JOURNAL OF BACTERIOLOGY, June 2002, p. 3203–3213 Vol. 184, No. 12
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.12.3203–3213.2002

mig-14 Is a *Salmonella* Gene That Plays a Role in Bacterial Resistance to Antimicrobial Peptides

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Received 29 January 2002/Accepted 25 March 2002

It was previously demonstrated that the *mig-14* gene of *Salmonella enterica* serovar Typhimurium is necessary for bacterial proliferation in the liver and spleen of mice following intragastric inoculation and that *mig-14* expression, which is induced within macrophages, is under the control of the global regulator PhoP. Here we demonstrate that the *mig-14* promoter is induced by growth in minimal medium containing low magnesium or acidic pH, consistent with regulation by PhoP. In addition, *mig-14* is strongly induced by polymyxin B, protamine, and the mammalian antimicrobial peptide protegrin-1. While *phoP* is necessary for the induction of *mig-14* in response to protamine and protegrin, *mig-14* is still induced by polymyxin B in a *phoP* background. We also demonstrate that *mig-14* is necessary for resistance of *S. enterica* serovar Typhimurium to both polymyxin B and protegrin-1. Gram-negative resistance to a variety of antimicrobial peptides has been correlated with modifications of lipopolysaccharide structure. However, we show that *mig-14* is not required for one of these modifications, the addition of 4-aminoarabinose to lipid A. Additionally, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of wild-type and *mig-14* lipopolysaccharide also shows no detectable differences between the two strains. Therefore, *mig-14* contributes to *Salmonella* resistance to antimicrobial peptides by a mechanism that is not yet fully understood.

Salmonella enterica serovar Typhimurium is a gram-negative facultative intracellular pathogen that causes self-limiting gastroenteritis in humans and a disease resembling systemic typhoid fever in mice. After being ingested, *Salmonella* traverses the stomach and invades M cells in the Peyer's patches of the small intestinal epithelium (46). After invasion, the bacteria are engulfed by host macrophages (5, 22) in which they survive and replicate within a specialized compartment known as the *Salmonella*-containing vacuole (2). The two-component regulatory system encoded by the *phoPQ* operon is required for the ability of *Salmonella* to survive and replicate within these phagocytes (7, 29).

Gram-negative bacteria frequently utilize two-component systems consisting of a sensor kinase and a DNA binding component to sense and respond to a variety of conditions, including changes in pH, ion concentration, and osmolarity. The survival of *Salmonella* in different host environments requires coordinate regulation of many gene products in response to these changing environmental conditions. The PhoQ sensor kinase responds to changes in the concentration of Mg^{2+} ions and phosphorylates its cognate response regulator PhoP in environments containing micromolar concentrations of Mg^{2+} (10, 41). PhoP in turn activates a number of genes, including those necessary for intracellular survival and antimicrobial peptide resistance (8, 31).

Antimicrobial peptides are an essential aspect of host innate immune defense and are produced by macrophages, neutrophils, and mucosal epithelial cells (18). These peptides are small (2 to 4 kDa) cationic molecules of various structural classes that bind to and permeabilize bacterial membranes. Within host tissues, *Salmonella* is likely to encounter antimicrobial peptides in the intestine, where Paneth cells constitutively secrete them, as well as within the host cell phagosomes that fuse with antimicrobial peptide-containing vesicles (34, 38). The ability of pathogenic bacteria to resist killing by host antimicrobial peptides present in different host niches may therefore contribute to their virulence.

Salmonella resistance to antimicrobial peptides is frequently mediated by modifications to the lipopolysaccharide (LPS) component of the bacterial outer membrane. Complete LPS consists of an endotoxic component, lipid A, which is linked to a polysaccharide molecule consisting of a core oligosaccharide and a repeating sugar structure known as the O antigen (35). Environmentally induced modifications to the structure of lipid A are correlated with resistance to several classes of antimicrobial peptides. For example, substitution of the 4′ phosphate with 4-aminoarabinose has been shown to be important for resistance to the antimicrobial peptide polymyxin B (PB) (13). Recently, it has been demonstrated that both 4′ and 1 phosphates can be substituted with aminoarabinose (47), although substitution at the 4′ phosphate is what has been correlated with resistance. PB is an amphipathic cationic molecule that binds to LPS and permeabilizes the outer and inner membranes of gram-negative bacteria by a mechanism thought to be similar to that of antimicrobial peptides (44, 49). Thus, resistance to PB often confers cross-resistance to some classes of mammalian antimicrobial peptides in vitro (39) and correlates with increased survival of *Salmonella* in neutrophils (43).

4-Aminoarabinose addition to lipid A and the addition of ethanolamine are controlled by a second two-component regulatory system encoded by *pmrAB* (13). The response regulator

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PmrA is regulated indirectly by PhoP through the PmrD protein (26). In addition, the PhoP-PhoQ system coordinately regulates several other unlinked loci involved in different LPS modifications that confer resistance to a number of structurally unrelated antimicrobial peptides (16, 17). Therefore, *phoP* mutants of *Salmonella* are highly sensitive to multiple classes of antimicrobial peptides due to an inability to generate several modified forms of LPS. In addition to LPS modifications, changes in expression of outer membrane proteins also play a role in conferring resistance to antimicrobial peptides. For example, the outer membrane protease PgtE has been shown to cleave alpha-helical antimicrobial peptides and is also regulated by the PhoP-PhoQ system (12). Furthermore, the *rck* gene found on the *S. enterica* serovar Typhimurium virulence plasmid encodes an outer membrane protein that confers increased resistance to complement killing when overexpressed in *Escherichia coli* (19). A recent screen in our laboratory for genes that can restore PB resistance to the *mig-14* mutant identified *rck* (unpublished data). Although *rck* is not known to be regulated by PhoP, it is coexpressed in vitro with *Salmonella* genes that are induced within the macrophage vacuole (C. Detweiler and S. Falkow, unpublished data).

We examined the regulation and function of the *Salmonella* gene *mig-14*, which is dependent on PhoP for transcriptional activation within cultured macrophages (53) and contributes to oral virulence in mice (51). We demonstrate here that the *mig-14* promoter is activated in minimal medium containing limiting concentrations of magnesium, low pH, or antimicrobial cationic peptides. The *mig-14* promoter is upregulated in response to PB in a *phoP*-independent manner, suggesting the existence of additional regulators of *mig-14*. The *mig-14* strain shows increased sensitivity to the antimicrobial peptides PB and protegrin-1 (PG-1), indicating that regulation of *mig-14* by these peptides is functionally important. Analysis of *mig-14* lipid A by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and crude LPS preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed no differences from the isogenic wild-type strain. Therefore, the mechanism by which Mig-14 contributes to antimicrobial peptide resistance remains to be elucidated.

