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Regulation of Polymyxin Resistance and Adaptation to Low-Mg^{2+} Environments

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The PmrA-PmrB two-component system of *Salmonella typhimurium* controls resistance to the peptide antibiotic polymyxin B and to several antimicrobial proteins from human neutrophils. Amino acid substitutions in the regulatory protein PmrA conferring resistance to polymyxin lower the overall negative charge of the lipopolysaccharide (LPS), which results in decreased bacterial binding to cationic polypeptides and increased bacterial survival within human neutrophils. We have now identified three PmrA-activated loci that are required for polymyxin resistance. These loci were previously shown to be necessary for growth on low-Mg^{2+} solid media, indicating that LPS modifications that mediate polymyxin resistance are responsible for the adaptation to Mg^{2+}-limited environments. Conditions that promote transcription of PmrA-activated genes—growth in mildly acidic pH and micromolar Mg^{2+} concentrations—increased survival in the presence of polymyxin over 16,000-fold in a wild-type organism but not in a mutant lacking *pmrA*. Our experiments suggest that low pH and low Mg^{2+} concentrations may induce expression of PmrA-activated genes within phagocytic cells and promote bacterial resistance to host antimicrobial proteins. We propose that the LPS is a Mg^{2+} reservoir and that the PmrA-controlled LPS modifications neutralize surface negative charges when Mg^{2+} is transported into the cytoplasm during growth in Mg^{2+}-limited environments.

The polymyxins are a group of cyclic, amphipathic, lipopeptide antibiotics that act preferentially on gram-negative bacteria (52). Polymyxins are cationic and show high affinity for negatively charged surfaces such as the lipopolysaccharide (LPS) in the outer membrane, and not surprisingly, microorganisms that are resistant to polymyxin have a modified LPS that binds less polymyxin due to a lower overall negative charge (53, 55). This is primarily due to neutralization of the acidic phosphate on lipid A (3, 24, 25, 36, 56). For example, polymyxin-resistant mutants of *Salmonella typhimurium* and *Escherichia coli* have a higher substitution of the ester-linked phosphate group in the lipid A portion of the LPS by 4-amino-4-deoxy-L-arabinose and show larger amounts of 2-aminoethanol esterifying phosphates in the core oligosaccharide (24, 29, 54). The 4-aminoarabinose substitution is almost stoichiometric in strains of *Proteus mirabilis* (47), *Chromobacterium violaceum* (22), and *Burkholderia cepacia* (5) that exhibit innate resistance to polymyxins.

Two regulatory loci—*pmrA* and *phoP*—control polymyxin resistance in *S. typhimurium*. The *pmrA* locus encodes a two-component system—PmrA-PmrB—and a putative membrane protein—PmrC—with no homologs in the sequence databases (41, 42). *S. typhimurium* strains harboring the *pmrA505* allele (a single amino acid substitution in the regulatory protein PmrA) are resistant to polymyxin and exhibit cross-resistance to several other cationic antimicrobial polypeptides that bind LPS, including the neutrophil-derived cationic antimicrobial proteins 57 and 37 (i.e., CAP57 and CAP37, also known as bactericidal/permeability-increasing protein and azurocidin, respectively) (41, 44, 45). Resistance to host antimicrobial proteins appears to be necessary for survival within human neutrophils because a polymyxin-resistant *pmrA* mutant of a rough (Rb LPS) *S. typhimurium* strain is more resistant to killing by intact human polymorphonuclear neutrophils than the isogenic *pmrA*$^+$ strain (51).

The *phoP* locus encodes a distinct two-component system—PhoP-PhoQ (14, 32)—that governs resistance to several amphipathic antimicrobial peptides, including defensins, magainins, and cecropins (9, 15, 16, 33), and was recently shown to be also required for resistance to polymyxin (19, 30). The PhoQ protein is a sensor for extracellular Mg^{2+} and Ca^{2+} (12, 13) that controls the activity of PhoP, a transcriptional regulator that governs expression of some 25 different loci (2, 17, 18, 26, 37, 38, 49). Transcription of PhoP-activated genes is induced in micromolar concentrations of Mg^{2+} and Ca^{2+} and repressed by growth in millimolar levels of these divalent cations (13, 49). A subset of PhoP-regulated loci, including the *pmrCAB* operon, is regulated via the PmrA protein (19, 23, 50). Expression of PmrA-regulated genes is modulated by the extracellular concentration of Mg^{2+} in a PhoP- and PhoQ-dependent manner and by pH in a mechanism that is independent of the PhoP and PhoQ proteins (50). Thus, activation of PmrA-regulated genes involves a regulatory cascade in which the PmrA-PmrB and PhoP-PhoQ two-component systems interact to integrate pH and Mg^{2+} signals.

