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Identification and Genetic Characterization of PmrA-Regulated Genes and Genes Involved in Polymyxin B Resistance in *Salmonella enterica* Serovar Typhimurium

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Salmonella enterica **serovar Typhimurium encounters antimicrobial peptides (AP) within the phagosomes of professional phagocytes and at intestinal mucosal surfaces. *Salmonella* serovar Typhimurium utilizes the two-component regulatory system PmrA-PmrB, which is activated in response to the environmental conditions encountered in vivo, to regulate resistance to several AP, including polymyxin B (PM). Random Mu*dJ* transposon mutagenesis was used to identify PmrA-PmrB-regulated genes, as well as genetic loci necessary for PM resistance. Three different phenotypic classes of genes were identified: those necessary for PM resistance and regulated by PmrA, those necessary for PM resistance and not regulated by PmrA, and PmrA-regulated genes not required for PM resistance. Loci identified as necessary for PM resistance showed between 6- and 192-fold increased sensitivities to PM, and transposon insertion sites include *surA*, *tolB*, and *gnd*. PmrA-regulated loci identified included *dgoA* and *yibD* and demonstrated 500- and 2,500-fold activation by PmrA, respectively. The role of the identified loci in aminoarabinose modification of lipid A was determined by paper chromatography. The *gnd* mutant demonstrated a loss of aminoarabinose from lipid A, which was suggested to be due to a polar effect on the downstream gene *pmrE*. The remaining PM^s mutants (*surA* and *tolB*), as well as the two PmrA-regulated gene (*yibD* and *dgoA*) mutants, retained aminoarabinose on lipid A. *yibD*, *dgoA*, and *gnd* (likely affecting *pmrE*) played no role in PmrA-regulated resistance to high iron concentrations, while *surA* and *tolB* mutations grew poorly on high iron media. All PM^s mutants identified in this study demonstrated a defect in virulence compared to wild-type *Salmonella* serovar Typhimurium when administered orally to mice, while the PmrA-regulated gene (*yibD* and *dgoA*) mutants showed normal virulence in mice. These data broaden our understanding of in vivo gene regulation, lipopolysaccharide modification, and mechanisms of resistance to AP in enteric bacteria.**

Amphipathic, cationic antimicrobial peptides (AP) are an important component of innate immunity (3, 60) and are present in macrophage phagosomes, granules of neutrophils, and secretions of mucosal epithelia (14, 29, 40). Functionally, AP have antiviral, antifungal, and antibacterial properties. In gram-negative bacteria, many cationic AP associate electrostatically with the bacterial outer membrane, namely the lipopolysaccharide (LPS), via ionic bonds with unsubstituted, anionic phosphate residues present on LPS lipid A and core oligosaccharide (50). Subsequent to binding, the AP permeabilize the outer membrane, translocate to the cytoplasmic membrane, which is the main cytotoxic target of most AP, and then form pores, resulting in leakage of cell constituents and cell death.

Salmonellae cause a diverse array of diseases in humans and animals and are capable of circumventing killing by the host immune system. In *Salmonella enterica* serovar Typhimurium, evasion of AP killing is directed in part by the PmrA-PmrB two-component regulatory system (17, 19, 40, 44, 47, 51, 52). Activation of PmrA-PmrB confers resistance to polymyxin B (PM), as well as the neutrophil cationic AP CAP-37 (azurocidin) and CAP-57 (bactericidal permeability-increasing protein). PmrA-PmrB accomplishes resistance to these AP by up-regulating genes involved in covalent modifications of the LPS (17, 19). The LPS modifications reduce the negative charge of LPS and consequently decrease attraction and binding of AP to the outer membrane. PmrA-induced modifications to LPS include the addition of 4-amino-4-deoxy-L-arabinose (Ara4N) to the 4′-phosphate of lipid A (and sometimes the 1-phosphate) and addition of phosphoethanolamine (pEtN) to the 1-phosphate of lipid A (and sometimes the 4′-phosphate), to 2-keto-3-deoxyoctulosonic acid, and to the first heptose residue of the core (22, 25, 62). A mutant of *Salmonella* serovar Typhimurium containing a point mutation in the *pmrA* gene (*pmrA*505, PmrA^c) that results in a constitutively active protein product expresses an LPS highly modified with Ara4N on lipid A and pEtN on lipid A and core (17, 22, 62). The PmrA^c mutant consequently has an increased MIC for PM which is some 80-fold higher than that of the wild type and a PmrA-null mutant.

PmrA-PmrB-regulated genes have been shown to be induced during infection of the vertebrate host (21). The PmrA-PmrB regulon can be activated by PhoP-PhoQ two-component system via the PmrD protein in response to low magnesium concentrations (15, 26). PmrD is thought to act upon the PmrA-PmrB system posttranscriptionally, possibly affecting the phosphorylation state of PmrA. PmrA-PmrB

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can also be activated independently of PhoP-PhoQ by mild acidic conditions or high iron concentrations, which are thought to be directly sensed by PmrB (42, 43, 57). Moreover, PmrA-PmrB has been shown to be necessary for resistance to high levels of iron (57). In this manner, salmonellae can react to in vivo conditions of low magnesium and mild acid pH, likely encountered within eukaryotic cell vacuoles or phagosomes, by up-regulating LPS modifications in preparation for encountering AP. While high iron environments are uncommon within vertebrate hosts, PmrA-PmrB may play a role in resistance to high iron levels in the environment (57).

Several PmrA-regulated loci involved in modification of LPS have been identified, including *pmrE* (*pagA*, *ugd*), which encodes a putative UDP-glucose dehydrogenase, and the *pmrHFIJKLM* operon (17). Products of these loci are involved in biosynthesis of the Ara4N modification to lipid A. In the putative pathway for Ara4N biosynthesis, supported by findings for the homologous gene products in *Escherichia coli*, PmrE converts UDP-glucose to UDP-glucuronate, and products of *pmrHFIJKLM* participate in the conversion of UDP-glucuronate to UDP-Ara4N and transfer of Ara4N to lipid A (7, 61). Trent et al. have recently identified the *pmrK* (designated *arnT* by the authors) gene product as an enzyme that transfers one or two Ara4N moieties from an undecaprenyl phosphate-α-L-Ara4N donor substrate to lipid A and certain lipid A precursors in *Salmonella* serovar Typhimurium and *E. coli* (48, 49). Like a *pmrA* mutant, mutants in *pmrE* or *pmrHFIJKLM* exhibit a virulence defect when administered orally to mice, presumably as a result of a lack of Ara4N modification to lipid A and loss of resistance to host AP (19).

