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PhoP-PhoQ Activates Transcription of *pmrAB*, Encoding a Two-Component Regulatory System Involved in *Salmonella typhimurium* Antimicrobial Peptide Resistance

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Antimicrobial cationic peptides are a host defense mechanism of many animal species including mammals, insects, and amphibians. *Salmonella typhimurium* is an enteric and intracellular pathogen that interacts with antimicrobial peptides within neutrophil and macrophage phagosomes and at intestinal mucosal surfaces. The *Salmonella* spp. virulence regulators, PhoP and PhoQ, activate the transcription of genes (*pag*) within macrophage phagosomes necessary for resistance to cationic antimicrobial peptides. One PhoP-activated gene, *pagB*, forms an operon with *pmrAB* (5′ *pagB-pmrA-pmrB* 3′), a two-component regulatory system involved in resistance to the antimicrobial peptides polymyxin, azurocidin (CAP37), bactericidal/permeability-increasing protein (BPI or CAP57), protamine, and polylysine. Expression of *pmrAB* increased transcription of *pagB-pmrAB* by activation of a promoter 5′ to *pagB*. *pmrAB* is also expressed from a second promoter, not regulated by PhoP-PhoQ or PmrA-PmrB, located within the *pagB* coding sequence. *S. typhimurium* strains with increased *pag* locus expression were demonstrated to be polymyxin resistant because of induction of *pagB-pmrAB*; however, PmrA-PmrB was not responsible for the increased sensitivity of PhoP-null mutants to NP-1 defensin. Therefore, PhoP regulates at least two separate networks of genes responsible for cationic antimicrobial peptide resistance. These data suggest that resistance to the polymyxin-CAP family is controlled by a cascade of regulatory protein expression that activates transcription upon environmental sensing.

Salmonella spp. cause a wide spectrum of disease in humans and animals and effectively avoid killing by the host immune system. One mechanism of immune-cell killing involves the actions of a group of amphipathic, cationic, antimicrobial peptides found within neutrophil granules, macrophage phagosomes, and secretions of mucosal epithelia. These antimicrobial factors include the defensins (10, 36), azurocidin (CAP37) (33, 37), and bactericidal/permeability-increasing protein (BPI or CAP57) (12, 37). Cationic antimicrobial peptides likely kill bacteria by binding to lipopolysaccharide (LPS) through ionic bonds with the unsubstituted, negatively charged phosphoryl groups of lipid A. After binding, permeabilization of the outer and inner membranes results in cell death. Other examples of compounds with diverse structures that likely kill bacteria by a similar mechanism include melittin, magainins, polymyxin B (PM), protamine sulfate, polylysine, cecropins, and serum complement (44).

One of the major control points of *Salmonella* sp. pathogenesis is the PhoP-PhoQ two-component regulator (13, 26). PhoP-PhoQ positively and negatively regulates the production of over 40 proteins (27). PhoQ is a membrane-bound kinase (17) that senses environmental signals and transfers phosphate to PhoP to activate *pag* (PhoP-activated gene) and repress *prg* (PhoP-repressed gene) transcription. *pag* loci are induced within acidified macrophage phagosomes where *Salmonella* spp. survive and replicate after phagocytosis (1, 4). It has been shown that increased levels of Mg^{2+} and Ca^{2+} can repress signaling by PhoQ to activate *pag* transcription and that low concentrations of these ions could be responsible for the activation of the PhoP regulon within macrophage phagosomes (11). Twenty-one *pag* loci, which encode a nonspecific acid phosphatase (21), magnesium transporters (11), the PhoP and PhoQ regulatory proteins (38), and proteins that produce active alkaline phosphatase fusions and therefore are membrane or secreted proteins (3, 26) have been described. The individual contributions of *pag* gene products to *Salmonella typhimurium* virulence have not been clearly demonstrated, but strains with null mutations in *phoP* or *phoQ* are attenuated for virulence in mice and humans (9, 20, 26), are unable to survive within macrophages (9, 26), and are sensitive to killing by antimicrobial cationic peptides (8, 14, 20, 28).

The observations that PhoP and PhoQ are essential to *S. typhimurium* virulence and that they mediate resistance to several different antimicrobial peptides suggest that the resistance itself is important to pathogenesis and most specifically to survival within professional phagocytes. The ability of *S. typhimurium* to survive within macrophages and its ability to express resistance to antimicrobial peptides have both been correlated with virulence in mice (9, 14). These correlations further support the possible linkage of these phenotypes. However, since no individual PhoP-regulated gene(s) has been determined to be essential to antimicrobial peptide resistance, both the mechanism and the significance of PhoP-PhoQ-mediated resistance remain undefined.

One mechanism of *S. typhimurium* resistance to antimicrobial peptides that is better understood is resistance to polymyxin. Polymyxin is a peptide antibiotic produced by *Bacillus polymyxa*. A derivative of this peptide antibiotic, PM, has been utilized to study bacterial antimicrobial peptide resistance and membrane biology (41). *S. typhimurium* mutants that are resistant to PM have been identified and characterized. These mutants map to a locus designated *pmrA* (polymyxin resistance) (22) and exhibit cross-resistance to neutrophil antimicrobial peptides CAP37 and CAP57, as well as to polylysine and protamine (37, 39, 42, 43). *pmrA* mutants (as well as LPS

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isolated from this strain) show decreased binding to PM, CAP37, and CAP57 (37, 46) and are more resistant than parental strains to intraphagocytic killing by polymorphonuclear neutrophils (40). Strains containing a *pmrA* mutation were shown to possess an increased level of phosphate substitution on the LPS core and a high degree of substitution of the 4′ phosphate of lipid A by 4-aminoarabinose (19, 45). These substitutions decrease the negative charge of LPS, which is thought to reduce the accessibility of the positively charged PM to lipid A. Roland et al. (34) cloned and characterized a *pmrA* locus mutant (*pmrA505*) responsible for the PM^r phenotype. DNA sequence analysis revealed a single base alteration that would result in an amino acid change in PmrA, which was predicted to be encoded by a member of an operon that includes *orf 1, pmrA*, and *pmrB* (34). PmrA-PmrB is similar to the OmpR-EnvZ family of two-component regulators (which includes PhoP-PhoQ), which suggested that PmrA-PmrB was a regulatory system controlling resistance to antimicrobial peptides. We present here the discovery that the *pmrAB* operon is regulated by PhoP-PhoQ, which was made in the process of characterizing the *pag* loci defined in our laboratory.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetics. Bacterial strains and plasmids used in this study are described in Table 1, with the exception of the strains containing transposon insertions defining *pagC* to *-P* (3) and *prgA* to *-C, -E,* and *-H* (2). Cultures were grown at 37°C with aeration in Luria broth (25) or N-minimal medium (30) with 8 μM $MgCl_2$, 38 mM glycerol, and 0.1% Casamino Acids. N-minimal medium with a low concentration of Mg^{2+} (8 μM $MgCl_2$) or Ca^{2+} (8 μM $CaCl_2$) is an activating condition for the PhoP regulon (11). Antibiotics were used at the following concentrations: chloramphenicol, 25 μg ml^{-1}; kanamycin, 45 μg ml^{-1}; tetracycline, 8 to 25 μg ml^{-1}; and ampicillin, 50 μg ml^{-1}. P22HT*int* bacteriophage was used in all transductional crosses (5, 7). All strains resulting from transductional crosses were routinely confirmed to be nonlysogens of P22.