In this paper, we demonstrate that polymyxin resistance is environmentally regulated by pH and Mg^{2+} in a PmrA-dependent manner, we identify three PmrA-regulated determinants responsible for polymyxin resistance, and we establish that they correspond to loci previously shown to be required to form colonies on low-Mg^{2+} solid media. We propose that the LPS is a Mg^{2+} reservoir and that the PmrA-controlled LPS modifications neutralize surface negative charges when Mg^{2+} is transported into the cytoplasm during growth in Mg^{2+}-limited environments.

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Description[a]	Reference or source
14028s	Wild type	10
EG7139	*pmrA1*::*cat*	50
EG9492	*pmrA505 zjd*::Tn*10d*-Cam	This work
EG9493	*zjd*::Tn*10d*-Cam	This work
EG9495	*phoP7953*::Tn*10 zjd*::Tn*10d*-Cam	This work
EG9496	*pmrA505 phoP7953*::Tn*10 zjd*::Tn*10d*-Cam	This work
EG9860	*pmrA505 ugd-9228*::MudJ *zjd*::Tn*10d*-Cam	This work
EG9862	*pmrA505 pbgE1*::MudJ *zjd*::Tn*10d*-Cam	This work
EG9868	*pmrA505 pbgP1*::MudJ *zjd*::Tn*10d*-Cam	This work
EG9880	*ugd-9228*::MudJ *zjd*::Tn*10d*-Cam	This work
EG9882	*pbgE1*::MudJ *zjd*::Tn*10d*-Cam	This work
EG9888	*pbgP1*::MudJ *zjd*::Tn*10d*-Cam	This work
MS7953s	*phoP7953*::Tn*10*	9

[a] Gene designations are as summarized by Sanderson et al. (43).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Strains were constructed by phage P22-mediated transductions as described elsewhere (6). Bacteria were grown at 37°C in Luria broth (LB) (31), in N-minimal medium containing 0.1% Casamino Acids and 38 mM glycerol (48) adjusted to pH 7.7 or 5.8 with HCl, or in a modified N-minimal medium containing 0.1% Casamino Acids and 38 mM glycerol in which the 100 mM Tris-HCl was replaced by a mixture of 50 mM bis-Tris and 50 mM Tris adjusted to pH 7.7 or 5.8 with HCl. $MgCl_2$ was added to a final concentration of 10 μM or 10 mM. Kanamycin was used at a final concentration of 50 μg/ml, chloramphenicol was used at 25 μg/ml, tetracycline was used at 10 μg/ml, and polymyxin was used at 2.5, 5.0, and 20 μg/ml.

Polymyxin resistance and β-galactosidase assays. The MIC of polymyxin was determined as described previously (41). One-hour polymyxin susceptibility assays were performed by a modification of a previously described procedure (15). In brief, overnight cultures of bacteria grown in N-minimal medium with 10 mM Mg^{2+} and at pH 7.7 were washed three times in N-minimal medium at pH 7.4 with no $MgCl_2$ and diluted 1:100 into N-minimal medium with either 10 μM $MgCl_2$ (pH 5.8), 10 μM $MgCl_2$ (pH 7.7), 10 mM $MgCl_2$ (pH 5.8), or 10 mM $MgCl_2$ (pH 7.7). After 3 to 4 h of incubation at 37°C, logarithmically growing bacteria were washed twice in N-minimal medium with 10 mM $MgCl_2$ (pH 7.7) and diluted to 5×10^4 to 1×10^5 CFU/ml in LB. Polymyxin was added to a final concentration of 2.5, 5.0, or 20 μg/ml, and after 1 h of incubation at 37°C with shaking, bacteria were diluted in phosphate-buffered saline (PBS) and plated onto LB agar plates. The number of CFU was determined after overnight incubation. Data are presented as percent survival relative to the original inoculum. β-Galactosidase activity was determined as described elsewhere (31).