To date, all known PmrA-regulated genes that are involved in modifying LPS affect Ara4N addition to lipid A, leaving a subset of PmrA-regulated genes involved in pEtN addition to the LPS core to be identified. Additionally, PmrA may affect loci with functions altogether distinct from those involved in resistance to AP, such as resistance to high levels of iron. We have undertaken a mutagenesis experiment to identify genes that are regulated by PmrA and/or are necessary for PM resistance. We report in this paper the identification and preliminary characterization of three non-PmrA-regulated loci that are necessary for PM resistance and two PmrA-regulated genes not required for resistance to PM.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. Salmonellae and *E. coli* cultures were grown in Luria-Bertani (LB) broth at 37°C with aeration. Antibiotics were used, when appropriate, at the following concentrations: PM, 8 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 45 μg/ml; tetracycline, 25 μg/ml; and ampicillin, 50 μg/ml. The chromogenic substrate for β-galactosidase, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase (X-Gal), was used in LB agar plates at a concentration of 40 μg/ml when needed. For high-iron growth experiments, strains were grown on solid N-minimal medium, pH 5.8, containing 10% Casamino Acids, 38 mM glycerol, 10 μM $MgCl_2$, and 0.1 mM $FeSO_4$ (57).

Mu*dJ* mutagenesis. *S. enterica* serovar Typhimurium strain JSG435 (*pmrA505*, $PmrA^c$ PM^r) was mutagenized with the Mu*dJ* transposon by infection with P22 bacteriophage that had been propagated with strain TT10288 (Fig. 1). Some Mu*dJ* transposon insertions generated active transcriptional fusions to the β-galactosidase-encoding gene contained within the transposon. After several independent transductions, ~10,000 chloramphenicol- and kanamycin-resistant mutants that showed utilization of the β-galactosidase substrate (resulting in blue colonies) were picked and replica plated on LB agar containing PM or kanamycin–X-Gal (LB-Kan-X-Gal). Colonies that grew on LB-Kan-X-Gal but not on LB-PM were retained as PM^s mutants, and the MIC of PM was ascertained. Each colony grown on LB-Kan-X-Gal was transduced with P22 phage propagated with strain JSG421 (*pmrA*::Tn*10*d) and selected on LB-Kan-Tet-X-Gal agar, in effect switching the regulatory background from $PmrA^c$ to PmrA-null. Colonies that appeared blue in the $PmrA^c$ background, but white or light blue in the PmrA-null background, were collected as mutants that contained a Mu*dJ* insertion in a PmrA-regulated locus. All mutants were transduced into a fresh $PmrA^c$ background to ensure that the phenotype was linked to the Mu*dJ* insertion. In addition, mutants were examined by Southern blot analysis to ensure the uniqueness of the Mu*dJ* insertion and to eliminate strains containing more than one insertion.

DNA cloning procedures and generation of mutants. In order to clone the genomic DNA adjacent to the Mu*dJ* insertion site for each mutant, a *Sal*I library of the mutant was constructed in plasmid pWSK29. A *Sal*I restriction site is present in Mu*dJ*, which allowed the kanamycin resistance marker to be used in selection of constructs containing a Mu*dJ*-chromosomal DNA junction (Fig. 1). The libraries were transformed into DH5α, and kanamycin- and ampicillin-resistant clones were sequenced using primer JG49 specific to Mu*dJ* DNA corresponding to the sequence at the 3′ end of the transposon (5′ CTA ATC CCA TCA GAT CCC G 3′). The sequence of the insertion site of one PM^s mutant, JSG1290, was obtained using direct genomic sequencing with primer JG49. Routine methods of determining the site of transposon insertion failed for identification of the Mu*dJ* insertion site in JSG1272. As an alternative, a "vectorette" method of obtaining the sequence for the Mu*dJ*-chromosomal DNA junction of JSG1272 was utilized (38). Chromosomal DNA was obtained using (i) the genomic DNA isolation protocol previously described, with proteinase K used in lieu of pronase (18); or (ii) the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Inc.). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.)

For generation of an independent fusion to *surA*, the *surA* gene was amplified from wild-type *Salmonella* serovar Typhimurium (JSG210) chromosomal DNA using primers JG350 (5′ GGAATTCGAACACCGAAGCGAAG 3′) and JG352 (5′ GAGGGTACCCCTTTATGCAGCTTCG 3′), which introduced *Eco*RI and *Kpn*I restriction sites (underlined), respectively. The PCR product was cloned into pGPL01 (18) to give plasmid pGPL350. pGPL350 was propagated in SM10λPir during cloning (JSG1320) and then introduced into wild-type *Salmonella* serovar Typhimurium JSG224 by mating, creating a luciferase fusion to the genomic copy of *surA* (JSG1321). The *surA*::*luc* fusion was then moved into $PmrA^c$ JSG435 by P22 phage-mediated transduction to yield JSG1325. For complementation studies, the *surA* gene was amplified from wild-type genomic DNA with primers JG355 (5′ GGAATTCCAACGTAATCCGCATTGCG 3′) and JG356 (5′ GGGGTACCGTTGCGCACTGCTCATTAG 3′), which introduced *Eco*RI and *Kpn*I restriction sites, respectively, for cloning into the expression vector pWKS29. The resulting construct was transformed into JSG851 by electroporation, creating JSG1327(pWKS355).