DNA-cloning procedures and vector construction. To clone the DNA adjacent to the Mu*d*J insertion that defined *pagB*, a *Sac*I library of strain CS013 was constructed in plasmid pBCSK+. *Sac*I cuts in the transposon 5′ to the kanamycin resistance gene; therefore, the *Sac*I fragment carrying the 3′ *pagB*-Mu*d*J fusion junction should carry the Kan^r marker of the transposon and chromosomal DNA 3′ to the insertion. Following transformation and kanamycin antibiotic selection, a transformant was identified and confirmed to contain *pagB* DNA by Southern blot hybridization experiments, as described in the Results section.

Chromosomal DNA was prepared as previously described except for the substitution of proteinase K for pronase (23). Restriction enzymes and PCR reagents were purchased from New England Biolabs (Beverly, Mass.) and used according to the recommendations of the manufacturer. Southern blots were accomplished with Genescreen Plus membranes (Dupont, Boston, Mass.). DNA probes were labeled with digoxigenin, and chemiluminescent detection was accomplished with an antidigoxigenin alkaline phosphatase-conjugated Fab fragment and Lumiphos (Boehringer Mannheim, Indianapolis, Ind.). All blotting conditions were as suggested by the manufacturer.

The firefly luciferase reporter and Pir-dependent suicide vector pLB02 was constructed as follows. From pMLB1109 (1), a *Pst*I-*Msc*I fragment that contained four transcriptional terminators 5′ to a multiple cloning site (MCS) and the *lacZ* gene 3′ to the MCS was cloned into the *Pst*I-*Sma*I site of plasmid pWPL33 to construct plasmid pWPL36. pWPL33 is a Pir-dependent derivative of pGP704 (29) that contains a 700-bp chromosomal region downstream of *pagC*. The firefly luciferase gene (*luc*) from pTKLUC was amplified by PCR with primers JG03 (5′ GAACCCGGGGTACCGTCGACTTTACTTTAAGAAGG AG 3′) and JG04 (5′ GCCGGATCCTAGAGTCGCGGCCG 3′) and cloned into the MCS of pWPL36 to create pLB02 (5′ transcriptional terminators, MCS of *Eco*RI-*Sal*I-*Kpn*I-*Sma*I, *luc lacZ pagC′* chromosomal region 3′). pLB02 is most useful in the construction of promoter-*luc* fusions that can be recombined onto the chromosome via the *pagC* downstream sequence to avoid promoter analysis in multicopy. Recombination of this vector onto the chromosome via the region downstream of *pagC* has no effect on *S. typhimurium* growth or virulence properties (data not shown).

The firefly luciferase reporter and Pir-dependent suicide vector pGPL01 was constructed from pLB02 as follows. A *Pst*I-*Bam*HI (the *Bam*HI site was filled in with the Klenow fragment of DNA polymerase I) fragment of pLB02 containing the terminators, the MCS, and the *luc* gene was moved from this vector into the *Pst*I-*Eco*RV sites of Pir-dependent suicide vector pGP704. The resultant vector, pGPL01, contains an MCS of *Eco*RI-*Sal*I-*Kpn*I-*Sma*I, and a promoterless *luc* gene useful for constructing chromosomal transcriptional *luc* fusions to a gene of interest via recombination with *Salmonella* DNA cloned into the MCS.

TABLE 1. Bacterial strains and plasmids and relevant properties

Strain or plasmid	Genotype or relevant phenotype	Source or reference[a]
Salmonellae		
CS019	*phoN2 zxx*::6251 Tn*10*d-cam	26
CS100	*phoP105*::Tn*10*d	16
ATCC 14028s	Wild type	ATCC
CS013	ATCC 14028s *pagB*::Mu*d*J	26
CS015	*phoP102*::Tn*10*d-cam	26
CS022	*pho-24* (PhoP-constitutive)	27
JSG421	*pmrA*::Tn*10*d	This work
JSG430	CS022 *pmrA*::Tn*10*d	This work
JSG435	ATCC 14028s *pmrA505 zjd*::Tn*10*d-cam	This work
JSG437	CS100 *pmrA505 zjd*::Tn*10*d-cam	This work
JSG438	ATCC 14028s with pHSG576	This work
JSG440	CS100 with pHSG576	This work
JSG442	ATCC 14028s with pKR100	This work
JSG444	CS100 with pKR100	This work
JSG465	*pagB*::Mu*d*J *phoP105*::Tn*10*d	This work
JSG420	*pagB*::Mu*d*J *pmrA*::Tn*10*d	This work
CS018	*pagC*::Tn*phoA phoP105*::Tn*10*d	This work
JSG468	*pagC*::Tn*phoA pmrA*::Tn*10*d	This work
CS586	*pagD*::Tn*phoA phoP105*::Tn*10*d	This work
JSG469	*pagD*::Tn*phoA pmrA*::Tn*10*d	This work
JSG205	*msgA*::Mu*d*J	16
JSG225	*msgA*::Mu*d*J *phoP105*::Tn*10*d	This work
JSG471	*msgA*::Mu*d*J *pmrA*::Tn*10*d	This work
JSG473	CS019 *pagB-pmrAB::luc*	This work
JSG474	CS013 *pagB-pmrAB::luc*	This work
JSG475	CS022 *pagB-pmrAB::luc*	This work
JSG476	CS015 *pagB-pmrAB::luc*	This work
JSG477	JSG421 *pagB-pmrAB::luc*	This work
JSG478	JSG435 *pagB-pmrAB::luc*	This work
JGS479	CS019 *zch::pLB8687*	This work
JSG240	CS019 *zch*::pLB02	This work
JSG463	Salmonella λ Pir pGPL01	This work
Plasmids		
pSG10	pBCSK+ with a 24-kb *SacI* fragment containing part of the *pagB*::Mu*d*J transposon (including the Kan^r gene) and downstream *Salmonella* DNA	This work
pGP704	Pir-dependent suicide vector, Amp^r	29
pGPL01	Firefly luciferase transcriptional fusion suicide vector, Amp^r	This work
pLB02	Firefly luciferase transcriptional fusion suicide vector, Amp^r	This work
pABL01	pGPL01 containing a 660-bp DNA fragment corresponding to the 3′ end of *pmrB*	This work
pLB8687	pLB02 containing a 343-bp DNA fragment 5′ to *pmrA*	This work
pKR100	pHSG576 containing the *pmrA505* allele	34
pHSG576	pSC101 derivative, Cam^r	K. Roland and J. Spitznagel
E. coli[b]		
DH5α	F$^-$ ϕ 80d*lac*ZΔM15 Δ(*lacZYA-argF*) U169*endA*1*recA*1*hsdR*17deoR *thi*-1*supE*441$^-$*gyrA*96*relA*1	BRL
SM10λ Pir	*thi*-1 *thr*-1 *leuB*6 *supE*44 *tonA*21*lacY*1*recA*::RP4-2-Tc::Mu	