MATERIALS AND METHODS

Media and culture conditions. Bacteria were grown overnight at 37°C with aeration in the N minimal medium of Nelson and Kennedy containing 10 mM $MgCl_2$ (32) with modifications as previously described (40), except where otherwise indicated. The pH of the medium was buffered with either 100 mM Tris-HCl, pH 7.4, or 10 mM methyl-ethyl sulfanilamide, pH 5.8. All marked mutations were moved into the SL1344 background by transduction with P22HT by standard methods (27). The strains and plasmids used in this study are listed in Table 1.

PB sensitivity assay. This assay was performed essentially as previously described (11). Briefly, bacterial strains were inoculated into modified N minimal medium at pH 7.4 containing 10 mM $MgCl_2$. Stationary-phase cultures were harvested and washed three times in N minimal medium, pH 7.4, without $MgCl_2$. Strains were then diluted 1:100 into one of four N minimal media: 10 mM $MgCl_2$, pH 7.4; 10 μM $MgCl_2$, pH 7.4; 10 mM $MgCl_2$, pH 5.8; or 10 μM $MgCl_2$, pH 5.8. Bacteria were incubated for 3 h at 37°C with aeration. Bacteria (5×10^4 to 1×10^5 per ml) from each culture condition were inoculated into Luria broth (LB) containing 2.5 μg of PB (8,070 U/g) (Sigma)/ml and grown for 1 h at 37°C with aeration. Serial dilutions of each culture were made in phosphate-buffered saline (PBS) and plated on selective agar. Percent survival was calculated either relative to the initial input or relative to the growth of each strain in LB in the absence of PB, as indicated in the text.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
SL1344	*rpsL hisG xyl*	21
RVY-5	SL1344 *mig-14*::Kan	51
phoP::Tn*10*	SL1344 *phoP*::Tn*10*Tet^r	36
LT2	Wild type	37
JKS1379	LT2 *pmrA508*::ΩKan	37
pmrA::Kan	SL1344 *pmrA*::Kan	This study
Plasmids		
pHSG576	pSC101 origin, Cm^r	45
pKR301	pHSG576, 1.2-kb *pmrD*-containing insert	37
pFPV25	ColE1 origin, promoterless GFP	52
pFPV25.1	pFPV25, *rpsM* promoter::GFP	52
p*mig14*::*GFP*	pFPV25, *mig-14* promoter::GFP	53
p*pmrD*::*GFP*	pFPV25, *pmrD* promoter::GFP	This study
p*pmrC*::*GFP*	pFPV25, *pmrCAB* promoter::GFP	This study

PG-1 sensitivity assay. Bacterial strains were grown as described above for the PB sensitivity assay. After the 3-h growth period, 5×10^4 to 1×10^5 bacteria per ml were added to 96-well plates containing 200 μl of LB with the appropriate concentration of peptide. Each 96-well plate was placed at 37°C on a platform shaker and gently agitated for 1 h. Each strain and peptide concentration was tested in triplicate. Appropriate dilutions were made in PBS and plated for CFU. Percent survival was calculated relative to bacteria treated in the absence of peptide.

Lipid A purification and MALDI-TOF analysis. Strains were grown overnight in the indicated medium, and LPS was purified by the Mg^{2+}-ethanol precipitation method as previously described (4). Lipid A was purified by hydrolysis in 1% SDS at pH 4.5 (3). Before being applied on a sample plate, the lyophilized lipid A was dissolved in 5 μl of 5-chloro-2-mercaptobenzothiazole MALDI matrix in chloroform-methanol (1:1). Negative-ion MALDI-TOF was performed as described previously (6).

Construction of *pmrD* reporter plasmids. The promoter regions of *pmrD* (290 bases upstream of the start ATG) and *pmrC* (208 bases upstream of the start ATG) were amplified in a Stratagene RoboCycler with PCRSupermix (Gibco BRL) and standard PCR conditions. The primers used for *pmrD* were pmrD5′, GCGAATTCAGTTTGGTC-ATCGTCCCGTTG, and pmrD3′, GCGGATC CGTTTCACGCTGATTGTTCATAAC. The primers used for *pmrC* were pmrC5′, GCGAATTCTCGAATTCGATCACCGCGCTG, and pmrC3′, GCGG ATCCGACTTCGGTGACGCTAATCGTG. The PCR products were cloned into the *Eco*RI-*Bam*HI sites of the green fluorescent protein (GFP) reporter plasmid pFPV25 (52) to generate p*pmrD*::*GFP* and p*pmrC*::*GFP*.

Fluorescence-activated cell sorter-based analysis of promoter activity. Bacterial strains harboring reporter constructs were grown overnight in N minimal medium, pH 7.4, plus 10 mM $MgCl_2$, harvested, washed in N minimal medium, pH 7.4, without $MgCl_2$, and diluted 1:100 into minimal medium, pH 7.4, plus 10 mM $MgCl_2$. Mid-log-phase bacteria (optical density at 600 nm, 0.5 to 0.6) were then inoculated into the indicated media and grown for 3 h to allow expression of GFP. Bacteria were diluted into PBS and analyzed by flow cytometry with a Becton Dickinson FACSCalibur and CellQuest acquisition and analysis software as previously described (52).

RESULTS

***mig-14* is induced by low Mg^{2+} concentrations.** In order to increase our understanding of *mig-14* regulation and investigate the role of *mig-14* in virulence, we used a *mig-14*::GFP transcriptional reporter fusion to determine the conditions necessary for induction or repression of *mig-14*. Previous work in our laboratory has demonstrated that *mig-14* expression in macrophages requires the two-component regulatory system PhoP-PhoQ (53). Since this system is activated by low Mg^{2+} concentrations (10), we first examined expression of *mig-14* in medium containing limiting concentrations of Mg^{2+}. For comparison to other PhoP-regulated genes involved in antimicro-

