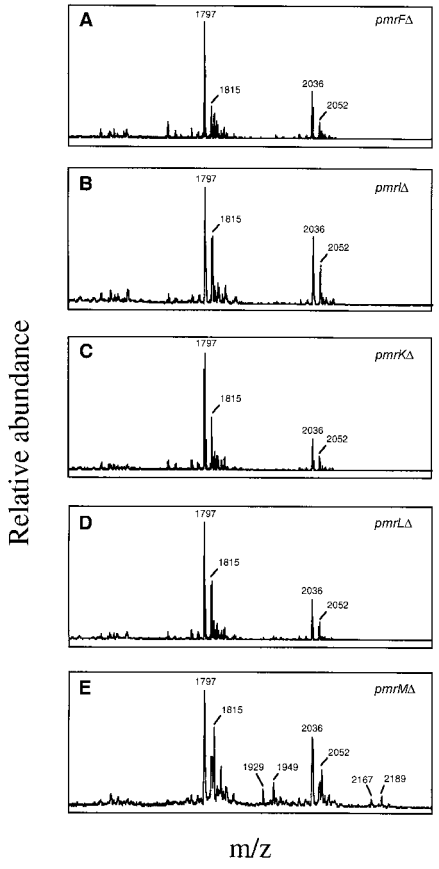

FIG. 7. Mass spectroscopy of the lipid A from *pmrF*, *pmrI*, *pmrK*, *pmrL,* and *pmrM* nonpolar deletion strains. The *m*/*z* ratios and related structures are as follows: 1,797, unmodified hexa-acyl lipid A; 1,815, hexa-acyl lipid A with 2-OH myristate; 1,929, hexa-acyl lipid A with Ara4N; 1,949, hexa-acyl lipid A with 2-OH myristate and Ara4N; 2,036, unmodified hepta-acyl lipid A; 2,052, hepta-acyl lipid A with 2-OH myristate; 2,167, hepta-acyl lipid A with Ara4N; 2,189, hepta-acyl lipid A with 2-OH myristate and Ara4N. The lipid A from strains with *pmrH* and *pmrJ* nonpolar deletions was also examined, and the results were identical to those shown in panels A through D but were not included due to differences in the scale of *m*/*z* values examined and the difficulty in comparing these tracings to those for the other samples. The PmrA^c strain that is the parental background of the strains with the examined deletions has been examined previously (8), and results for it are identical to those shown in panel E.

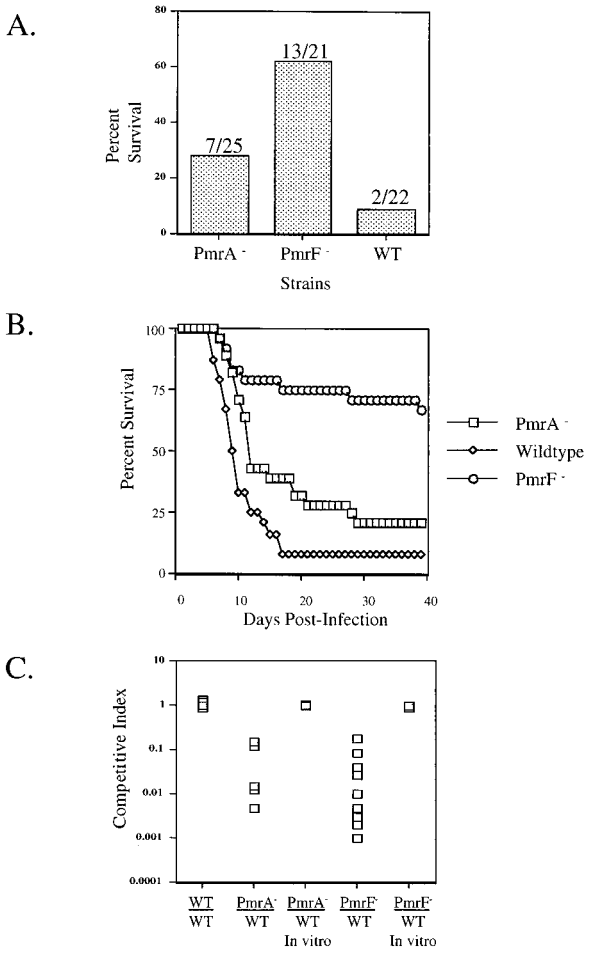

FIG. 8. Mutations in *pmrA* or the *pmrHFIJKLM* operon affect virulence by the oral route. Mice were inoculated orally with a dose of 10^6 CFU. BALB/c mice were infected with ATCC 14028s (WT), WT with a *pmrA*::Tn*10d* insertion (PmrA$^-$), WT with a *pmrF*::Tn*10d* insertion (PmrF$^-$), or a combination of two of these strains. (A) Percent survival of BALB/c mice receiving the WT, PmrA$^-$, or PmrF$^-$ strain. The numbers above the bars represent the number of surviving mice per the number of mice tested. (B) Percent survival of mice over 40 days postinfection. (C) Competition assays of WT versus PmrA$^-$ and WT versus PmrF$^-$ strains. The competitive index is the ratio of CFU in the livers of mice infected with a PmrA$^-$, WT, or PmrF$^-$ strain to CFU in livers of mice infected with the WT strain. The WT-versus-WT experiment involved two WT strains marked with different antibiotic resistances. The competitions labeled in vitro were performed by growth overnight in LB medium to ensure that any in vivo effects were not due to a growth disadvantage. Both liver and spleen were examined in the competition assays, but only the data for the liver are shown.

moter fragments than the WT PmrA protein, suggesting that the constitutive activity of this protein may be due to increased promoter affinity.