[a] ATCC, American Type Culture Collection; BRL, Bethesda Research Laboratory.
[b] *E. coli*, *Escherichia coli.*

Plasmid pABL01 was constructed by cloning a 669-bp PCR fragment (made with the primer JG84 [5′ GGAATTCCACCACGCTCGACAATGAACG 3′] containing an *Eco*RI site at the 5′ end and JG85 [5′ GGGGTACCTTATGCC TTTTTCAACAGC 3′] containing a *Kpn*I site at the 5′ end) of the 3′ end of

pmrB into pGPL01. Strain SM10λ Pir containing this plasmid was mated with strain CS019, and Amp^r Cam^r transconjugates were identified. Recombination of the plasmid into *pmrB* was confirmed by Southern blot hybridization experiments. This luciferase fusion was moved by P22HT*int* transduction into several strains, and recombination within *pmrB* was again confirmed by Southern blot hybridization. These strains were then assayed for luciferase activity as described below.

Plasmid pLB8687 was constructed by cloning a 333-bp PCR fragment (amplified with primers JG86 [5′ GGGGTACCTGCACGGTTGCAG 3′] containing a *Kpn*I site at the 5′ end and JG87 [5′ GGAATTCTATATTGTAGATAAAGCC 3′] containing an *Eco*RI site at the 5′ end) into the *Eco*RI-*Kpn*I sites of pLB02. Strain SM10λ Pir containing pLB8687 was mated with CS019, and recombination of pLB8687 downstream of *pagC* was confirmed by Southern blot hybridization. The chromosomal region containing the recombined vector was transduced by P22HT*int* into various *Salmonella* strains, and the chromosomal location of pLB8687 was confirmed by Southern blot hybridization to be identical to the location of the plasmid in CS019.

DNA sequencing and analysis. DNA sequence analysis was performed on double-stranded templates by the dideoxy chain termination method (35) with the Sequenase enzyme and kit reagents (U.S. Biochemicals Corp., Cleveland, Ohio). Computer analysis of the DNA sequence was accomplished with the Wisconsin package (GCG, version 7) program.

Enzyme assays. Determination of the alkaline phosphatase activity of *pag*::Tn*phoA* fusions was performed as previously described with the substrate 5-bromo-4-chloro-3-indolylphosphate (XP) (24). The β-galactosidase activities of *pag*::Mu*dJ* fusions were determined using *o*-nitrophenyl β-D-galactoside as the substrate (25). The results were expressed in units as calculated for β-galactosidase by using the formula of Miller (25).

Luciferase assays were performed as follows. Ninety microliters of a 100-Klett-unit (optical density, 0.6) culture was mixed with 10 μl of a solution containing 1M K_2HPO_4 (pH 7.8) and 20mM EDTA (pH 8.0) and subjected to one freeze-thaw cycle. Nine hundred microliters of luciferase buffer (1× buffer; 25mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol, 2 mM EDTA [pH 8.0], 2.5 mg of bovine serum albumin per ml, 10% glycerol, 1% Triton X-100) was added, and this cell suspension was briefly sonicated to lyse all remaining cells. Cell debris were pelleted briefly in a microfuge, and a 10-μl aliquot of this lysate was assayed for 10 s in a Berthold (Pittsburgh, Pa.) LB9501 luminometer (100-μl injector) by using the luciferase assay reagents from Promega Corp. (Madison, Wis.).

Transposon mutagenesis. A pool of random Tn*10*d insertions was transduced into strain CS013 to identify a transposon insertion within the *pmrA* locus. Transductants were screened on Tet XP plates for white colonies. These transductants should have resulted from the displacement via recombination of the Mu*dJ* insertion in *pagB* with a linked Tn*10*d. These colonies were confirmed to have a Tet^r Kan^s phenotype and were further analyzed by Southern blot and PCR to identify the location of the Tn*10*d insertion. One strain, JSG421, was identified that contained a Tn*10*d insertion within *pmrA*.

Antimicrobial peptide resistance assays. To assay *Salmonella* strains for polymyxin resistance, strains were grown to mid-log phase (50 Klett units) and diluted to a concentration of approximately 2,500 CFU ml^{-1} in tryptone-saline solution. Cells (200 μl) were mixed on a microtiter plate with various concentrations of PM (U.S. Biochemicals; 8,040 U mg^{-1}) and incubated at 37°C for 1 h. Then, 100 μl of PM-treated cells was directly plated on Luria-Bertani medium. Relative strain resistances and 50% lethal doses (LD_{50}s) were similar regardless of whether or not cells were washed following incubation with PM. After overnight growth, colony counts of cells incubated with the various concentrations of polymyxin were compared to colony counts of cells not exposed to polymyxin.

NP-1 defensin assays were accomplished by diluting exponential cultures to approximately 10^6 CFU ml^{-1} and incubating 100 μl of these cultures at 37°C in 0.5% tryptone–0.05% acetic acid for 1 h with or without 35 μg of NP-1 defensin per ml. The cultures were diluted and plated onto Luria-Bertani medium, and colony counts were determined following overnight growth.

Nucleotide sequence accession number. The sequence characterized in this study, has been previously assigned GenBank accession number L13395 (34).

RESULTS

***pagB* is located at centisome 93.5 and is immediately 5′ to *prmAB*.** The *pagB* gene, defined by Miller et al. (26), was shown to be dependent on PhoP for expression and was transcriptionally induced within macrophage phagosomes ([1], see also Table 3). To clone the gene defined by the *pagB*::Mu*dJ* insertion, a *Sac*I library of DNA from strain CS013 was constructed and transformed into DH5α. After being plated on selective media, Amp^r (carried by the vector) and Kan^r (carried by the Mu*dJ* transposon) transformants were identified. One clone, pSG10, contained an insert of approximately 24 kb and was chosen for further study. A fragment of pSG10 was used as a probe in Southern blot hybridization experiments to confirm that the cloned DNA represented *pagB*. The chromosomal DNA hybridization patterns of the wild-type strain (ATCC 14028s) and strain CS013 (*pagB*::Mu*dJ*) to the pSG10 probe were compared. The use of restriction endonucleases that had no recognition sites within the transposon resulted in hybridizing fragments of CS013 that were 13 kb larger than that of ATCC 14028s which corresponded to the size of the Mu*dJ* transposon (data not shown). These results clearly demonstrated that pSG10 contained *pagB*.