RESULTS

Identification of PmrA-regulated loci required for polymyxin resistance. We have recently established that the *pbgE*, *pbgP*, and *ugd* loci of *S. typhimurium* are transcriptionally controlled by the PmrA-PmrB regulatory system (50). We hypothesized that these PmrA-regulated loci may be required for polymyxin resistance because strains in which either one of these genes was inactivated could not grow on low-Mg^{2+} solid media (50). Since polymyxin must displace Mg^{2+} and Ca^{2+} ions from the LPS in order to reach its target, we reason that LPS modifications allowing growth in Mg^{2+}-limited conditions may also confer resistance to polymyxin and could be mediated by the same set of genes.

To ascertain the role of PmrA-regulated genes in polymyxin resistance, we determined the MIC of polymyxin B for strains harboring both the *pmrA505* polymyxin resistance allele (a His81 Arg substitution in the PmrA protein [41]) and a MudJ transposon insertion in either *pbgE*, *pbgP*, or *ugd*. Inactivation of these PmrA-activated genes rendered the *pmrA505* resistant mutant as susceptible as wild-type *pmrA*$^+$ *S. typhimurium* (Table 2), indicating that the *pbgE*, *pbgP*, and *ugd* loci encode products necessary for polymyxin resistance.

TABLE 2. MIC of polymyxin B for strains harboring mutations in the *pmrA* locus and/or PmrA-regulated genes

Strain	Relevant genotype	MIC (μg/ml)
EG9492	*pmrA505*	32
EG9493	*pmrA*$^+$	4
EG7139	*pmrA1*::*cat*	4
EG9860	*pmrA505 ugd-9228*::MudJ	4
EG9862	*pmrA505 pbgE1*::MudJ	4
EG9868	*pmrA505 pbgP1*::MudJ	4

The *pmrA505* allele promotes high transcriptional activity of PmrA-regulated genes. It has been hypothesized that the PmrA505 protein confers resistance to polymyxin by promoting transcription of PmrA-regulated determinants to high levels in the absence of the environmental signal controlling the PmrA-PmrB system (41). To test this hypothesis, we determined the transcriptional activity of the PmrA-activated genes *pbgE*, *pbgP*, and *ugd* in isogenic *pmrA505* and *pmrA*$^+$ strains by measuring the β-galactosidase activity of strains harboring *lac* fusions to these loci. When the strains were grown in LB, transcription of PmrA-activated genes was four to eight times higher in the *pmrA505* mutant than in the *pmrA*$^+$ strain (Fig. 1). In contrast, the transcriptional activity of the PmrA-independent *mgtA* gene was not affected by the *pmrA* allele. Taken together with the results from the MIC determinations (Table 2), these experiments support a model in which the PmrA505 protein confers polymyxin resistance by increasing transcription of PmrA-activated genes.

Under normal growth conditions the MICs of polymyxin for *pmrA*$^+$ and *pmrA* mutant strains are the same. During growth in LB, transcription of PmrA-activated genes is 15 to 140 times higher in a *pmrA*$^+$ strain than in a mutant harboring a chloramphenicol resistance cassette in the *pmrA* gene—*pmrA*::*cat* (50). Thus, we anticipated that the MIC of polymyxin for wild-type *S. typhimurium* would be higher than the MIC for the *pmrA*::*cat* mutant. Unexpectedly, MICs for the *pmrA*$^+$ and *pmrA*::*cat* strains were identical (4 μg/ml versus 32 μg/ml for the *pmrA505* mutant), which suggests that polymyxin resistance demands expression of PmrA-activated genes to levels higher than those achieved by a wild-type organism grown in LB.