For generation of an in-frame deletion in *gnd*, the open reading frame (ORF) of *gnd*, plus 800 bp of DNA flanking both the 5′ and 3′ ends of the ORF, were amplified from wild-type (JSG210) chromosomal DNA by PCR using JG370 (5′ GTCGAGCTCCGTTCTGTTACTGTAGTCCCCTCG 3′) and JG371 (5′ ACA TGCATGCCGACTCGCATAGCGAGATAAGTATTGG 3′). The primers introduced *Sac*I and *Sph*I restriction sites, respectively, which allowed for cloning of the *gnd*-containing PCR fragment into pUC19 to yield pUC370 in JSG1543. Primers JG372 (5′ GGACTAGTCGTACTGATAAAGAAGGC 3′) and JG373 (5′ GGACTAGTCATCACTGCCATACCGACGAC 3′) were used for PCR amplification with pUC370 as a template, resulting in a product 4.3 kb in size consisting of all pUC370 DNA except the majority of *gnd*. Primers JG372 and JG373 each introduced *Spe*I sites, permitting circularization of the amplified product (pUC372, JSG1548). The *gnd* deletion fragment was then subcloned into *rpsL* suicide vector pKAS32 (41), creating pKAS397. pKAS397 was propagated in SM10λPir (JSG1607) and moved into a streptomycin-resistant, $PmrA^c$ *Salmonella* serovar Typhimurium background (JSG844) by conjugation to generate JSG1686. Colonies obtained from the transduction were screened for double homologous recombination on LB agar containing 1 mg of streptomycin/ml. Strains incorporating the deletion were identified by PCR (JSG1724).

For experiments concerning cotranscription of *gnd* and *pmrE*, a *gnd*::Tn*10*d mutation was moved via P22 phage-mediated transduction into JSG547, which displays the $PmrA^c$ background and contains a *pmrE*::Mu*dJ* fusion.

MIC and transcriptional assays. The MIC of PM for each mutant was obtained as previously described (46), testing serial dilutions of PM (U.S. Biochemicals; 8,040 U/mg) in 0.2% bovine serum albumin–0.01% acetic acid. β-Ga-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant phenotype	Source
Salmonellae		
JSG210	ATCC 14028s; wild type	ATCC
JSG224	*phoN2 zxx*::6251 Tn*10*d-cam (CS019)	32
TT10288	Mu*dJ* donor strain	23
JSG425	Salmonella λ Pir *phoP*::Tn*10*d-TET	
JSG844	*pmrA505* with streptomycin resistance allele	19
JSG435	ATCC 14028s *pmrA505 zjd*::Tn*10*d-cam	18
JSG421	*pmrA*::Tn*10*d	18
JSG851	JSG435 with Mu*dJ* insertion in *imp* (affect on *surA*); PM^s	This work
JSG895	JSG435 with Mu*dJ* insertion in *tolB;* PM^s	This work
JSG1272	JSG435 with Mu*dJ* insertion in *gnd;* PM^s	This work
JSG1290	JSG435 with Mu*dJ* insertion in *gnd;* PM^s	This work
JSG897	JSG435 with Mu*dJ* insertion in *yibD;* PmrA regulated	This work
JSG898	JSG421 with Mu*dJ* insertion in *yibD;* PmrA regulated	This work
JSG899	JSG435 with Mu*dJ* insertion in *dgoA;* PmrA regulated	This work
JSG900	JSG421 with Mu*dJ* insertion in *dgoA;* PmrA regulated	This work
JSG901	JSG435 with Mu*dJ* insertion in *pmrC;* PmrA regulated, PM^s	This work
JSG918	JSG435 with Mu*dJ* insertion in *pmrC;* PmrA regulated, PM^s	This work
JSG973	JSG435 with Mu*dJ* insertion in *pmrC;* PmrA regulated, PM^s	This work
JSG1036	JSG435 with Mu*dJ* insertion in *pmrC;* PmrA regulated, PM^s	This work
JSG923	JSG435 with Mu*dJ* insertion in *pmrI;* PmrA regulated, PM^s	This work
JSG1036	JSG435 with Mu*dJ* insertion in *pmrI;* PmrA regulated, PM^s	This work
JSG1521	ATCC 14028s with Mu*dJ* insertion in *imp* (affect on *surA*)	This work
JSG1522	ATCC 14028s with Mu*dJ* insertion in *tolB*	This work
JSG1523	ATCC 14028s with Mu*dJ* insertion in *gnd*	This work
JSG1524	ATCC 14028s with Mu*dJ* insertion in *gnd*	This work
JSG1525	ATCC 14028s with Mu*dJ* insertion in *yibD*	This work
JSG1526	ATCC 14028s with Mu*dJ* insertion in *dgoA*	This work
JSG1325	ATCC 14028s *pmrA505 zjd*::Tn*10*d-cam *surA*::*luc*	This work
JSG1329	ATCC 14028s *pmrA505 zjd*::Tn*10*d-cam *surA*::*luc imp*::Mu*dJ*	This work
JSG1337	JSG851 complemented with *surA* carried on pWSK350	This work
JSG547	ATCC 14028s *pmrA505 pmrE*::Mu*dJ*	This work
JSG1319	LT2 *gnd*::Tn*10*d (Tc)	Gift of D. M. Downs; 11
JSG1330	*pmrE*::Mu*dJ gnd*::Tn*10*d (Tc)	This work
JSG1686	JSG844 with pKAS397, single recombinant	This work
JSG1724	JSG844 with in-frame deletion of *gnd*	This work
E. coli		
DH5α	F^- ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*) U169*endA* 1 *recA* 1 *hsdR*17deoR *thi*-1 *supE*441$^-$ *gyrA*96*relA*1	BRL
SM10λPir	*thi*-1 *thr*-1 *leuB*6 *supE*44 *tonA*21*lacY*1 *recA*::RP4-2-Tc::Mu	
JSG1320	SM10λPir containing pGPL350	This work
JSG1336	DH5α containing pWKS350	This work
JSG1543	DH5α containing pUC370	This work
JSG1548	DH5α containing pUC372	This work
JSG1567	SM10λPir containing pCVD370	This work
Plasmids		
pWSK29	Low-copy expression vector, Amp^r	55
pGPL01	Firefly luciferase transcriptional fusion suicide vector, Amp^r	18
pUC19	High-copy cloning vector	58
pKAS32	*rpsL* suicide vector	41
pWSK350	pWSK29 containing ~1.3-kb fragment corresponding to *surA* gene	This work
pGPL350	pGPL01 containing ~1.3-kb fragment corresponding to *surA* gene	This work
pUC370	pUC19 containing ~2.9-kb fragment consisting of *gnd*, 800 bp upstream and 80-bp downstream	This work
PKAS397	pKAS32 containing 1.6-kb insert consisting of 800 bp 5′ and 3′ of *gnd*	This work

lactosidase assays were performed as described previously (18). Firefly luciferase assays were performed using 100 μl of cells grown to mid-logarithmic phase (optical density at 600 nm, ~0.6). Cells were suspended in luciferase buffer (25 mM Tris phosphate, pH 7.8; 2 mM dithiothreitol; 2 mM EDTA, pH 8.0; 10% glycerol; 1% Triton X-100) and then sonicated briefly. Cell debris was removed by centrifugation. Assays were performed using 10 μl of lysate, using an EG&G Berthold Lumat LB 9507 luminometer and luciferase assay reagents from Promega (Luciferase Assay System). All MIC and transcriptional assays were performed in triplicate.