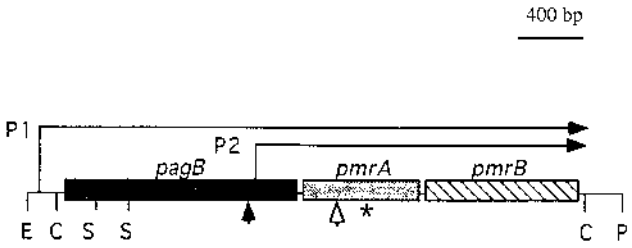

FIG. 1. Partial restriction map of the chromosomal region encoding PagB and the two-component regulatory system, PmrA-PmrB. The location of the Mu*dJ* insertion that defined *pagB* is denoted by a solid arrowhead, and a Tn*10*d insertion in *pmrA* is denoted by an open arrowhead. The asterisk denotes the location of the *pmrA505* mutation identified by Roland et al. (34) that is thought to result in a PmrA-constitutive phenotype. Promoters P1 and P2 are shown; arrows above the map show the direction of transcription. Restriction sites are abbreviated as follows: C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sac*II.

A primer 50 bp from the end of Mu*dJ* (in the left end of Mu) was used to sequence the *pagB*::Mu*dJ* fusion junction. DNA sequence analysis indicated that Mu*dJ* was inserted at bp 1541 of the *S. typhimurium pmrAB* operon at centisome 93.5 on the *S. typhimurium* chromosome (34) (Fig. 1). *pmrA* and *pmrB* were previously defined as encoding a two-component regulatory system that affects resistance to polymyxin and other antimicrobial cationic peptides. The location of the Mu*dJ* insertion is within *orf1* upstream of *prmA*; therefore, *orf1* is *pagB*: an expressed gene positively regulated by PhoP-PhoQ.

***pmrAB* is regulated by PhoP-PhoQ and forms an operon with *pagB*.** Roland et al. (34) demonstrated by phenotypic analysis of polymyxin resistance that *orf1 (pagB), pmrA*, and *pmrB* were likely to be expressed and to form an operon. However, we wished to confirm that *pmrA* and *pmrB* were transcriptionally linked to *pagB* and were PhoP regulated. To accomplish this, a derivative of the Pir-dependent suicide vector pGP704 containing the firefly luciferase gene as a transcriptional reporter was constructed (pGPL01). A 660-bp PCR fragment corresponding to the 3′ end of *pmrB* (bp 2944 to 3604) was cloned into pGPL01 to make a transcriptional fusion to luciferase (pABL01). SM10λ Pir containing pABL01 was mated with strain CS019. Recombination with the homologous chromosomal region created a luciferase transcriptional fusion immediately following *pmrB*, such that neither *pagB*, nor *pmrA*, nor *pmrB* was disrupted. P22HT*int* transduction was utilized to move this fusion into various strains including CS015 (PhoP null) and CS022 (PhoP constitutive) to create strains JSG476 and JSG475, respectively. Luciferase activity (measured in relative light units, RLU) in JSG476 and JSG475 was monitored and showed a 24-fold increase in expression in JSG475 (PhoP-constitutive background) compared with that in JSG476 (PhoP-null background) (Table 2).

To determine if the transcript detected by this *luc* fusion began upstream of *pagB* and contained *pagB, pmrA*, and *pmrB*, strains were constructed that contained the *pagB-pmrAB*::*luc* fusion (described above) as well as *pmrA*::*Tn10*d and *pagB*::*Mud*J transposon insertions. SM10λPir containing pABL01

TABLE 2. Analysis of transcriptional regulation of *pagB-pmrAB* by using an operon fusion to firefly luciferase

Strain	Relevant strain mutations and/or fusions	Luciferase activity (RLU)[a]
JSG473	*pagB-pmrAB*::*luc*[b]	125,337 ± 10,125
JSG476	*phoP105*::*Tn10*d-tet *pagB-pmrAB*::*luc*	15,517 ± 1,903
JSG475	*pho-24 pagB-pmrAB*::*luc*	365,934 ± 25,865
JSG477	*pmrA*::*Tn10*d-tet *pagB-pmrAB*::*luc*	834 ± 61
JSG474	*pagB*::*Mud*J *pagB-pmrAB*::*luc*	6,423 ± 155
JSG478	*pmrA505 pagB-pmrAB*::*luc*	496,931 ± 16,645
JSG497	*zch*::*pLB8687*[c]	20,617 ± 816
JSG240	*zch*::*pLB02*	263 ± 51
Salmonella λ Pir	pGPL01	115 ± 81

[a] Values are means ± standard errors.

[b] *pagBpmrAB*::*luc* is a chromosomal transcriptional fusion of the firefly luciferase gene (*luc*) to the 3′ end of the *pmrB* gene. The *pagB, pmrA*, and *pmrB* genes are not disrupted in this construct.

[c] JSG497 is CS019 containing the plasmid pLB8687 (*pmrAB* promoter-*luc* fusion) recombined on the chromosome downstream of *pagC* (centisome 26). JSG240 is the vector control for this construct.

was mated into strain JSG421 containing a Tn*10*d insertion in *pmrA*, and strain CS013 containing the *pagB*::*Mud*J insertion and recombinants were identified by selection for ampicillin resistance. Strain JSG473 containing the *pagB-pmrAB*::*luc* fusion in a wild-type background had an activity of 125,337 RLU, while there was a 150-fold decrease in activity (834 RLU) of the *luc* fusion in JSG477 (*pmrA*::*Tn10*d *pagB-pmrAB*::*luc*), and an 19-fold reduction in JSG474 (*pagB*::*Mud*J *pagB-pmrAB*::*luc*) (6,423 RLU). These data indicate that transposon insertions in *pagB* and *pmrA* markedly attenuate transcription of downstream genes, demonstrating that *pagB, pmrA*, and *pmrB* are cotranscribed and that this operon is regulated by PhoP-PhoQ at a promoter 5′ to *pagB*.

Examination of *pag* regulation by PmrA-PmrB: the *pagB-pmrAB* operon is positively regulated by PmrA. Because PmrA-PmrB is predicted to be a two-component regulatory system regulated by PhoP-PhoQ, it was possible that previously identified *pag* loci were regulated by PhoP-PhoQ through PmrA-PmrB. To examine this hypothesis, strains containing transcriptional and translational reporters of the *pagA* to *-P* loci were transduced with P22HT*int* phage propagated on strain JSG421 containing *pmrA*::*Tn10*d. Overnight cultures (in which *pag* loci were transcriptionally activated) were compared for alkaline phosphatase or β-galactosidase activity in the presence or absence of *pmrA*::*Tn10*d. Only expression of the *pagB*::*lacZ* fusion was significantly altered by the presence of the *pmrA*::*Tn10*d (sevenfold decrease), suggesting that the *pagB-pmrAB* operon was positively regulated by PmrA (data not shown; see Table 3 for the effect of *pmrA*::*Tn10*d on *pagB* expression). This effect can also be seen in the comparison of *pagB-pmrAB*::*luc* fusion activity between JSG473 (wild-type background) and JSG478 (*pmr505* background) (Table 2), which shows that there is a fourfold increase in luciferase activity due to the *pmrA505* mutation, a mutation that confers increased polymyxin resistance on *S. typhimurium*, most likely through increased expression of PmrA-regulated genes. Five *prg* genes identified in our laboratory (*prgA* to *-C, -E*, and *-H*) and the *phoN* gene, which is a PhoP-regulated nonspecific acid phosphatase, were also examined for regulation by PmrA-PmrB; however, as with the *pag* loci examined, none were regulated by PmrA-PmrB (data not shown).