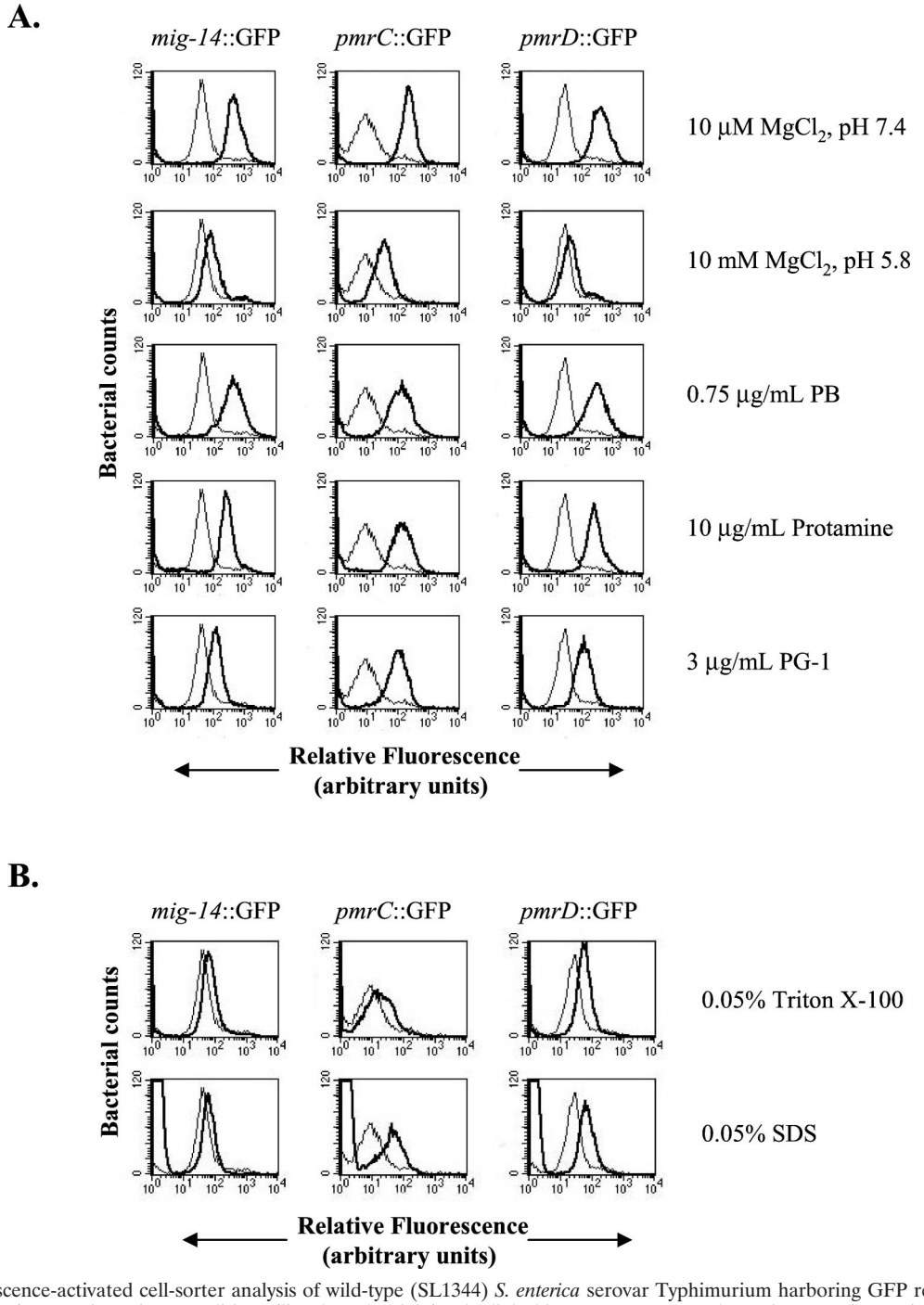

FIG. 1. Fluorescence-activated cell-sorter analysis of wild-type (SL1344) *S. enterica* serovar Typhimurium harboring GFP reporter constructs (indicated at the top) grown in various conditions (listed on the right). The light histograms represent bacteria grown in N minimal medium, pH 7.4, and 10 mM $MgCl_2$; the bold histograms represent bacteria grown in identical N minimal medium with additions or modifications as indicated. The activity of all three promoters is significantly induced by low Mg^{2+} and by antimicrobial peptides. (A) Induction by low Mg, low pH, and cationic antimicrobial peptides. (B) Induction by membrane-active detergents.

bial peptide resistance, we made reporter fusions to the *pmrC* and *pmrD* promoters. *pmrD* is activated by PhoP, and the PmrD protein is thought to posttranscriptionally regulate the activity of PmrA (26). *pmrC* is the first gene of the *pmrCAB* operon and is cotranscribed with *pmrAB* (14). The *pmrC* promoter has been shown to be regulated by both PhoP and PmrA (14, 55). Therefore, the transcriptional activity of *pmrD* should be PhoP dependent but PmrA independent, whereas the activity of *pmrC* should be both PhoP and PmrA dependent.

Transcription from the *mig-14* promoter was induced approximately 10-fold more in 10 μM $MgCl_2$ than in 10 mM $MgCl_2$ (Fig. 1A and Table 2). The *pmrC* and *pmrD* promoters were similarly induced in this low-magnesium medium. These data indicate that, like other PhoP-activated genes, *mig-14* is

TABLE 2. Fold induction of promoter activity in indicated medium condition[a]

Strain	Medium condition	Result for promoter fusion					
		mig-14::GFP		*pmrC*::GFP		*pmrD*::GFP	
		Mean peak fluorescence (au[e])	Fold induction	Mean peak fluorescence (au[e])	Fold induction	Mean peak fluorescence (au[e])	Fold induction
SL1344 (wild type)[b]	10 μM $MgCl_2$	399	11.7	199	19.9	361	14.4
	pH 5.8	70	2.1	34	3.4	35	1.4
	PB	333	9.8	129	12.9	266	10.6
	Protamine	218	6.4	107	10.7	214	8.6
	PG-1	112	3.3	107	10.7	101	4.0
	Triton X-100	57	1.7	13	1.3	45	1.8
	SDS	56	1.6	39	3.9	53	2.1
phoP::Tn*10*[c]	10 μM $MgCl_2$	6	1	1	1	4	1
	pH 5.8	6	1	5	5	3	0.75
	PB	26	4.3	1	1	9	2.25
	Protamine	5	0.8	1	1	5	1.25
	PG-1	4	0.7	1	1	4	1
	Triton X-100	6	1.0	1	1	4	1
	SDS	6	1.0	1	1	5	1.25
pmrA::Kan[d]	10 μM $MgCl_2$	798	20.5	1	1	509	11.6
	pH 5.8	67	1.7	1	1	42	1
	PB	805	20.6	1	1	212	4.8
	Protamine	286	7.3	1	1	222	5.0
	PG-1	104	2.7	1	1	158	3.6
	Triton X-100	56	1.4	1	1	58	1.3
	SDS	54	1.4	1	1	74	1.7

[a] Mean peak fluorescence values are for the populations grown under the condition indicated. Fold inductions were calculated by dividing the mean peak fluorescence values for growth under the condition indicated by the mean peak fluorescence values for growth in 10 mM $MgCl_2$ at pH 7.4 (values given in footnotes *b, c*, and *d*).
[b] Graphic representations of these results are shown in Fig. 1. Mean peak fluorescence values of the bacterial population grown in 10 mM $MgCl_2$ at pH 7.4 are as follows: for *mig-14*::GFP, 34; for *pmrC*::GFP, 10; and for *pmrD*::GFP, 25.
[c] Graphic representations of these results are shown in Fig. 2. Mean peak fluorescence values of the bacterial population grown in 10 mM $MgCl_2$ at pH 7.4 are as follows: for *mig-14*::GFP, 6; for *pmrC*::GFP, 1; and for *pmrD*::GFP, 4.
[d] Graphic representations of these results are shown in Fig. 3. Mean peak fluorescence values of the bacterial population grown in 10 mM $MgCl_2$ at pH 7.4 are as follows: for *mig-14*::GFP, 39; for *pmrC*::GFP, 1; and for *pmrD*::GFP, 44.
[e] au, arbitrary units.

induced by low concentrations of Mg^{2+}. Also like other PhoP-activated genes, *mig-14* promoter activity was constitutively induced to high levels in a strain containing constitutively activated PhoP even in medium with 10 mM $MgCl_2$ (data not shown).