Virulence assays. The Mu*dJ* fusions identified in the mutagenesis experiment were moved into a wild-type *Salmonella* serovar Typhimurium background via P22 phage-mediated transduction (JSG1521 to -1526). Survival assays were performed as described previously (19). Briefly, each mutant, as well as a PmrA-null control, 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 μl of phosphate-buffered saline, pH 7.4. Female BALB/c mice (weighing 16 to 18 g) were inoculated orally using the swallowing reflex of the mouse. Dilutions of the stationary-phase cultures were plated to determine the number of bacteria present in the inocula. Infected mice were observed for 21 days postinoculation.

LPS and lipid A isolation and paper chromatography. LPS isolation was accomplished by a hot phenol-water method as previously described (2). Lipid A was obtained from whole LPS by hydrolysis in 1% sodium dodecyl sulfate–10 mM sodium acetate, pH 4.5, for 18 h at 100°C (8). One-milligram samples were loaded onto Whatmann paper and allowed to run in descending fashion overnight in a solvent consisting of isopropanol-ethyl acetate-water (7:1:2). Dry

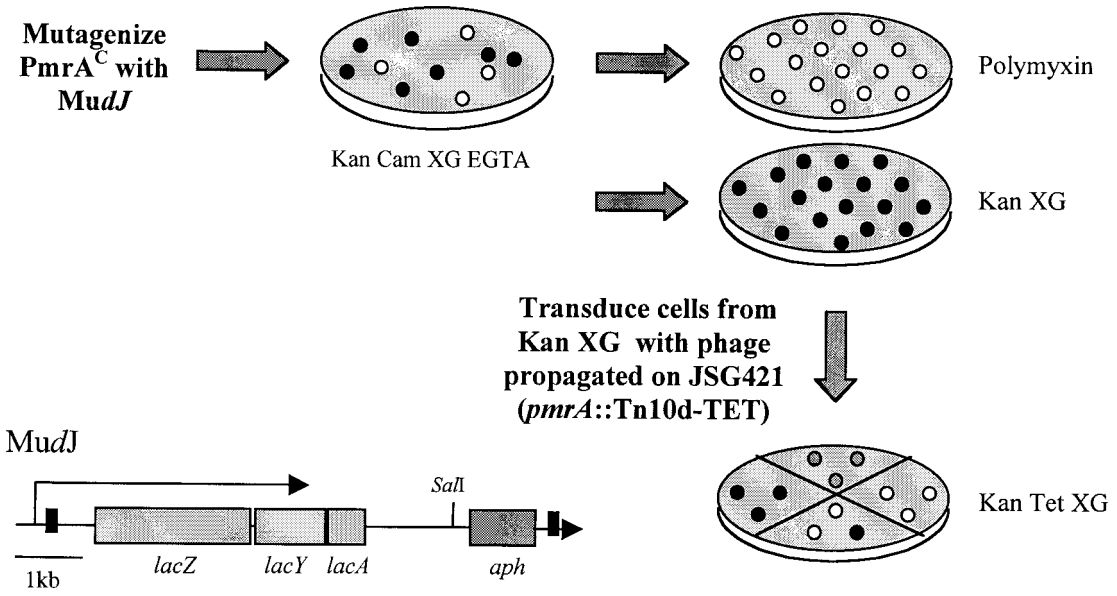

FIG. 1. Schematic of the strategy used to mutagenize JSG435 (PmrA^c) with Mu*dJ* in order to obtain mutants in PmrA-regulated genes and/or genes involved in PM resistance. The Mu*dJ* transposon encodes genes involved in β-galactosidase production, as well as a kanamycin resistance cassette. The *Sal*I site used in cloning the Mu*dJ*-chromosomal DNA junction for sequencing is shown. Black circles represent blue colonies resulting from active fusion of *lacZ* in the Mu*dJ* transposon to an ORF. White circles symbolize Kan^r colonies that contain inactive fusions. Light grey colonies represent a light blue phenotype corresponding to low-level transcription of *lacZ*. Abbreviations: Kan, kanamycin; Tet, tetracycline; Cam, chloramphenicol; XG, X-Gal.

chromatograms were treated with ninhydrin spray reagent (Sigma) to detect aminoarabinose as yellow spots.

RESULTS

Mu*dJ* mutagenesis. *Salmonella* serovar Typhimurium strain JSG435 was used for mutagenesis because it expresses a constitutively active PmrA, rendering the use of inducing conditions for growth unnecessary. The PmrA^c strain also expresses LPS modified with Ara4N and pEtN, which results in resistance to PM (17, 22, 62). PM-sensitive mutants and mutants in PmrA-regulated genes were obtained using Mu*dJ* mutagenesis of JSG435, as detailed in Materials and Methods. The strategy for identification of mutants is depicted in Fig. 1. Kanamycin-resistant colonies containing an active transcriptional fusion to *lacZYA*, i.e., blue colonies, were chosen for further analysis as described in Materials and Methods. A total of ~10,000 β-galactosidase-positive mutants were screened for both PM sensitivity and regulation by PmrA. Mutants were divided into three categories: those with an insertion in a PmrA-regulated gene not affecting PM resistance, those with an insertion in a gene regulated by PmrA but not involved in PM resistance, and those with a mutation in a PmrA-regulated gene necessary for PM resistance (see Table 2 for a summary of the results).

Two PmrA-regulated gene mutants that retained PM resistance were identified: JSG897 and JSG899. In the former, Mu*dJ* was inserted in *yibD*, which encodes a putative glycosyl transferase (31). In JSG899, the insertion occurred in *dgoA*, which is predicted to encode 2-oxo-3-deoxygalactonate-6-phosphate aldolase–galactonate dehydratase (31).