***pmrAB* is also transcribed from a constitutive promoter within the *pagB* coding sequence.** The data presented above demonstrate a marked effect of the *pmrA*::*Tn10*d insertion on *pagB* transcription in JSG420 (*pagB*::*Mud*J *pmrA*::*Tn10*d). However, the *pagB*::*Mud*J insertion was not completely polar on *pmrA* and *pmrB* transcription. This suggested that transcription of *pmrAB* also occurred by means of a promoter directly upstream of *pmrA* or a functional promoter formed by insertion of Mu*d*J. Further evidence suggesting the presence of a promoter directly upstream of *pmrA* can be observed by examination of the data in Table 2. Although both the *pagB*::*Mud*J insertion and the *pmrA*::*Tn10*d insertion dramatically decreased expression of the *pagB-pmrAB*::*luc* fusion, 6,423 RLU was observed even in the presence of the *pagB*::*Mud*J insertion, and this activity was approximately eightfold greater than the activity seen with the *pmrA*::*Tn10*d insertion. To determine if a promoter existed immediately 5′ to *pmrA*, a 343-bp fragment (bp 1502 to 1845) of DNA upstream of the start of translation of *pmrA* and extending beyond the site of the Mu*d*J insertion, was amplified by PCR. This fragment was cloned into the vector pLB02, which, like pGPL01, is a suicide vector and luciferase transcriptional reporter with transcriptional terminators located 5′ to the MCS. The terminators ensure that luciferase activity will be detected only if a functional promoter is inserted into the MCS. This vector also contains a 700-bp region of *S. typhimurium* chromosomal DNA located downstream of *pagC* that can be used to recombine the vector onto the chromosome, thereby avoiding the potential problems of promoter analysis in multicopy. Following the mating of this plasmid (pLB8687) into CS019 to create strain JSG497 and the confirmation of recombination within the proper chromosomal region, luciferase activity was monitored. Significant levels of luciferase activity were observed when compared with those of the vector control, demonstrating the presence of a functional promoter within the *pmrA* 5′ region cloned into pLB02 (Table 2). The activity of this promoter, labeled P2, was sixfold less than the activity detected for the *pagB-pmrAB*::*luc* fusion and was unaffected when the promoter was moved by P22HT*int* transduction into PhoP^c (JSG490), PhoP-null (JSG489), PmrA-null (JSG491), and PmrA505 (JSG492) backgrounds (data not shown). Therefore, this region contains a relatively weak promoter that directs transcription of *pmrA* and *pmrB* and that is not regulated by PmrA-PmrB or PhoP-PhoQ.

***pagB-pmrAB* expression is regulated by Mg^{2+} and Ca^{2+}.** Garcia-Vescovia et al. (11) recently demonstrated that PhoP-mediated transcription could be repressed by Mg^{2+} and Ca^{2+}. Therefore, we examined the effect of Mg^{2+} and Ca^{2+} on *pagB* expression as well as the effect of Mg^{2+} and Ca^{2+} on the expression of selected *pag* loci in a PmrA-null background. The results, described in Table 3, reflect the use of Ca^{2+} to examine the effect of the cations on gene expression and were nearly identical to those observed when the effect of Mg^{2+} on gene expression was examined (data not shown). *pagB* expression increased approximately fourfold when cells were grown in N-minimal medium containing low levels of Ca^{2+} (8 μM) compared with the expression in Luria-Bertani broth and was nearly completely repressed by the addition of 2 mM Ca^{2+}. Expression of *pagB* in JSG465 (*pagB*::*Mud*J *phoP*::*Tn10*d) was not activated in N-minimal medium containing low levels of Ca^{2+}, demonstrating that PhoP-PhoQ was necessary for Ca^{2+} responsiveness. Two PhoP-regulated genes, *pagC* and *pagD*, were examined for regulation by Ca^{2+} and for the potential effects of PmrA-PmrB on the levels of Ca^{2+} regulation. Both *pagC* and *pagD* were 50- to 80-fold repressed by growth in the presence of 2 mM Ca^{2+}. As expected, *pagC* and *pagD* containing a PhoP-null mutation were not expressed in the presence of low or high levels of Ca^{2+} in the growth medium. The introduction of a *pmrA*::*Tn10d* insertion into the *pagC*::*TnphoA* and *pagD*::*TnphoA* strains had no effect on Ca^{2+} responsiveness.

TABLE 3. Effect of *phoP* and *pmrA* mutations and Ca^{2+} on transcriptional regulation of the promoter 5′ to *pagB*

Strain	Relevant mutations	Growth conditions[a]	Fusion activity[b]
CS013	*pagB*::*MudJ*	LB	122 ± 6
JSG465	*pagB*::*MudJ phoP102*::*Tn10*d	LB	8 ± 2
CS025	*pagB*::*MudJ pho24*	LB	380 ± 53
JSG420	*pagB*::*MudJ pmrA*::*Tn10*d	LB	18 ± 4
CS013	*pagB*::*MudJ*	8 μM Ca^{2+}	590 ± 23
CS013	*pagB*::*MudJ*	2 mM Ca^{2+}	<10
JSG465	*pagB*::*MudJ phoP102*::*Tn10*d	8 μM Ca^{2+}	<10
JSG465	*pagB*::*MudJ phoP102*::*Tn10*d	2 mM Ca^{2+}	<10
CS119	*pagC*::*TnphoA*	LB	74 ± 10
CS119	*pagC*::*TnphoA*	8 μM Ca^{2+}	883 ± 19
CS119	*pagC*::*TnphoA*	2 mM Ca^{2+}	18 ± 3
CS018	*pagC*::*TnphoA phoP102*::*Tn10*d	8 μM Ca^{2+}	15 ± 4
CS018	*pagC*::*TnphoA phoP102*::*Tn10*d	2 mM Ca^{2+}	<10
JSG468	*pagC*::*TnphoA pmrA*::*Tn10*d	8 μM Ca^{2+}	1,022 ± 78
JSG468	*pagC*::*TnphoA pmrA*::*Tn10*d	2 mM Ca^{2+}	29 ± 6
CS585	*pagD*::*TnphoA*	LB	47 ± 5
CS585	*pagD*::*TnphoA*	8 μM Ca^{2+}	788 ± 30
CS585	*pagD*::*TnphoA*	2 mM Ca^{2+}	<10
CS586	*pagD*::*TnphoA phoP102*::*Tn10*d	8 μM Ca^{2+}	<10
CS586	*pagD*::*TnphoA phoP102*::*Tn10*d	2 mM Ca^{2+}	<10
JSG469	*pagD*::*TnphoA pmrA*::*Tn10*d	8 μM Ca^{2+}	754 ± 22
JSG469	*pagD*::*TnphoA pmrA*::*Tn10*d	2 mM Ca^{2+}	<10
JSG205	*msgA*::*MudJ*	LB	203 ± 6
JSG205	*msgA*::*MudJ*	8 μM Ca^{2+}	256 ± 7
JSG205	*msgA*::*MudJ*	2 mM Ca^{2+}	228 ± 6
JSG225	*msgA*::*MudJ phoP102*::*Tn10*d	8 μM Ca^{2+}	241 ± 9
JSG225	*msgA*::*MudJ phoP102*::*Tn10*d	2 mM Ca^{2+}	233 ± 7
JSG471	*msgA*::*MudJ pmrA*::*Tn10*d	8 μM Ca^{2+}	234 ± 5
JSG471	*msgA*::*MudJ pmrA*::*Tn10*d	2 mM Ca^{2+}	228 ± 6