Because *mig-14* is induced in the macrophage phagosome, we asked whether low pH would also induce *mig-14* transcription. Indeed, we observed that low pH in the absence of low Mg^{2+} could induce *mig-14* expression, albeit at lower levels than medium containing low Mg^{2+} alone (Table 2). There was no synergistic effect between magnesium and pH, as pH 5.8 medium also containing low Mg^{2+} did not show a greater fold induction of *mig-14* than the low Mg^{2+} medium alone (data not shown).

***mig-14* is induced by antimicrobial peptides.** Recent work has demonstrated that PhoP activates a number of genes, including the two-component response regulator encoded by *pmrA*, that are important in *Salmonella* resistance to PB and some mammalian antimicrobial peptides (13). It is likely that *Salmonella* encounters antimicrobial peptides within host microenvironments during in vivo infection. These peptides may contribute to environmental signals that trigger changes in bacterial gene expression. We therefore examined the cationic peptides PB, protamine, and PG-1 to determine whether any or all of these could stimulate *mig-14* gene expression. Protamine is a polycationic molecule present in sperm nuclei, and PG-1 is a mammalian antimicrobial peptide originally isolated from porcine leukocytes (25). In all cases, we observed that expression of GFP from the *mig-14* reporter fusion was significantly higher in medium containing antimicrobial peptide than in medium without peptide. Similarly, the *pmrC* and *pmrD* reporters were also induced in media containing these antimicrobial peptides (Fig. 1A and Table 2). PB induced the greatest level of expression in all cases, with the *pmrC* promoter exhibiting the highest overall levels of induction. Expression of the *phoP*-independent gene *rpsM* was unaltered in all of the above conditions, demonstrating that these conditions specifically induce the expression of genes that are PhoP regulated (data not shown).

Exposure of bacteria to high concentrations of cationic peptides in most cases results in membrane damage and bacterial death. We therefore examined the membrane-active detergents Triton X-100 and SDS to determine whether exposure to membrane-damaging agents per se would induce expression of the *mig-14* and *pmr* GFP reporters. In contrast to both *pmrC* and *pmrD*, *mig-14* was only slightly induced by these detergents (Fig. 1B and Table 2). Interestingly, *pmrC* was markedly induced in the presence of SDS. *phoP* was required for the induction of these reporters in Triton X-100 and SDS, as this activation was not observed in the *phoP*::Tn*10* mutant (Fig.

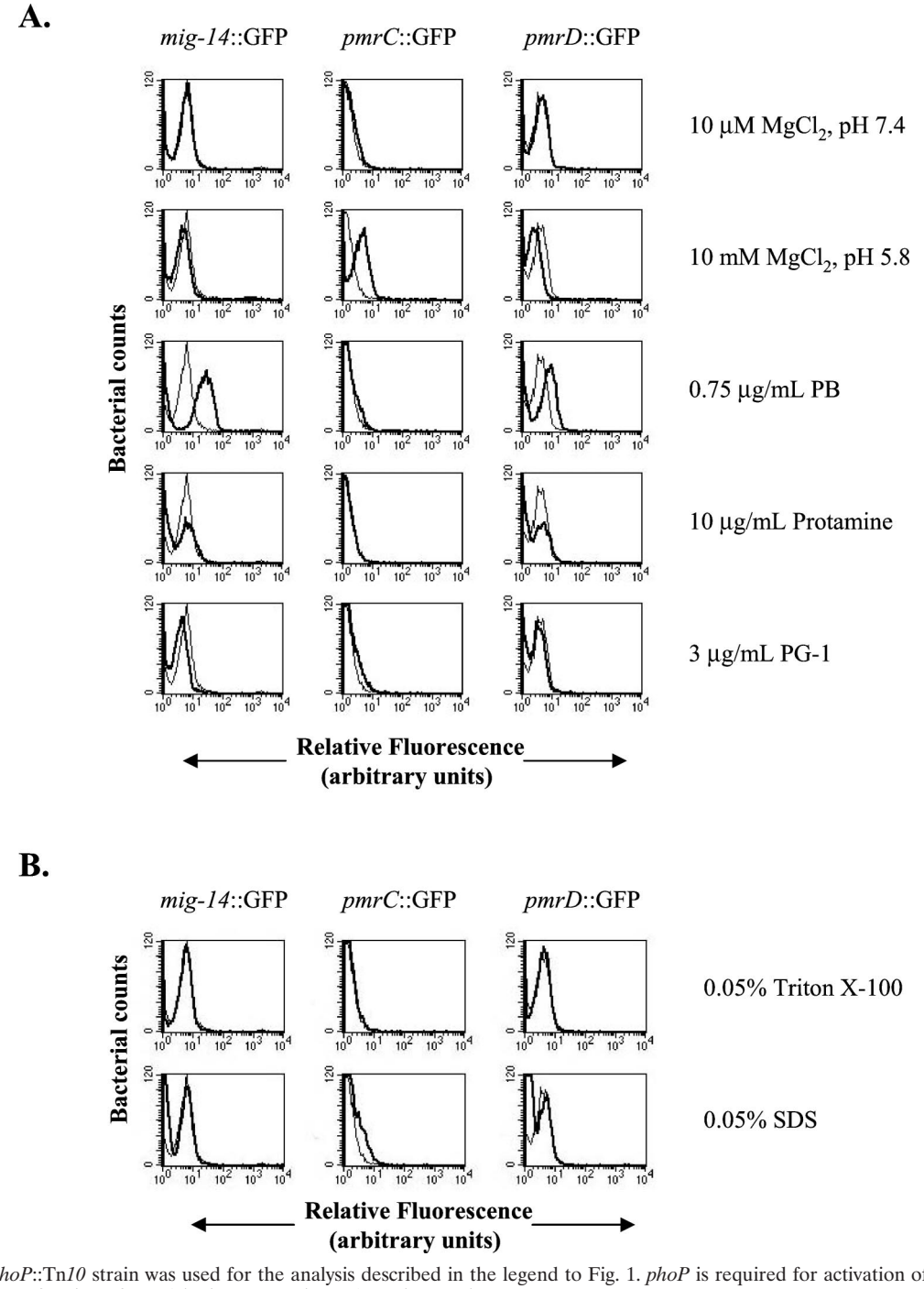

FIG. 2. The *phoP*::Tn*10* strain was used for the analysis described in the legend to Fig. 1. *phoP* is required for activation of all promoters by all conditions except for that of *pmrC* by low pH and *mig-14* and *pmrD* by PB.

2B). However, we found that induction of the reporter fusions by these detergents was only observed in log-phase-grown bacteria. The nondetergent conditions induced expression of all three reporters regardless of the growth phase (data not shown). This is consistent with previous results that PhoP-regulated genes do not play a role in resistance to killing by Triton X-100 or SDS (54). These data suggest that in addition to *pmrA*, *pmrD*, and other PhoP-regulated genes, *mig-14* may play a role in adaptive responses to membrane perturbations caused specifically by antimicrobial peptides.