Four PM-sensitive mutants with insertions in non-PmrA-regulated genes were obtained. In JSG851, Mu*dJ* was inserted near the 3′ end of *imp*, an ortholog of an *E. coli* gene involved in resistance to organic solvents (31). JSG895 contained a Mu*dJ* insertion in *tolB*, which plays a role in tolerance to colicins and affects overall membrane stability in a variety of bacteria (5, 6, 9, 10, 24, 30, 36, 37, 39). The third PM-sensitive mutant obtained, JSG1272, contained an insertion in *gnd*, which encodes a 6-phosphogluconate dehydrogenase with a primary role in the pentose-phosphate pathway (45). The PM-sensitive mutant JSG1290 also contained a Mu*dJ* insertion within the *gnd* gene.

Mutations identified in loci that are both PmrA regulated and necessary for PM resistance were in previously identified genes, namely *pmrC* and *pmrI* (also called *arnA* and *pbgP3*) (17, 49, 57). Four independent mutations in *pmrC*, which is upstream of and cotranscribed with *pmrAB*, were identified: JSG901, JSG918, JSG973, and JSG1036. Two additional mutants, JSG923 and JSG925, contained insertions in *pmrI*, a

TABLE 2. Mutants identified in the Mu*dJ* transposon mutagenesis screen

Description	Strain	Tn insertion site
PM sensitive	JSG851	*imp/surA*
	JSG895	*tolB*
	JSG1272	*gnd*
	JSG1290	*gnd*
PmrA regulated	JSG897	*yibD*
	JSG899	*dgoA*
PmrA regulated, PM sensitive	JSG901	*pmrC*
	JSG918	*pmrC*
	JSG973	*pmrC*
	JSG1036	*pmrC*
	JSG923	*pmrI*
	JSG925	*pmrI*

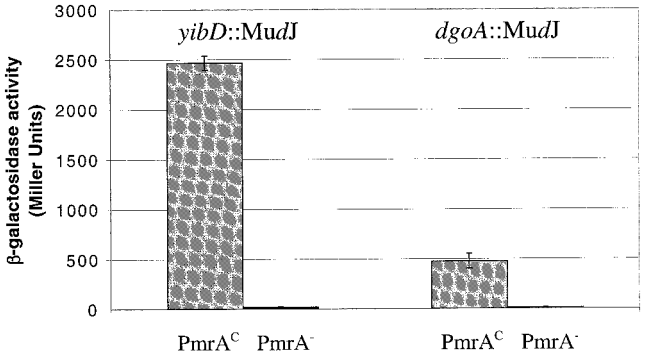

FIG. 2. Regulation of *yibD* and *dgoA* by PmrA. Transcription of the loci was measured by the production of β-galactosidase from the *lacZ* gene in the Mu*d*J transposon. Strains compared were *yibD*::Mu*d*J in a PmrA^c and a PmrA-null background and *dgoA*::Mu*d*J in a PmrA^c and a PmrA-null background. Loci were transcribed effectively in a PmrA^c background (grey bars); transcription of the loci was essentially eliminated in a PmrA-null background (black bars, barely visible).

gene included in the pathway for Ara4N biosynthesis and addition to lipid A.

Analysis of PmrA-regulated genes. The regulatory effect of PmrA on newly identified PmrA-activated genes was examined. Transcription of the *lacZ* gene fusions created by insertion of the Mu*d*J transposon was measured for *yibD*::Mu*d*J and *dgoA*::Mu*d*J in PmrA^c and PmrA-null regulatory backgrounds. Figure 2 shows the results of these assays. Transcription of *yibD* was activated approximately 2,500-fold in the presence of PmrA. Similarly, PmrA activated transcription of the *dgoA* locus by nearly 500-fold. In PmrA-null backgrounds, transcription of these genes was negligible.

The PmrA-regulated gene mutants JSG897 (*yibD*) and JSG899 (*dgoA*) were also analyzed for sensitivity to PM by MIC assay. The MIC of PM for each mutant was 12 μg/ml (data not shown), equivalent to the MIC for the parental PmrA^c *Salmonella* serovar Typhimurium strain, indicating that the PmrA-regulated genes identified in the screen may have functions other than those ascribed to genes regulated by PmrA, i.e., they may not be required for PM resistance.

Wosten et al. have demonstrated that PmrA is necessary for resistance of *Salmonella* serovar Typhimurium to high iron concentrations (57). To address the potential role of the PmrA-regulated genes identified in the screen in iron resistance, JSG897 (*yibD*) and JSG899 (*dgoA*) were grown on solid N-minimal medium supplemented with 100 μM $FeSO_4$. While a PmrA-null mutant was sensitive to this level of iron, JSG897 and JSG899 were able to grow as well as a PmrA^c strain (data not shown), demonstrating that neither of these PmrA-regulated genes is essential for resistance to the toxic effects of iron.

TABLE 3. MICs of PM for the PM^s mutants

Strain	Description	MIC (μg/ml)
JSG435	PmrA^c	12
JSG421	PmrA-null	0.0625
JSG851	*imp*::Mu*d*J (*surA*)	2.0
JSG895	*tolB*::Mu*d*J	0.0625
JSG1272	*gnd*::Mu*d*J	0.125
JSG1290	*gnd*::Mu*d*J	0.125

For further characterization of the PmrA-regulated gene mutants, the promoter regions of *yibD* and *dgoA* were analyzed for the presence of a putative PmrA-binding site, which was defined by the occurrence of the consensus binding sequence for PmrA, YTTAAK-N_5-YTTAAK (1). For *yibD*, a strong match to the consensus binding sequence was identified 50 bp upstream of the translational start codon (Fig. 3). Moreover, the YTTAAK repeats described by Aguirre et al. (1) are conserved. No consensus PmrA-binding sequence was identified in this manner for *dgoA*, neither within the putative promoter region upstream of *dgoA* nor within the putative promoter upstream of the predicted *dgoKAT* operon. Lack of a consensus PmrA-binding site for JSG899 suggests that regulation of this gene by PmrA may be indirect.