[a] Assays in which the level of Ca^{2+} in the medium was varied were accomplished in N-minimal media. LB, Luria-Bertani medium.

[b] Fusion activity represents the assay of β-galactosidase or alkaline phosphatase produced from Mu*dJ* or Tn*phoA* transposon fusions, respectively.

The promoter of a defined, non-PhoP-regulated gene, *msgA* (16), was used as a control, and the expression of this gene was unaffected by any of the growth conditions or transposon insertions tested. Therefore, these data confirm and extend the results of Garcia-Vescovia et al. (11) that Mg^{2+} and Ca^{2+} repress the transcription of *pag* loci through the PhoP-PhoQ proteins and that under laboratory growth conditions, PmrA-PmrB does not mediate the regulatory effect of the cations Ca^{2+} or Mg^{2+} on *pag* locus transcription.

PhoQ-Ile-48 (PhoP-constitutive) mutants and wild-type strains grown in low levels of Mg^{2+} are polymyxin resistant as a result of increased expression of *pmrAB*. A point mutation in *pmrA (pmrA505)*, thought to result in a PmrA-constitutive phenotype, was shown to confer on *S. typhimurium* a marked increase in PM resistance (34). Because PhoP-PhoQ activates transcription of *pmrAB*, environmental conditions or mutations that activate PhoP-PhoQ may also result in PM resistance. Therefore, we examined the PM^r phenotype of the PhoQ-Ile-48 mutant singly and in combination with a *pmrA* mutation.

As shown in Fig. 2, CS022, which has increased expression of *pag* loci as a result of a PhoQ mutation, showed a dramatic increase in PM resistance compared with wild-type *S. typhimurium* (LD_{50}, 10 versus 0.5 to 1 μg ml^{-1}; 100% killing at >50 versus 10 to 25 μg ml^{-1}). The *phoP*::*Tn10*d strain (CS015) and the *pmrA*::*Tn10*d strain (JSG421) are slightly more sensitive to PM than the wild-type strain (LD_{50}, 0.25 to 0.5 μg ml^{-1}; 100% killing at 10 μg ml^{-1}). To determine if the *pmrAB* locus was responsible for the observed increase in the PM resistance of CS022, the *pmrA*::*Tn10*d allele was moved into CS022 by P22HT*int* transduction and the resulting strain, JSG430, was assayed for PM resistance. This strain had a PM phenotype similar to that of the PhoP-null and PmrA-null strains, demonstrating that the PM^r phenotype of CS022 was due to increased expression of the *pagB-pmrAB* operon.

This effect was also examined in strains carrying chromosomal or plasmid copies of the *pmrA505* allele (Fig. 3). CS100 (PhoP null) carrying a chromosomal or plasmid copy of the *pmrA505* allele was less resistant to PM (LD_{50}, 7 to 9 μg ml^{-1}; 100% killing at 50 μg ml^{-1}) than ATCC 14028s carrying the same allele (LD_{50}, 20 to 40 μg ml^{-1}; 100% killing at >100 μg ml^{-1}). JSG437 (PhoP null, *pmrA505*) was more resistant to PM than CS015 (PhoP null), suggesting that the *pmrA505* gene must be transcribed at a low level and translated in the absence of PhoP (likely from promoter P2) and that this subsequent low level of PmrA505 is enough to increase resistance to PM.

To examine the effect on PM resistance of environmental stimuli that activate PhoP-PhoQ, wild-type bacteria with or without a *pmrA*::*Tn10*d mutation were grown in PhoP-PhoQ-activating conditions (low Mg^{2+}) and examined for PM resistance. ATCC 14028s grown in medium with low Mg^{2+} was shown to be resistant to PM (LD_{50}, 25 μg ml^{-1}) at a level which is higher than that for CS022 and similar to that for a *pmrA505* mutant (Fig. 4). The introduction of a PmrA-null mutation resulted in a dramatic decrease in PM resistance (LD_{50}, 1 μg ml^{-1}), demonstrating again that PmrA-PmrB is necessary for the PM^r phenotype of *S. typhimurium* observed in PhoP-activating conditions.

Defensin and polymyxin resistance phenotypes are mediated by distinct PhoP-regulated genes or networks. *S. typhimurium* is resistant to another class of cationic peptides, defensins (8, 28). PhoP-null mutants are sensitive to defensins, suggesting that a *pag*(s) is responsible for this phenotype (8,

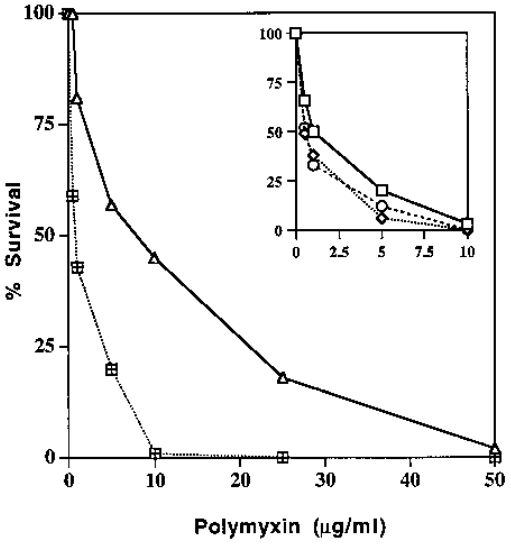

FIG. 2. Effect of PhoP-PhoQ and PmrA-PmrB on polymyxin resistance of *S. typhimurium*. The insert graph shows the Pm^s strains 14028s (wild type) (□), CS015 ($PhoP^-$) (◇), and JSG421 ($PmrA^-$) (○), which have LD_{50}s of 1.25, 0.5, and 0.5 μg ml^{-1}, respectively. CS022 (PhoQ-Ile-48) (△) demonstrated increased PM resistance (LD_{50}, 9 to 10 μg ml^{-1}), and introduction of a PmrA-null mutation into CS022 (JSG430 [PhoQ-Ile-48 $PmrA^-$]) (⊞) returned resistance levels to that of JSG421.