PB induces *mig-14* in a *phoP*-independent manner. *phoP* was previously shown to be required for the induction of *mig-14* in macrophages. We therefore asked whether *phoP* was also required for the induction of *mig-14* in response to these in vitro conditions. As expected, we found that in a *phoP*::Tn*10* strain background, expression of all three PhoP-regulated genes was

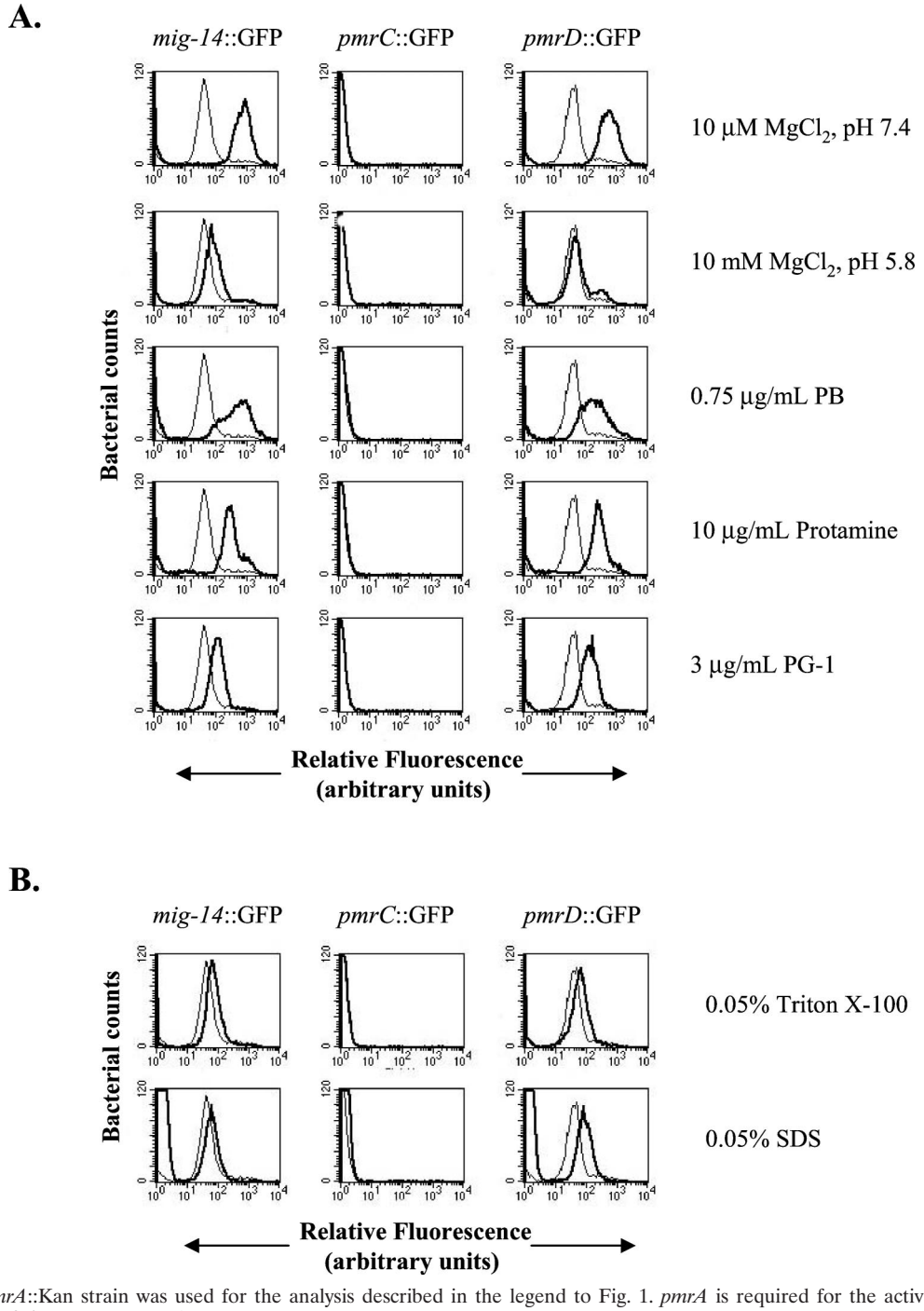

FIG. 3. The *pmrA*::Kan strain was used for the analysis described in the legend to Fig. 1. *pmrA* is required for the activation of the *pmrC* promoter but not of the *pmrD* or *mig-14* promoters.

reduced compared to that of the wild type (compare Fig. 1A and 2A; Table 2). We also found that *phoP* was required for induction of all three reporter constructs in low Mg^{2+}, protamine, and PG-1. *pmrC* was still induced by low pH in a *phoP*::Tn*10* background, consistent with previous results that the PmrA-PmrB system responds to low pH independently of PhoP-PhoQ (42). We also found that in contrast to the other antimicrobial peptides, PB induced expression of both *mig-14* and *pmrD* reporter constructs even in the *phoP*::Tn*10* background, although absolute levels of induction were lower than in the isogenic wild type (Fig. 2A and Table 2). This suggests that an additional system regulates gene expression in response to PB and possibly other antimicrobial peptides.

***pmrA* is not required for regulation of *mig-14*.** It has been demonstrated that a subset of PhoP-regulated genes is directly controlled by an alternate two-component regulatory system encoded by *pmrAB*. We therefore examined the transcriptional responses of the *mig-14*, *pmrC*, and *pmrD* reporter fusions in a

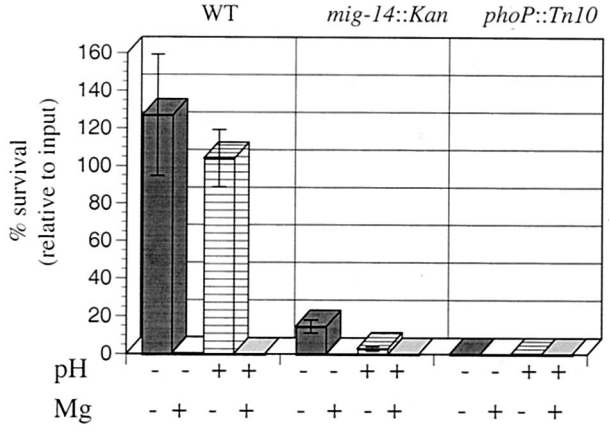

FIG. 4. Survival of *S. enterica* serovar Typhimurium strains after a 1-h PB challenge. Bacterial strains were exposed to 2.5 μg of PB/ml in LB for 1 h after being grown in the following N minimal media: 10 μM $MgCl_2$, pH 5.8 (dark grey bars); 10 mM $MgCl_2$, pH 5.8 (white bars); 10 μM $MgCl_2$, pH 7.4 (lined bars); and 10 mM $MgCl_2$, pH 7.4 (light grey bars). WT refers to the SL1344 strain. Data are expressed relative to the input bacterial CFU. For ease of analysis, Mg^{2+} and pH conditions are indicated below the corresponding bars. For Mg^{2+}, a plus sign refers to 10 mM and a minus sign refers to 10 μM $MgCl_2$. For pH, a plus sign indicates pH 7.4 and a minus sign indicates pH 5.8. The *mig-14*::Kan strain is 10- to 20-fold more sensitive to polymyxin than the corresponding wild-type strain. The survival of the *phoP*::Tn*10* strain was less than 1% for all conditions. Experiments were performed at least three times in triplicate, and the data presented are from a representative experiment.