Analysis of PM-sensitive mutants. The degree of PM sensitivity of PM^s, non-PmrA-regulated gene mutants JSG851 (*imp*::Mu*d*J), JSG895 (*tolB*::Mu*d*J), JSG1272 (*gnd*::Mu*d*J), and JSG1290 (*gnd*::Mu*d*J) was obtained by MIC assay. Results are summarized in Table 3. The MIC of PM for these mutants ranged from 0.0625 to 2.0 μg/ml, which equates to a 6- to 192-fold increased sensitivity to PM when compared to the PmrA^c parental strain.

β-Galactosidase assays were used to determine if the PM-sensitive mutants contained insertions in non-PmrA-regulated genes, as suggested by the preliminary screen (Fig. 1). The β-galactosidase activity of *imp*::Mu*d*J, *tolB*::Mu*d*J, and

FIG. 3. Promoter analysis of PmrA-regulated loci identified in screen. The consensus PmrA-binding sites shown for *pmrE* (*ugd*) and *pmrCAB* were identified previously by Aguirre and colleagues (1). The YTTAAK direct repeats are marked in bold and underlined. The predicted −10 regions are italicized and marked in bold. Transcriptional start sites are labeled with arrows for *pmrCAB* and *pmrE* (56). For *yibD*, the putative PmrA consensus binding site begins 67 bp upstream of the translational start site. Ambiguous codes used are as follows: Y, C/T; K, G/T.

gnd::Mu*dJ* fusions were measured in PmrA^c and PmrA-null backgrounds. Each of these loci expressed essentially the same levels of β-galactosidase with or without a constitutively active PmrA, with less-than-twofold increases in expression in a PmrA^c background (data not shown). These data confirmed that PmrA did not affect transcription of these loci.

The transposon insertion that occurred in JSG851 interrupted the *imp* gene 23 bp from the termination codon. Because *imp* is immediately followed by *surA*, with only a 43-bp intervening sequence, and *surA* has been shown to affect membrane integrity, the possibility was investigated that the PM^s phenotype of the mutant resulting from the Mu*dJ* insertion in *imp* was a consequence of a polar effect on *surA*. An intact *surA* gene was reintroduced into the PM^s mutant JSG851 on a low-copy expression vector to create JSG1337. Full complementation for PM resistance was obtained in JSG1337, which displayed a PM MIC of 12 μg/ml, which was equivalent to that displayed by the PmrA^c strain. To further support that the insertion in *imp* is actually affecting production of SurA, cotranscription of *imp* and *surA* was demonstrated by measuring transcription of a *surA*::*luc* fusion in a strain with an intact *imp* gene (JSG1325) or a strain with an interrupted *imp* (JSG1329). Disruption of *imp* caused a 46-fold reduction of *surA* transcription (data not shown.)

JSG1272 and JSG1290 contained Mu*dJ* insertions in *Salmonella* serovar Typhimurium *gnd*. The ORF for *pmrE* is 237 bp downstream of *gnd*, with no other ORFs present in the intervening DNA. The possibility arose that the two genes are cotranscribed and that the PM^s phenotype resulting from the Mu*dJ* insertion may be due to a polar effect on *pmrE* rather than a disruption of *gnd* itself, particularly since a *pmrE* mutant has been demonstrated to be PM^s (17). To address the issue of cotranscription of *gnd* and *pmrE*, transcription of a *pmrE*::Mu*dJ* fusion was measured in a strain containing an intact *gnd* gene and in a strain containing a *gnd*::Tn*10*d mutation (11). Transcription of *pmrE*::Mu*dJ* was consistently about 1.5-fold higher in a background with *gnd* intact (data not shown), but this result may not indicate operonic arrangement of these genes; this is supported by the fact that *pmrE* is strongly activated by PmrA, but *gnd* is not PmrA regulated. To further examine the role of *gnd* in PM resistance, an independent, in-frame deletion in *gnd* was created and introduced into JSG844 (PmrA^c Str^r). The new Δ*gnd* mutant, JSG1724, was examined for PM resistance. JSG1724 demonstrated a PM MIC of 12 μg/ml, equivalent to the PM resistance exhibited by the PmrA^c parental strain. Deletion of *gnd* therefore does not result in PM sensitivity, indicating that the Mu*dJ* insertion in *gnd* in JSG1290 is likely affecting *pmrE*.

The four PM^s mutants were tested for sensitivity to high iron concentrations, as mutations that result in loss of LPS modifications that affect sensitivity to cationic AP may also result in sensitivity to ferric ions (57). JSG851 (*surA*) and JSG895 (*tolB*) grew poorly on high iron, whereas JSG1272 (*gnd*) and JSG1290 (*gnd*) were as resistant to 100 μM $FeSO_4$ as was PmrA^c *Salmonella* serovar Typhimurium. It may be that *surA* and *tolB* mutations cause a destabilization of the bacterial envelope and in this manner lead to increased sensitivity to high iron concentrations. A transposon insertion in *gnd*, however, which may affect *pmrE* transcription and in this way alter LPS structure, does not affect resistance to high iron concentrations.

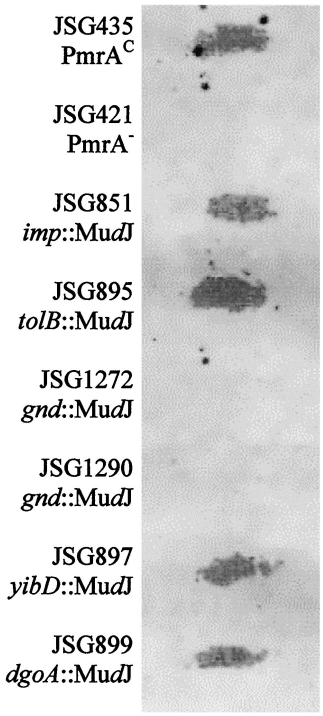

FIG. 4. Analysis of lipid A for the presence of aminoarabinose. Paper chromatography was performed on lipid A from PmrA^c and PmrA-null *Salmonella* serovar Typhimurium, as well as lipid A from the four PM^s mutants and two PmrA-regulated gene mutants identified in this study. The chromatograms were run with a solvent system of isopropanol-ethyl acetate-water (7:1:2). Yellow color upon development with ninhydrin is characteristic of 4-aminoarabinose (yellow color was converted to grey).