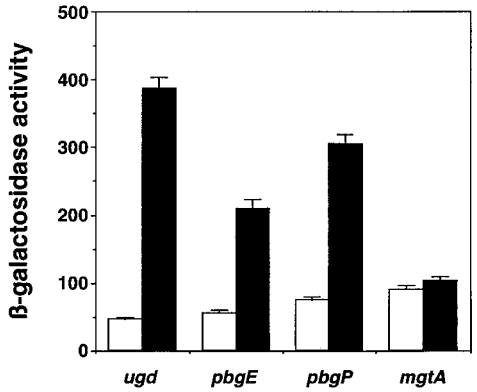

FIG. 1. The PmrA505 allele enhances expression of PmrA-activated genes. β-Galactosidase activities (Miller units) expressed by strains grown in LB were determined for mutants harboring a *lac* transcriptional fusion to the PmrA-dependent PhoP-activated genes *ugd*, *pbgE*, and *pbgP* and to the PmrA-independent PhoP-activated *mgtA* gene. Transcriptional activity was investigated in two different backgrounds: wild type (open bars) and strains harboring the *pmrA505* allele (solid bars). The data correspond to mean values from two independent experiments done in duplicate.

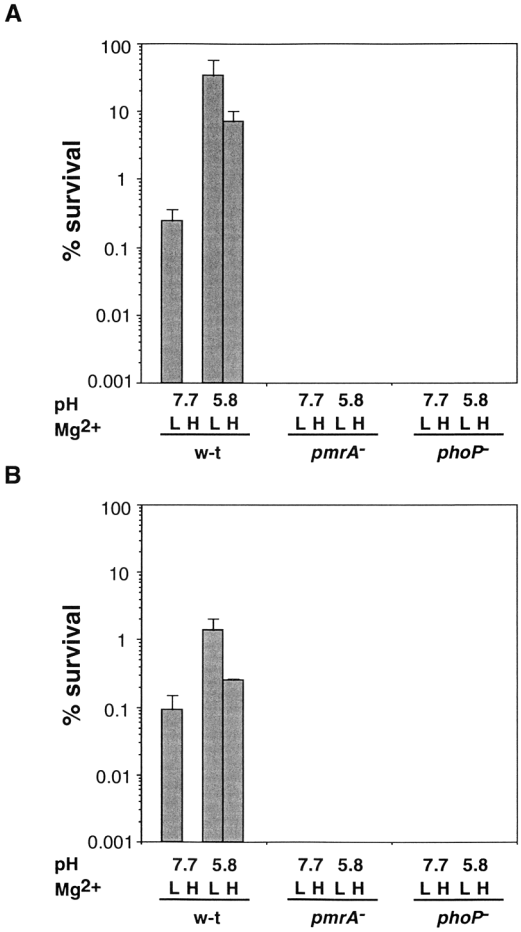

FIG. 2. Growth in low Mg^{2+} and low pH renders wild-type *S. typhimurium* resistant to polymyxin B. Wild-type (14028s), *pmrA*::*cat* (EG7139), and *phoP* mutant (MS7953s) strains were grown to logarithmic phase in N-minimal medium with 10 μM (L) or 10 mM (H) $MgCl_2$ and at either pH 7.7 or 5.8. Polymyxin B was added to washed bacteria at a final concentration of 2.5 (A) or 5.0 (B) μg/ml, and the bacteria were incubated for 1 h at 37°C. Samples were diluted in PBS and plated on LB agar plates to assess bacterial viability. Survival values are relative to the original inoculum. No bacteria were recovered following treatment of the *pmrA* and *phoP* mutants. Note the logarithmic scale on the *y* axis. Data correspond to mean values from three independent experiments done in duplicate.

(Roland and coworkers have reported similar findings using a strain in which both the *pmrA* and *pmrC* genes were deleted [40].)

pH and Mg^{2+} modulate polymyxin resistance in wild-type *Salmonella*. We have recently established that expression of PmrA-activated genes is modulated by pH and the concentration of extracellular Mg^{2+}: PmrA-activated genes are transcriptionally induced by mild acidification and growth in micromolar concentrations of Mg^{2+}, whereas growth at pH 7.7 and millimolar concentrations of Mg^{2+} represses expression of PmrA-activated genes (50). We hypothesized that environmental conditions modulating transcription of PmrA-activated genes would control the phenotypic display of polymyxin resistance, and indeed, the surviving fraction of wild-type *Salmonella* exposed to polymyxin was >16,000-fold higher when grown at pH 5.8 and 10 μM Mg^{2+} than when grown at pH 7.7 and 10 mM Mg^{2+} (Fig. 2). Growth at mildly acid pH and high Mg^{2+} (i.e., pH 5.8 and 10 mM Mg^{2+}) promoted polymyxin resistance but not to the levels achieved by incubation at low Mg^{2+} and mildly acid pH (Fig. 2). On the other hand, wild-type *Salmonella* displayed intermediate levels of polymyxin resistance when grown at pH 7.7 and 10 μM Mg^{2+} (Fig. 2). The regulatory effects of pH and Mg^{2+} were mediated by the PmrA protein because the *pmrA*::*cat* mutant was sensitive to polymyxin regardless of the pH and Mg^{2+} concentration used to grow the microorganism (Fig. 2). Cumulatively, these results indicate that resistance to polymyxin can be phenotypically modulated by pH and the concentration of extracellular Mg^{2+} in a mechanism that requires a functional PmrA protein.