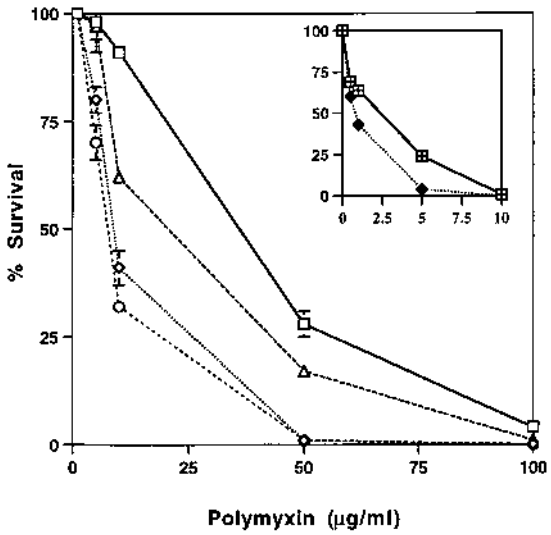

FIG. 3. Effect of PhoP-PhoQ mutants on the polymyxin resistance of *S. typhimurium* strains containing a chromosomal or plasmid-borne copy of the *pmrA505* allele. The insert graph shows the survival percentages of the controls JSG439 and JSG440 which contained the vector only. The LD_{50}s of these strains are similar to those of the strains without the vector (Fig. 2). A chromosomal copy of the *pmrA505* allele results in a dramatic increase in Pm resistance (LD_{50}, 35 μg ml^{-1}), and this resistance is markedly reduced in a PhoP-null background (LD_{50}, 8 μg ml^{-1}). A similar effect can be observed with ATCC 14028s and $PhoP^-$ strains containing a plasmid-borne copy of *pmrA505* (LD_{50}, 22 and 8 μg ml^{-1}, respectively). Symbols: □, JSG435 (ATCC 140285 *pmrA505*); △, JSG437 ($PhoP^-$ *pmrA505*); ○, JSG444 ($PhoP^-$ pKR100); △, JSG442 (ATCC 14028s pKR100); ⊞, JSG439 (ATCC 14028s pHSG576); ◆, JSG440 ($PhoP^-$ pHSG576).

14, 28). To date, none of the *pag* loci that have been defined have been found to be responsible for defensin resistance. However, since PmrA-PmrB regulates genes that modify phosphate groups of LPS to eliminate charge-to-charge interactions thought to allow access of both defensins and polymyxins through the outer membranes of gram-negative bacteria, we examined the effect of PmrA-PmrB on the defensin resistance phenotype of *S. typhimurium*. Exponential-phase cultures of strains containing various PhoP-PhoQ or PmrA-PmrB mutations were diluted to approximately 10^5 CFU ml^{-1} and incubated in the presence of 35 μg of NP-1 defensin per ml. As shown in Fig. 5, a PhoP-null mutant is, as expected, sensitive to this concentration of NP-1 defensin; however, the *pmrA*::*Tn10*d mutation had no effect on the defensin resistance phenotype of ATCC 14028s or CS022 (PhoQ-Ile-48). Therefore, PmrA-PmrB alone is not responsible for the sensitivity to defensin exhibited by PhoP-PhoQ mutants.

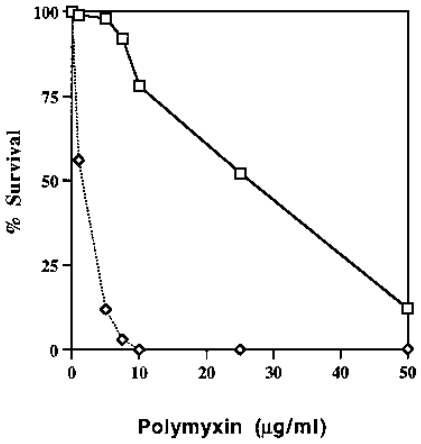

FIG. 4. Polymyxin resistance of strains grown in minimal medium with a low level of Mg^{2+} (8 μM), a condition known to activate *pag* gene transcription. ATCC 14028s (wild type) is resistant to high levels of polymyxin when grown in these conditions (LD_{50}, 25 μg ml^{-1}), and the introduction of a PmrA-null mutation results in a dramatic decrease in PM resistance (LD_{50}, 1 μg ml^{-1}). Symbols: □, ATCC 14028s; ◇, JSG421 ($PmrA^-$).

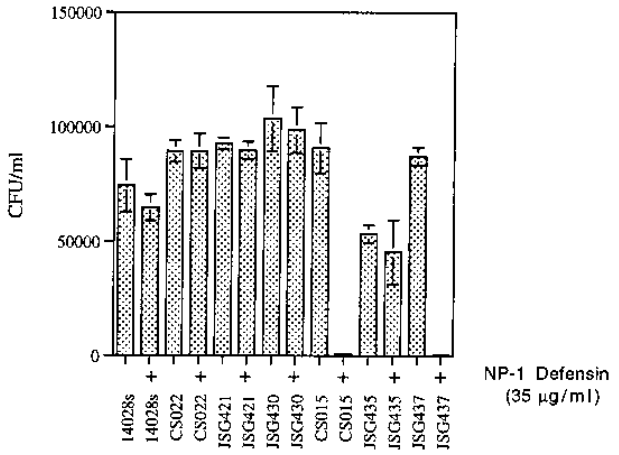

FIG. 5. Examination of the effect of *pmrA* mutations on the defensin resistance phenotype of *S. typhimurium*. A plus sign above the strain name indicates the addition of 35 μg ml^{-1} of NP-1 defensin. The strains examined were ATCC 14028s (wild type), CS022 [PhoQ-Ile-48 ($PhoP^c$)], JSG421 ($PmrA^-$), JSG430 (PhoQ-Ile-48 $PmrA^-$), CS015 ($PhoP^-$), JSG435 (PmrA505), and JSG437 ($PhoP^-$ PmrA505). Standard errors are shown for each individual bar.

DISCUSSION

The ubiquitous occurrence of antimicrobial cationic peptides in nature suggests that these compounds are a formidable host defense against microbial infection. Many successful pathogens have developed methods to evade host defenses. Salmonellae, which are thought to encounter cationic peptides during passage through the intestine and within phagocytic vacuoles, have evolved mechanisms to resist attack by antimicrobial peptides, and at least two of these mechanisms appear to be regulated by PhoP-PhoQ.