Analysis of lipid A for presence of Ara4N. Ara4N modification of lipid A, which is a consequence of the function of a number of PmrA-regulated genes, is associated with a PM-resistant phenotype of gram-negative bacteria. Accordingly, the lipid A of each mutant identified in the screen, including four PM^s, non-PmrA-regulated gene mutants and two PM^r, PmrA-regulated gene mutants, was analyzed for the presence of Ara4N. Purified lipid A was examined using a paper chromatography method in which ninhydrin is the detection agent. Staining of Ara4N with ninhydrin results in a unique yellow color, whereas other lipid A components containing amino groups yield a purple color. Both of the PM^s, non-PmrA-regulated gene mutants with insertions in *gnd* (JSG1272 and JSG1290) lacked Ara4N modification to lipid A (Fig. 4). However, based on the data derived from the *gnd* deletion strain, it is likely that the Ara4N loss can be attributed to an effect of the Mu*dJ* insertion on *pmrE* transcription. PM^s mutants JSG851 (*surA*) and JSG895 (*tolB*) retained Ara4N on lipid A in similar amounts to the parental PmrA^c strain, suggesting that the Mu*dJ* insertions in these mutants affect PM resistance without influencing Ara4N modification to LPS. JSG897 and JSG899, with mutations in PmrA-regulated genes *yibD* and *dgoA*, respectively, also still possessed Ara4N modification of lipid A in similar amounts to the parental PmrA^c strain. Therefore, these genes fall into a minor class of PmrA-regulated genes that alone do not affect LPS modification or AP resistance.

TABLE 4. Virulence of PmrA-regulated gene mutants and mutants with increased sensitivity to PM

Strain	Description	No. of mice surviving[a]
JSG210	Wild type	1/4
JSG1521	*imp*::Mu*d*J (*surA*), PM^s	4/4
JSG1522	*tolB*::Mu*d*J, PM^s	4/4
JSG1523	*gnd*::Mu*d*J, PM^s	3/4
JSG1524	*gnd*::Mu*d*J, PM^s	3/4
JSG1525	*yibD*::Mu*d*J, PmrA-reg[b]	0/4
JSG1526	*dgoA*::Mu*d*J, PmrA-reg	0/4

[a] Mice were inoculated orally with 5×10^6 bacteria.
[b] PmrA-reg, PmrA-regulated mutant.

Virulence analysis of mutants. The PmrA-null mutant has been demonstrated to have a virulence defect when administered orally to mice (19). Additionally, mutants that are sensitive to AP, including those lacking Ara4N on lipid A, possess similar virulence defects (17). Consequently, the mutants identified by Mu*d*J mutagenesis were examined for attenuation of virulence in mice. Experiments were performed using strains with Mu*d*J insertion mutations in a wild-type background rather than a PmrA^c background. PmrA-regulated gene mutants JSG1525 (*yibD*) and JSG1526 (*dgoA*) were as virulent as wild-type *Salmonella* serovar Typhimurium, so the genes interrupted have no essential role in the infection process (Table 4). However, mutants JSG1521 (*surA*), JSG1522 (*tolB*), JSG1523 (*gnd*, affecting *pmrE*), and JSG1524 (*gnd*, affecting *pmrE*), all of which showed increased sensitivity to PM, were less virulent than the wild type. Since these mutants (*surA*, *tolB*, and *gnd*, affecting *pmrE*) are also PM sensitive, these data may support the hypothesis that AP resistance is required for *Salmonella* serovar Typhimurium virulence. However, the membrane-destabilizing effects of the *surA* and *tolB* mutants may result in a more generalized in vivo sensitivity.

DISCUSSION

Modifications to LPS have been suggested to play an important role in pathogenesis of *Salmonella* serovar Typhimurium by increasing resistance to cationic AP (4, 12, 13, 16, 20, 33, 47, 53, 54, 59). *Salmonella* serovar Typhimurium extensively modifies its LPS in response to signals that the organism is likely to encounter in the host (15, 42, 43, 57). These modifications are mediated by the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems (4, 16–19, 22, 52, 62). The PmrA-PmrB two-component system plays a key role in responding to the host microenvironment by up-regulating the genes involved in adding Ara4N to lipid A in response to mild acidic pH and alterations in the external concentrations of iron and magnesium (17, 18, 42, 57). Mutants in *pmrE* or *pmrF*, two PmrA-activated loci, lack Ara4N modification to lipid A and demonstrate reduced virulence when administered orally to mice (19). The Ara4N modification results in neutralization of the net negative charge of LPS, resulting in a decreased affinity of cationic AP to LPS. While several PmrA-regulated loci identified to date are involved in addition of Ara4N to lipid A, other potential targets for regulation by PmrA are proposed: PmrA-regulated genes responsible for pEtN addition to LPS have not been found, and PmrA-regulated genes involved in resistance to high iron (in addition to *pmrL*/*pbgE2* and *pmrM*/*pbgE3*) remain to be identified (57).

Mu*d*J transposon mutagenesis was utilized to find PmrA-regulated genes, genes necessary for PM resistance, and genes that exhibit both properties. Three transposon insertion sites were identified as affecting PM resistance. JSG851 contained a Mu*d*J insertion within the *imp* ORF. Cotranscriptional and complementation analyses demonstrated that the transposon insertion in this mutant caused the PM-sensitive phenotype by affecting *surA*, the gene downstream of *imp*. In *E. coli*, SurA has been shown to be required for correct folding of certain outer membrane proteins, including OmpA, OmpF, and LamB (27). The inability of a *surA* mutant to efficiently fold these important outer membrane proteins would likely cause a destabilization of the outer membrane. For instance, an *E. coli surA* mutant shows sensitivity to compounds such as vancomycin, bacitracin, and bile salts (27); a stable outer membrane contributes to resistance to these compounds. The envelope defects of a *Salmonella* serovar Typhimurium *surA* mutant may thus result in increased sensitivity to PM. Similarly, the finding that JSG851 grew poorly on high concentrations of iron suggests that deficiencies in outer membrane integrity can affect sensitivity to ferric ions. The fact that JSG851 retained its ability to modify its lipid A with Ara4N and still showed PM sensitivity supports the conclusion that factors other than LPS modifications are responsible for the PM sensitivity of this mutant.