pmrA::Kan strain background. We found that induction of *mig-14* and *pmrD* still occurred, in contrast to the *pmrC* reporter, which was no longer induced under any of the conditions tested (Fig. 3A). PmrB has been shown to sense both iron and pH, and it activates PmrA in response to high iron (56). We did not see induction of *mig-14* in the presence of iron, confirming that *mig-14* is not regulated directly by PmrA-PmrB (data not shown). However, in the case of the *mig-14* reporter, expression levels in low Mg^{2+} and PB were significantly higher in the *pmrA*::Kan strain than in the wild type (Table 2). Furthermore, uninduced levels of *pmrD* were approximately twofold higher than in the wild-type strain. These data suggest that although *pmrA* is not required for induction of *mig-14* or *pmrD*, it may be involved in feedback regulation.

***mig-14* is necessary for inducible polymyxin resistance.** We next investigated the functional significance of the ability of antimicrobial peptides to induce *mig-14* promoter activity by examining the resistance of the *mig-14*::Kan strain to PB. Figure 4 shows survival data for *Salmonella* strains grown in various conditions followed by a 1-h exposure to 2.5 μg of PB/ml. Consistent with previously reported results, we observed that the wild-type strain, SL1344, was resistant to PB after growth in medium containing low concentrations (10 μM) of Mg^{2+}. The *mig-14*::Kan mutant was approximately 5-fold more sensitive to PB in low-pH medium containing low Mg^{2+} and approximately 20-fold more sensitive in low-Mg^{2+} medium alone than the wild-type strain. As expected, the isogenic *phoP*::Tn*10* strain was completely susceptible to PB challenge following growth in all of the conditions tested. Nearly 100% of the *phoP*::Tn*10* strains were killed within the first 15 min of PB challenge (data not shown).

***mig-14*::Kan sensitivity to polymyxin is complemented by overexpression of PmrD.** Previous work has shown that overexpression of *pmrD* confers increased resistance to PB (37). This has been hypothesized to be due to increased expression of genes regulated by PmrA, which is positively regulated by PmrD (26). We transformed SL1344, *mig-14*::Kan, and *pmrA*::Kan strains with a plasmid, pKR301, that expresses *pmrD* from its native promoter to determine whether *pmrD* overexpression could restore wild-type levels of PB resistance to the *mig-14* and *pmrA* strains. Overexpression of *pmrD* did indeed restore nearly wild-type levels of resistance to the *mig-14*::Kan mutant strain (Fig. 5). This effect was not due to nonspecific activity of the plasmid, since overexpression of *pmrD* failed to complement the *pmrA*::Kan strain, and the parent plasmid pHSG576 also did not confer increased PB resistance. The ability of *pmrD* overexpression to complement the PB sensitivity of the *mig-14* strain suggests that *mig-14* could participate in modification of LPS along with other genes regulated by PhoP-PhoQ and PmrA-PmrB. Alternatively, the complementation could be due to overexpression of genes regulated by PmrD and PmrA-PmrB which can bypass the defect in the *mig-14* strain. In addition to restoring PB resistance with *pmrD*, we could also partially restore PB resistance to the *mig-14* strain with a *mig-14*-expressing plasmid (data not shown). This plasmid had been previously used to complement the *mig-14* mutant for virulence in oral infection of mice (51).

***mig-14* is not required for addition of 4-aminoarabinose to LPS.** A major mechanism of resistance to PB that is controlled by the PmrA-PmrB system is the addition of 4-aminoarabinose to the 4′ phosphate group of lipid A (13, 20), though some evidence suggests that PB resistance can be restored to a sensitive mutant even in the absence of 4-aminoarabinose (13). To examine if Mig-14 participates in the addition of 4-aminoarabinose to lipid A, we analyzed lipid A of wild-type and *mig-14* mutant strains by MALDI-TOF mass spectrometry as de-

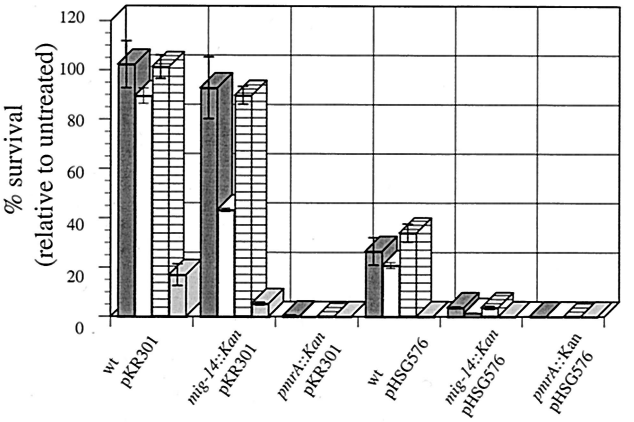

FIG. 5. Survival of *S. enterica* serovar Typhimurium strains overexpressing *pmrD* after a 1-h PB challenge. Data are expressed relative to identical strains grown without PB for 1 h. Dark grey bars, 10 μM $MgCl_2$, pH 5.8; white bars, 10 mM $MgCl_2$, pH 5.8; lined bars, 10 μM $MgCl_2$, pH 7.4; light grey bars, 10 mM $MgCl_2$, pH 7.4. Bacterial strains harboring the *pmrD*-expressing plasmid pKR301 were treated as described in Materials and Methods. Overexpression of *pmrD* restored PB resistance to the *mig-14*::Kan strain but not to the *pmrA*::Kan mutant.