pH and Mg^{2+} modulate polymyxin resistance in the *pmrA505* polymyxin-resistant mutant. We examined whether the polymyxin-resistant *pmrA505* allele was truly constitutive by investigating whether polymyxin resistance could be modulated in this mutant by environmental cues regulating polymyxin resistance in the $pmrA^+$ strain. The surviving fraction of the *pmrA505* mutant exposed to polymyxin was >2,800 times lower when grown at pH 7.7 and 10 mM Mg^{2+} than when grown at pH 5.8 and 10 μM Mg^{2+} (Fig. 3). Still, the *pmrA505* mutant displayed higher levels of polymyxin resistance than the isogenic $pmrA^+$ strain under all examined conditions. These results demonstrate that the PmrA505 protein is not blind to environmental cues and that it responds to the same signals controlling polymyxin resistance in wild-type *Salmonella*.

Role of the PhoP-PhoQ system in polymyxin resistance. The PhoP-PhoQ regulatory system is absolutely required for the transcriptional induction of PmrA-activated genes that results from growth in micromolar Mg^{2+}. However, PhoP-PhoQ is not essential for the acid-pH-promoted expression of PmrA-activated genes, since 20- to 30-fold transcriptional induction is still observed in strains mutated in either the *phoP* or *phoQ* gene (50). While this suggested that the PhoP-PhoQ system might not be essential for polymyxin resistance, a *phoP* mutant was as susceptible to polymyxin as the *pmrA* null mutant regardless of the pH and Mg^{2+} concentration used to grow the microorganism (Fig. 2).

To further examine the role of the PhoP-PhoQ system in polymyxin resistance, we investigated the polymyxin susceptibility of a *phoP pmrA505* double mutant. When grown under conditions that promote expression of PmrA-activated genes—pH 5.8 and 10 μM Mg^{2+}—the surviving fraction of *phoP pmrA505* double-mutant cells exposed to polymyxin was >10,000-fold lower than that of the isogenic $phoP^+$ *pmrA505* strain (data not shown). As predicted, polymyxin resistance was not modulated by changes in the extracellular concentration of Mg^{2+} in the *phoP pmrA505* double mutant, but it could still be induced over fivefold by growth in mildly acid pH (data not shown). These results indicate that polymyxin resistance requires a functional PhoP-PhoQ system and support the notion that the PmrA505 protein responds to environmental cues.

The *pmrC* gene is not required for polymyxin resistance. Because transcription of *pmrC* (the first gene in the *pmrCAB* operon) is regulated in the same manner as that of other PmrA-activated genes (50), we investigated the possibility of *pmrC* being required for polymyxin resistance. When grown under conditions promoting polymyxin resistance (i.e., pH 5.8 and 10 μM Mg^{2+}), a strain harboring a MudJ transposon insertion in the *pmrC* gene was as resistant to polymyxin as the wild-type parent (data not shown). In contrast, the polymyxin susceptibility of the *pbgE*, *pbgP*, and *ugd* mutants was similar to that of the *pmrA*::*cat* mutant (data not shown). Cumulatively, these results indicate that the PmrC protein is not essential for polymyxin resistance and are in agreement with both the MIC data (Table 2) and our previous findings that inactivation of the *pmrC* gene does not eliminate PmrA-dependent phenotypes such as growth on low-Mg^{2+} solid media (50). (As pre-