The PM^s mutant JSG895 contained an insertion in *tolB*. The Tol–peptidoglycan-associated lipoprotein (Pal) system of *E. coli* is necessary for uptake of group A colicins and is believed to anchor the outer membrane to the cytoplasmic membrane (5). The various components of the Tol/Pal complex are associated with the cytoplasmic membrane (TolQ, TolR, and TolA), the periplasm (TolB), and the outer membrane (PAL) (28). These proteins interact with the various membrane components and with one another to stabilize the envelope of several gram-negative bacteria, and homologues have been studied in *E. coli*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* (5, 6, 9, 10, 24, 30, 36, 37, 39). Mutations in the *Salmonella* serovar Typhimurium *tol* genes result in increased sensitivity to antibiotics and detergents such as bile salts (35). The transposon insertion in *Salmonella* serovar Typhimurium *tolB* could therefore result in hypersensitivity to PM as a consequence of the loss of outer membrane stability. In addition, JSG895 showed sensitivity to high iron. Like the *surA* mutant, iron sensitivity may be due to outer membrane structural defects. Susceptibility to PM and high iron concentrations is not due to loss of Ara4N substitutions to lipid A, as this modification is still present in JSG895.

In two PM-sensitive Mu*d*J mutants, JSG1272 and JSG1290, the transposon insertion site was determined to interrupt *gnd*, which encodes a gluconate-6-phosphate dehydrogenase involved in the pentose-phosphate metabolic pathway (45). *gnd* is the final gene in the *rfb* locus involved in LPS biosynthesis (34) and is located upstream of *pmrE*, a PmrA-regulated gene shown to be necessary for PM resistance and wild-type virulence (17). Previous analyses of the *rfb* locus suggest the presence of a strong transcriptional terminator following *gnd* and preceding *pmrE* (34). Nonetheless, the potential for a polar effect of the transposon insertion in *gnd* on the transcription of

pmrE was investigated using cotranscription studies and an independent mutation in *gnd*. Assays analyzing the effect of a Mu*d*J insertion in *gnd* on transcription of a *pmrE*::Mu*d*J fusion showed a slight decrease in *pmrE* transcription in a *gnd*::Tn*10*d background. Because these results are not conclusive, we proceeded to generate an in-frame deletion of *gnd* to determine whether elimination of *gnd* specifically causes sensitivity to PM. MIC analysis of the Δ*gnd* mutant (JSG1724) showed resistance to PM equivalent to that of the parental PmrA^c strain, suggesting that *gnd* does not play a role in PM resistance. It may be postulated that the Mu*d*J transposon insertion in *gnd* was capable of interrupting *pmrE* transcription, thus causing PM sensitivity, while a deletion of *gnd* precluded this effect. This interpretation is supported by previous studies showing that a PmrA^c strain containing a *pmrE* mutation does not express Ara4N modification of lipid A (17), as this study demonstrated a lack of Ara4N on lipid A in both *gnd*::Mu*d*J mutants.

Virulence analysis of the PM^s mutants showed that insertions in *imp* (affecting *surA*), *tolB*, and *gnd* (likely affecting *pmrE*) resulted in a decrease in virulence (i.e., fewer mice died) in the mouse model, compared to wild-type *Salmonella* serovar Typhimurium. In light of the findings suggesting that the PM sensitivity shown by the *gnd*::Mu*d*J mutants (JSG1272 and JSG1290) is likely a result of a polar effect of Mu*d*J on *pmrE*, it may be interpreted that the virulence defect in JSG1272 and JSG1290 can be attributed to the polarity of the transposon insertion as well. As discussed above, mutations in *tolB* and *surA* may cause a destabilization of the bacterial envelope. In addition to the resulting sensitivity to PM and high iron concentrations, the potential effect of the mutations on membrane integrity may also affect the ability of these strains to survive within the host, due to increased sensitivities to other membrane-active agents or osmotic changes.

Several mutants were identified in which the transposon insertion occurred in PM resistance genes regulated by PmrA. However, these insertions occurred in known loci, including the *pmrCAB* and *pmrHFIJKLM* operons. The fact that these loci were repeatedly identified in the screen suggests completeness of the mutagenesis. However, there was specificity for Mu*d*J insertion in both the *pmrCAB* and *pmrHFIJKLM* operons, as all insertions recovered were in *pmrC* or *pmrI*.

The transposon insertion in JSG897 identified a PmrA-regulated gene, *yibD*, which encodes a putative glycosyl transferase. Sequencing of the Mu*d*J insertion site of JSG899 identified *dgoA* as a PmrA-regulated gene. *dgoA* is predicted to encode a 2-oxo-3-deoxygalactonate-6-phosphate aldolase–galactonate dehydratase involved in galactonate metabolism. Minimal research has been performed on these two loci. Neither *dgoA* nor *yibD* affect PM resistance, which is supported by the finding that JSG897 and JSG899 are capable of modifying lipid A with Ara4N. In addition, neither *yibD* nor *dgoA* affect resistance to high concentrations of iron or virulence in the mouse model. Therefore, it is not clear if these genes affect unknown functions of the PmrA-PmrB regulon or if redundant functions exist for these genes and they do affect AP resistance and/or Ara4N modification. While it is possible that these PmrA-regulated genes (as well as the genes involved in PM resistance) could affect the other known PmrA-induced LPS modification, pEtN, we feel that this is unlikely based on what is known about the identified genes (*surA*, *tolB*, and *pmrE*) and because *yibD* and *dgoA* do not affect virulence or PM resistance, which are predicted phenotypes of the loss of pEtN modification from the core (59).

A strong match to the consensus PmrA-binding site was identified upstream of *yibD*, indicating direct regulation by PmrA. However, no consensus site was apparent in *dgoA*, suggesting indirect activation by PmrA. PmrA has been shown to bind the promoters of other PmrA-regulated loci, including *pmrCAB*, *pmrHFIJKLM*, and *pmrE* (*ugd*) (1, 56). Future studies involving *Salmonella* serovar Typhimurium *yibD* and *dgoA* mutants will entail determining whether PmrA is capable of directly binding the promoters of these loci and identifying the putative regulatory binding sites in these regions.

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