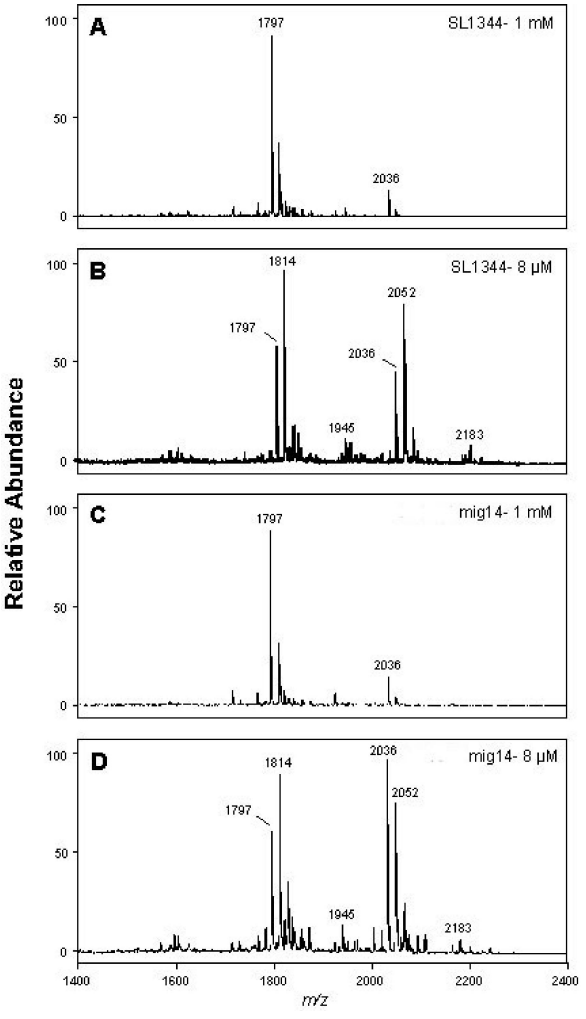

FIG. 6. MALDI-TOF mass spectra of purified lipid A from wild-type (SL1344) and *mig-14*::Kan (mig14) strains. Strains were grown in N minimal medium containing either 1 mM (A and C) or 8 μM (B and D) $MgCl_2$. The growth of both strains in 1 mM $MgCl_2$ shows the expected peaks at an *m/z* of 2,036 (hepta-acylated wild-type LPS) and at an *m/z* of 1,797 (hexa-acylated LPS lacking palmitate). Analysis of LPS from the wild-type and *mig-14*::Kan strains grown in 8 μM $MgCl_2$ yielded peaks at *m/z* of 2,183 and 1,929, which is consistent with the presence of hepta- and hexa-acylated forms of LPS containing the 4-aminoarabinose modification (B and D).

scribed in Materials and Methods. Lipid A was isolated from wild-type and isogenic *mig-14*::Kan strains after growth in N minimal medium containing 1 mM or 8 μM $MgCl_2$. As expected, neither SL1344 nor *mig-14*::Kan strains grown in 1 mM $MgCl_2$ contained detectable amounts of 4-aminoarabinose-modified LPS (Fig. 6A and C). However, when grown in limiting Mg^{2+} conditions, both strains synthesized 4-aminoarabinose lipid A as indicated by the presence of peaks at *m/z* of 1,945 and 2,183 (Fig. 6B and D). These data suggest that the increased sensitivity of the *mig-14*::Kan strain to PB is not due to a defect in 4-aminoarabinose modification of lipid A. Because MALDI-TOF (mass spectrometry) analysis is not quantitative, it is formally possible that there may be an overall reduction in the amount of 4-aminoarabinose containing lipid A in the outer membrane of the *mig-14*::Kan mutant strain.

We next examined the global LPS profiles of the *mig-14*::Kan mutant by SDS-PAGE of crude bacterial preparations from wild-type, *mig-14*, *phoP*, and *galE* strains as described by Tsai and Frasch (48). Once again, no detectable differences in the LPS profiles of the wild-type and *mig-14* strains were observed (data not shown). It is possible that there are other differences in the LPS structure or differences in the expression of outer membrane proteins that could account for the observed sensitivity of the *mig-14*::Kan strain to PB.

***mig-14* is necessary for resistance to mammalian peptide PG-1.** Sensitivity of *Salmonella* to PB has been shown to correlate with sensitivity to mammalian antimicrobial peptides. This finding has significant consequences for the infection process of this pathogen, as it encounters various antimicrobial peptides throughout the process of colonization within its host. It was recently demonstrated that strains of *Proteus mirabilis* selected for PB sensitivity also had increased sensitivity to PG-1 as well as defects in LPS structure (28). We therefore examined the sensitivity of wild-type, *mig-14*::Kan, and *phoP*::Tn*10* strains to a panel of PG-1 concentrations. We observed a marked loss of resistance to PG-1 in the *phoP* mutant strain and saw intermediate levels of sensitivity to PG-1 in the *mig-14* mutant (Fig. 7). While not as dramatic as that of the *phoP* strain, the sensitivity of the *mig-14* mutant was nonetheless significantly lower than that of the isogenic wild-type strain, even at the lowest peptide concentration examined. The results were similar whether bacteria were grown in low Mg^{2+} alone or both in low Mg^{2+} and at low pH (data not shown). These results indicate that *mig-14* is regulated by antimicrobial peptides and low-Mg^{2+} conditions that are likely to be present in some host microenvironments, and they suggest a functional link between this regulation and a role for *mig-14* in resistance of *S. enterica* serovar Typhimurium to antimicrobial peptides.

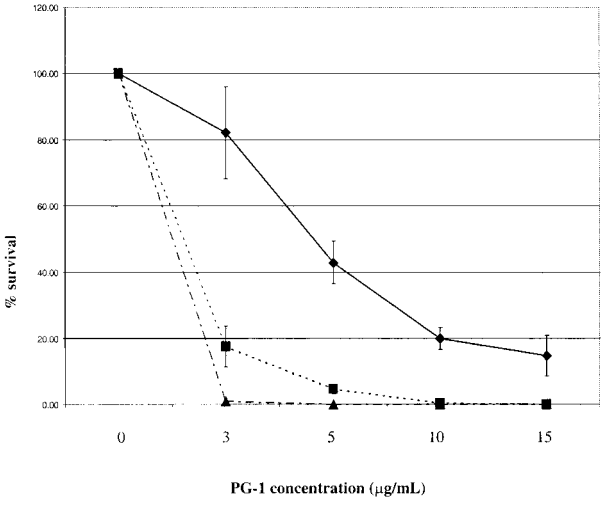

FIG. 7. Sensitivity of *S. enterica* serovar Typhimurium strains to PG-1. Wild-type (◆), *mig-14*::Kan (■), and *phoP*::Tn*10* (▲) strains were grown overnight as described, washed, diluted in N minimal medium, pH 7.4, and 10 μM $MgCl_2$, and grown for 3 h prior to being inoculated into LB containing the indicated concentrations of PG-1. The experiment was performed 3 times in triplicate, and the data shown are from a representative experiment. Data are expressed relative to bacteria treated without PG-1. *mig-14*::Kan is markedly attenuated in its ability to survive PG-1 treatment compared to the wild type, although not as drastically as *phoP*::Tn*10*.

DISCUSSION

mig-14 is a PhoP-activated gene of *S. enterica* serovar Typhimurium required for virulence in orally infected mice (51). It is induced within the macrophage vacuole in a *phoP*-dependent manner but is not required for bacterial survival and replication within macrophages in tissue culture (53). To better understand the role of *mig-14* in *Salmonella* pathogenesis, we have characterized the regulation of *mig-14* in a variety of in vitro conditions. *mig-14* expression, as measured by *mig-14*::GFP reporter activity, was most strongly upregulated in medium containing low Mg^{2+} and cationic antimicrobial peptides such as PB, protamine, and PG-1. It is interesting that protamine as well as PB and PG-1 induced the expression of the reporter constructs, as it has been suggested that the antimicrobial action of protamine is mechanistically different than the membrane permeabilizing activity of PB and PG-1 (1).