Nonpolar deletions were constructed in each gene of the *pmrHFIJKLM* operon (except *pmrM*, which was disrupted by insertion), and the effect of these mutations on PM resistance was measured. All of the genes except *pmrM* had an effect on PM resistance, which resulted in an MIC level similar to that of a PmrA$^-$ strain (MIC of PmrA^c and Δ*pmrM* strains, 4 μg/ml; MIC of *pmrHFIJKL* deletion strains, 0.1 μg/ml). Based on protein homologies and known sugar biosynthesis pathways, a model involving the *pmrHFIJKLM* operon and *pmrE* has been proposed for Ara4N biosynthesis and addition to lipid A (1). This pathway begins with the conversion of UDP-glucose to UDP-glucuronic acid by the *pmrE* gene product, followed by the conversion to UDP-4-amino-L-deoxyarabinose and attachment of this moiety to lipid A by the products of the *pmrHFIJKLM* operon. This model, however, was unable to identify functions for PmrL and PmrM, as neither of these small proteins has any strong similarities to proteins in GenBank. From this study, it is clear that PmrL does belong at some point in the pathway but PmrM does not. Therefore, the function of PmrM remains unknown.

Previous studies showed that the gene upstream of the *pmrHFIJKLM* operon, *pmrG*, was regulated by PmrA-PmrB (8). Because the effect of this gene on the PM resistance phenotype had not been previously examined, a *pmrG* mutant was assayed for PM resistance in a PmrA^c background and the *pmrG* mutation was shown to have no effect. The gene downstream of the operon, *pmrD*, however, had previously been shown to have an effect on PM resistance only in high copy number. To see if it too was regulated by PmrA-PmrB, a firefly luciferase fusion was created and expression was measured when strains were grown in various backgrounds. This data surprisingly demonstrated that *pmrD* was regulated by PhoP-PhoQ and not PmrA-PmrB. Interestingly, work by others accomplished concurrently with our studies also showed that *pmrD* was regulated by PhoP-PhoQ and that the *pmrD* gene product mediates interaction of the PhoP-PhoQ system with the PmrA-PmrB system, as PmrA-regulated genes are not expressed in a *pmrD* mutant grown under PhoP-PhoQ-inducing conditions (low magnesium) (14). PmrD is thought to exert its effect on PmrA-PmrB through a posttranscriptional mechanism, possibly involving its effect on PmrA phosphorylation. These results likely explain why *pmrD* had an effect on PM resistance only when in multicopy and when strains were grown in LB medium (non-phoP-PhoQ-inducing conditions).

Loss of expression of the *pmrHFIJKLM* operon eliminates Ara4A addition to lipid A and PM resistance. To determine if this loss played a role in *S. enterica* serovar Typhimurium pathogenesis, a *pmrF* mutant was examined in mice and was shown to have markedly reduced virulence by the oral route but not by the intraperitoneal route. In addition, for most of the mice that did eventually die, the time from inoculation to death was much longer than for mice infected with a WT strain. A *pmrA* mutant showed similar results, but did not display as severe of a virulence defect as the *pmrF* mutant strain. This was surprising, as PmrA activates the *pmrF*-containing operon and therefore was expected to give similar results. These data suggest that the *pmrHFIJKLM* operon may be expressed in vivo by a mechanism independent of PmrA-PmrB.

At this time, it is unclear at which stage of infection the *pmrHFIJKLM* locus plays a role. The PmrA-PmrB system (and the PhoP-PhoQ system, which can activate transcription of *pmrAB*) is thought to be induced within macrophages, and if the role of the PmrA-PmrB regulatory system is to promote survival within macrophages, then a defect should have been observed in mice by both the intraperitoneal and oral routes. Therefore, the data suggest that the defect is prior to the interaction with macrophages. In vitro experiments show no defect in invasion or type III secretion with *pmrA* or *pmrF* mutants (J. S. Gunn, unpublished results). Therefore, it is intriguing to speculate that PmrA-PmrB may be activated by unknown environmental signals within the small intestine and that the PmrA-PmrB-induced LPS modifications play a role in resistance to intestinal AP or other intraintestinal antimicrobial factors.

The results of this work further characterize a highly regulated island of genes necessary for LPS modification, AP resistance, survival in mice, and two-component system interactions. Furthermore, these data demonstrate that in vivo-regulated modifications of LPS are an important part of

Salmonella pathogenesis and suggest that the PhoP-PhoQ and PmrA-PmrB regulatory systems may be important at locations other than within host cells.

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