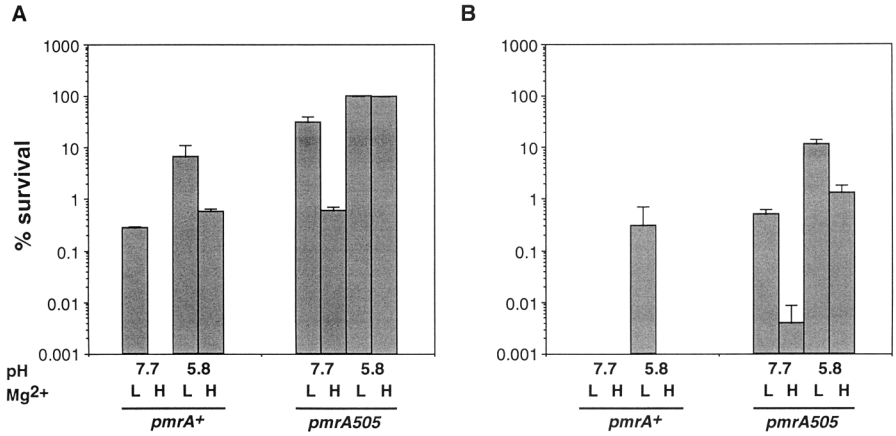

FIG. 3. Growth in high Mg^{2+} and high pH renders the polymyxin-resistant *pmrA505* mutant of *S. typhimurium* sensitive to polymyxin B. Wild-type (EG9493) and *pmrA505* (EG9492) strains were grown to logarithmic phase in N-minimal medium with 10 μM (L) or 10 mM (H) $MgCl_2$ and at either pH 7.7 or 5.8. Polymyxin B was added to washed bacteria at a final concentration of 5.0 (A) or 20 (B) μg/ml, and bacteria were incubated for 1 h at 37°C. Samples were diluted in PBS and plated on LB agar plates to assess bacterial viability. Survival values are relative to the original inoculum. No bacteria were recovered following treatment of the wild-type strain with 20 μg of polymyxin B per ml. Note the logarithmic scale on the *y* axis. Data correspond to mean values from three independent experiments done in duplicate.

viously suggested, a promoter within the *pmrC* gene or the MudJ transposon may be responsible for transcription of the *pmrA* gene in the *pmrC*::MudJ mutant [19, 50].)

DISCUSSION

We have established that resistance to the cationic peptide antibiotic polymyxin B is environmentally regulated by pH and the concentration of Mg^{2+}: growth in mildly acid pH and micromolar Mg^{2+}—conditions that promote expression of PmrA-activated genes—rendered wild-type *Salmonella* resistant to polymyxin. The regulatory effects of pH and Mg^{2+} required a functional PmrA-PmrB system, because these signals failed to induce polymyxin resistance in a *pmrA* null mutant. Moreover, we identified three PmrA-activated loci that are necessary for polymyxin resistance and determined that they correspond to loci previously shown to be required for growth on low-Mg^{2+} solid media.

Modulation of polymyxin resistance. Environmental conditions, genetic factors, and microbial growth phase modulate the phenotypic display of polymyxin resistance in *S. typhimurium*. Growth in mildly acid pH and micromolar Mg^{2+} increased the surviving fraction of wild-type *Salmonella* exposed to polymyxin >16,000 times more than organisms grown in mildly alkaline pH and millimolar concentrations of Mg^{2+} (Fig. 2). Survival in the presence of polymyxin could be induced >120-fold by growing wild-type *Salmonella* in micromolar Mg^{2+}, whereas mild acidification increased survival in the presence of polymyxin >3,500 fold (Fig. 2). These results are in agreement with the increased bacterial tolerance towards polymyxin resulting from either adaptation of wild-type *S. typhimurium* to pH 5.8 for one or two cell doublings (27) or growth in the presence of very low concentrations of divalent cations (cited in reference 24).

Our experiments with *pmrA* and *phoP* mutants suggest a model in which polymyxin resistance requires a threshold level of transcription of PmrA-activated genes below which a wild-type organism cannot resist polymyxin-mediated killing. First, $pmrA^+$ and *pmrA* strains were equally susceptible to polymyxin when grown in LB (Table 2) despite the 15- to 140-fold difference in transcription of PmrA-activated genes (50). Second, a *phoP* mutant was as susceptible to polymyxin as the *pmrA* null mutant (Fig. 2) even though transcription of PmrA-activated genes can be stimulated >20-fold by growth in mildly acid pH in a *phoP* mutant (50). Finally, wild-type *Salmonella* displayed polymyxin resistance only when grown under conditions that promoted high levels of transcription of PmrA-activated genes (50).