The upregulation of *mig-14*::GFP and *pmrD*::GFP by PB showed a *phoP*-independent component as induction occurred even in the absence of an intact PhoP-PhoQ system. This observation suggests that other regulatory systems may activate *mig-14*, and perhaps other antimicrobial peptide resistance genes, directly in response to the presence of antimicrobial peptides. We further demonstrate that in addition to being induced by antimicrobial peptides, *mig-14* is also required for bacterial resistance to killing by PB and PG-1.

The results of our PB resistance assays indicated that growth of bacteria in low Mg^{2+} was the strongest signal to induce PB-resistant adaptations, as we did not see significant resistance in bacteria grown at pH 5.8 in high Mg^{2+} (Fig. 4). This is in contrast to the results of Groisman et al. (11), who observed significant levels of resistance in pH 5.8 medium alone. The reasons for these differences are not fully understood, but one possible explanation is that there are subtle strain-specific differences in response to low-pH conditions. It is possible that these conditions did not produce optimal induction of the pH-responsive PmrA-PmrB system in our strain, resulting in reduced resistance. This is supported by the regulation studies, where we observed significantly higher levels of induction by Mg^{2+} than by low pH, even of the *pmrC*::GFP reporter, suggesting that we may not have achieved maximal induction of the *pmrAB* regulon. In the complementation experiments overexpressing *pmrD*, we did in fact observe significant levels of resistance in pH 5.8 medium alone. Regardless of the reasons for this subtle difference, the *mig-14*::Kan strain showed attenuated resistance to antimicrobial peptides in comparison to the wild type in all of our assays. Together, our data suggest that *mig-14* participates with other PhoP-regulated genes in the adaptive responses of *Salmonella* to environmental conditions that include magnesium depletion and damage by cationic peptides.

PhoP and PhoQ comprise a two-component system that regulates a large number of genes in *S. enterica* serovar Typhimurium in response to a variety of nutrient starvation conditions (24). It has been appreciated for a number of years that the *phoPQ* regulon is necessary for *Salmonella* replication in macrophages and virulence in mouse models of infection (8, 29). Investigators also demonstrated that *phoP*-null mutants were attenuated in resistance to a number of antimicrobial peptides (7, 31). More recently, it has become clear that one of the functions of PhoP-regulated genes is to remodel the bacterial envelope in response to changing environmental conditions. This remodeling occurs both at the level of outer membrane protein expression (12, 30) and at the level of structural modifications of LPS (16). Both *phoP* mutants that are fixed in the constitutively active state and *phoP*-null mutants are unable to remodel the outer membrane in response to environmental signals and are avirulent in animal infections (30), suggesting that this remodeling is crucial for survival within the host environment.

A consensus PhoP binding site has been identified in *E. coli* by using the promoters of the genes *mgtA* and *mgrB* (23). We were not able to identify this consensus sequence in the promoter region of *mig-14*, suggesting that PhoP could regulate *mig-14* indirectly. A number of PhoP-regulated genes, several of which are regulated indirectly through a second two-component system, PmrA-PmrB (14, 42) also lack this consensus sequence (41). PhoP activates PmrA via PmrD, which is thought to posttranscriptionally activate PmrA (26). Our results indicated that *mig-14* is not regulated by PmrA-PmrB (Fig. 3). This suggests either that PhoP directly regulates *mig-14* and binds to the *mig-14* promoter at an uncharacterized binding site or that *mig-14* may be regulated by as-yet-unidentified upstream regulatory genes. The induction of *mig-14* by PB in a *phoP* background indicates that it is likely that there are other regulators of *mig-14*.

The PhoP-PhoQ and PmrA-PmrB regulatory systems participate in activating the transcription of gene products that generate several different modified forms of LPS. Substitution of lipid A with 4-aminoarabinose at one of the two phosphate groups is directly controlled by PmrA-PmrB and is an important mechanism of resistance to PB and several other antimicrobial peptides. Our data indicate that *mig-14* is not required for the addition of 4-aminoarabinose to lipid A. Furthermore, we observed no changes in global LPS profiles of *mig-14* bacteria by SDS-PAGE and silver staining (data not shown). This suggests that another mechanism is responsible for the contribution of the Mig-14 protein to antimicrobial resistance. Mig-14 could be involved in the addition of ethanolamine to the LPS core, which has also been shown to correlate with polymyxin resistance (20, 50). However, as ethanolamine addition takes place in *E. coli* K-12 (33, 57), which does not possess *mig-14* (51), it is at least the case that ethanolamine addition can take place in the absence of *mig-14* function.

While *mig-14* is not necessary for colonization of the Peyer's patches, it plays a role in colonization of the spleen and liver following oral inoculation in a mouse model of typhoid (51). This observation and our results that *mig-14* strains are more sensitive to antimicrobial peptides are consistent with the findings of Gunn et al., who demonstrate that certain polymyxin-sensitive mutants of *Salmonella* were strongly out-competed in replication by the wild-type strain in the liver and spleen following oral infection (15). The reduced virulence of the *mig-14*::Kan mutant is therefore consistent with the idea that the Mig-14 protein is necessary for resistance to killing by antimicrobial peptides within the host environment and contributes to *Salmonella* survival during in vivo infection. The sensitivity of the *mig-14* strain to antimicrobial peptides could

account for the attenuation of its virulence in mice following oral infection.

The precise role of Mig-14 in conferring protection from antimicrobial peptides is as yet unknown. However, our data suggest that *mig-14* is likely to contribute to antimicrobial peptide resistance via a mechanism other than direct modification of LPS. A sequence present in Mig-14 shares homology with the helix-loop-helix motif of the AraC family of transcriptional regulatory proteins, suggesting that it could be a regulatory protein. Interestingly, a large number of AraC family members are involved in antibiotic resistance, stress response, and carbohydrate metabolism (9). We are pursuing further studies to establish the precise function of the Mig-14 protein and determine the mechanism by which it contributes to antimicrobial peptide resistance.

ACKNOWLEDGMENTS

We thank Corrie Detweiler, Erin Gaynor, Denise Monack, Scotty Merrell, and Sunny Shin for critical readings of the manuscript and members of the Falkow lab for helpful discussion. Michael Starnbach was very helpful in providing protocols for SDS-PAGE analysis of LPS. *S. enterica* serovar Typhimurium strain JKS 1379 and the pKR301 plasmid were generously provided by Kenneth Roland (Meganhealth, Inc.). pHSG576 was kindly provided by Jeff Stock (Princeton University). PG-1 was a kind gift of Tom Parr and Tim Falla (Intrabiotics).

This work was supported by grant AI26195 from the National Institutes of Health to S.F., grant RO1 AI30479 to R.K.E. and S.I.M., and Molecular Basis of Host Parasite Interaction training grant 5 T32 AI07328 to I.E.B.

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