In this work, we demonstrate that *pagB*, a PhoP-activated gene previously shown to be induced when *S. typhimurium* is within macrophage phagosomes (1), forms an operon with *pmrAB* and that all three genes are regulated by PhoP-PhoQ. The *pagB-pmrAB* operon is positively autoregulated by PmrA at a promoter 5′ to *pagB*. In addition, a second weakly active, constitutively expressed promoter was found 5′ to *pmrA*.

PmrA and PmrB are similar to members of the OmpR-EnvZ family of two-component regulators, and on the basis of the isolation and characterization of what appears to be a constitutive mutation in the *pmrA* locus (*pmrA505*) (34), PmrA-PmrB regulates genes that promote resistance to the antimicrobial peptides polymyxin, CAP37, CAP57, protamine, and polylysine. Consistent with the regulation of *pmrAB* by PhoP-PhoQ, a *phoQ* locus mutant (CS022) with a PhoP-constitutive phenotype and wild-type *S. typhimurium* grown in conditions that activate *pag* transcription were shown to display a marked increase in PM resistance. Resistance in both cases was dependent on *pmrAB* because a transposon insertion in *pmrA* abrogated PM resistance. In addition, strains carrying the *pmrA505* mutation require PhoP-PhoQ to express full resistance. There-

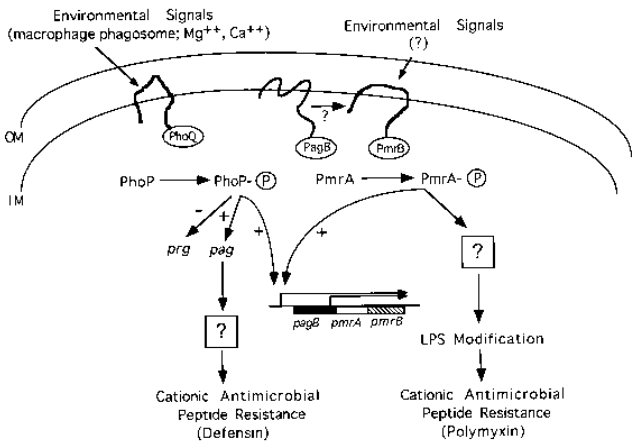

FIG. 6. Diagram of the regulatory cascade involving PhoP-PhoQ and PmrA-PmrB that results in lipid A modifications and cationic peptide resistance. Upon entry of *S. typhimurium* into a macrophage phagosome, signals are sensed by PhoQ, which activates PhoP through a phosphorelay system. Phospho-PhoP represses the transcription of *prg* loci and activates the transcription of *pag* loci, one of which is the *pagB-pmrAB* operon. Increased transcription of this operon results in lipid A modifications and cationic peptide resistance, likely by activation of PmrA-regulated genes. Pag or Pag-mediated functions, such as LPS modifications not directed by PmrA-PmrB, affect resistance to other cationic antimicrobial peptides including defensins. Because *pagB*, which encodes a putative membrane protein, is cotranscribed with *pmrAB*, it is interesting to speculate that PagB could play a role in PM resistance by interacting with PmrB in the inner membrane. The environmental signals that activate the PmrA-PmrB system are unknown, as are the genes regulated by PmrA-PmrB that modify LPS.

fore, a regulatory cascade involving PhoP-PhoQ and PmrA-PmrB is necessary for maximal resistance to antimicrobial, cationic peptides (Fig. 6).

Resistance of *S. typhimurium* to antimicrobial peptides has been correlated with virulence in mice. This correlation was established in studies of transposon mutants susceptible to protamine (14). Loci identified included the *sapABCDF* operon, which encodes proteins required for virulence in mice and which belongs to the "ATP binding cassette" family of peptide transporters (31), and the *sapG* gene product, a member of a low-affinity K^+ transport system required for wild-type virulence in mice (32). These data suggest that PmrA-PmrB functions may be essential for virulence in mice. However, intraperitoneal injection of the PmrA-null strain JSG421 into mice had no significant effect on the LD_{50}, indicating that the loss of PmrA-PmrB does not dramatically alter *S. typhimurium* virulence in mice (15).

Mutations in *pmrA* had no effect on the resistance of *S. typhimurium* to another antimicrobial cationic peptide, NP-1 defensin, hence the ability to define two distinct PhoP-PhoQ regulated cationic peptide resistance pathways (Fig. 6). PhoP-PhoQ mutants are hypersensitive to a number of antimicrobial peptides, including defensins and magainins, most likely as a result of lack of expression of *pag*(s) (8, 14, 28). To date, no PhoP-regulated gene(s) has been defined as responsible for the PhoP-null mutant cationic peptide hypersensitivity phenotype. It is unclear which antimicrobial peptide resistance mechanism regulated by PhoP-PhoQ is most relevant to virulence in mice. A PmrA-null mutant appears to have wild-type virulence in mice, which may suggest that resistance to defensins (or defensin-like antimicrobial factors) is more important to the survival of salmonellae within phagocytic cells. Alternatively, it is possible that both resistance mechanisms are redundant and both protect against the actual relevant antimicrobial peptide(s) encountered by *S. typhimurium* in vivo within macrophage phagosomes. The number of genes involved in resistance and the involvement of at least two regulatory systems induced in vivo upon infection suggests that cationic peptides represent an important obstacle for *Salmonella* spp. to overcome in order to cause disease.

A mutation (*pmrA505*) closely linked to *pagB-pmrAB* and resulting in PM resistance was shown to have an increase in LPS phosphate substitutions of both core and lipid A (19, 45). These covalent LPS modifications were not demonstrated for the *pmrA505* mutation, which is closely linked to but which has not been shown to be identical to the PM^r mutant for which lipid A was analyzed. A mass spectrometry examination of lipid A from JSG435 (*pmrA505*), CS022 (PhoQ-Ile-48), and a wild-type strain grown in defined medium with low Mg^{2+} demonstrated the presence of 4-aminoarabinose modifications of lipid A phosphate groups that were not found on lipid A of PhoP-null mutants (18). This result demonstrates that PhoP-PhoQ activation of *pmrAB* results in LPS covalent modifications. These modifications likely occur in vivo since transcription of *pagB* is activated within the macrophage phagosome (1), and *pmrA* and *pmrB* have been identified as in vivo-expressed genes in a screen that utilized the in vivo expression technology (IVET) system (6). The studies of Guo et al. (18) also demonstrated unique structural alterations of LPS that are mediated by PhoP-PhoQ but not PmrA-PmrB. Therefore, a major function of PhoP-PhoQ activation after phagocytosis by macrophages may be the alteration of lipid A structure and a reduction of cell envelope net negative charge. It seems plausible that lipid A alterations (or a combination of lipid A alterations and production of Pag products) not mediated by PmrA-regulated genes are responsible for resistance to other cationic antimicrobial peptides including defensins.

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