The PhoP-PhoQ system has been shown to control lipid A modifications independently of the PmrA protein (20). While the role that these modifications play in resistance to polymyxin remains unknown, it raises the possibility of the PhoP-PhoQ system contributing to polymyxin resistance not only by promoting high levels of expression of PmrA-activated genes but also by governing transcription of PmrA-independent resistance determinants.

In addition to the regulatory signals promoting polymyxin resistance in logarithmically growing cells, bacteria that reach stationary phase in LB or that stop growing because of starvation for carbon, nitrogen, or phosphorus exhibit heightened resistance to polymyxin (30). Starvation-promoted resistance does not require the stationary-phase RpoS sigma factor but is dependent on the *phoP* gene shortly after the onset of starvation (30). It is presently unknown whether PhoP-mediated resistance is dependent on a functional PmrA protein, because starvation-promoted polymyxin resistance has not been examined in mutants lacking the *pmrA* gene.

The multiple regulatory signals controlling polymyxin resistance indicate that the LPS structure is not static but rather is modifiable in response to environmental cues. That these LPS modifications may take place within host cells and enhance resistance to antimicrobial proteins and peptides is suggested by the following. First, the phagosomes harboring *Salmonella* are known to be acidic (1) and low in Mg^{2+} (11). Second, the PmrA-regulated gene *ugd*/*pagA* is activated within acidified phagosomes (1). And third, growth within cultured mammalian cells promotes changes in the peptidoglycan structure of *S. typhimurium* (39).

The PmrA505 protein can respond to external cues. It has been hypothesized that cells harboring the *pmrA505* allele (a His81 Arg substitution in the regulatory protein PmrA) are resistant to polymyxin because they constitutively overexpress PmrA-regulated determinants (41), and indeed, transcription of PmrA-activated genes is four- to eightfold higher in the *pmrA505* mutant than in the $pmrA^+$ strain (Fig. 1). However, survival of the *pmrA505* mutant in the presence of polymyxin

was >2,800-fold lower when the strain was grown in alkaline pH and millimolar Mg^{2+} concentrations (Fig. 3), indicating that this mutant responds to the same environmental cues that modulate polymyxin resistance in wild-type *Salmonella* (Fig. 2). These results suggest that the PmrB protein—the putative cognate sensor-kinase for the PmrA protein—may exhibit phosphatase activity towards the PmrA protein. Alternatively or in addition, the phosphorylated state of the PmrA protein may be environmentally controlled by phosphoprotein phosphatases analogous to those modulating the activity of the CpxR-CpxA and RcsB-RscC two-component systems in *E. coli* K-12 (34).

Role of PmrA-regulated determinants in resistance to antimicrobial peptides and proteins. Polymyxin-resistant *pmrA* mutants bind less polymyxin than wild-type organisms (53, 55) because their LPS has a higher substitution of the ester-linked phosphate group in lipid A by 4-amino-4-deoxy-L-arabinose and contains larger amounts of 2-aminoethanol esterifying phosphates in the core oligosaccharide (24, 29, 54). Since different enzymes are anticipated to participate in the biosynthesis and/or incorporation of these substituents into the LPS, Roland and coworkers suggested that at least two PmrA-regulated determinants would be required for polymyxin resistance (41). We have now identified three PmrA-activated loci that are essential for polymyxin resistance: *pbgE*, *pbgP*, and *ugd*. These loci are in addition to *pmrD*, a gene that confers polymyxin resistance when present in a multicopy plasmid and that appears to be regulated by the PmrA-PmrB system because *pmrD*-mediated polymyxin resistance is not observed in a mutant lacking the *pmrA* gene (40).

Consistent with a potential role in LPS modification, the *ugd* gene maps to one of the LPS biosynthetic gene clusters in enteric bacteria and encodes a protein that is homologous to the UDP-glucose dehydrogenase of *Streptococcus pneumoniae*, where it is essential for capsule production (7, 8). On the other hand, the *pmrD* gene encodes an 85-amino-acid polypeptide that exhibits similarity to a protease of Rous sarcoma virus (40). The *pmrD* gene does not confer resistance to CAP57 (40), which, like polymyxin, targets the lipid A portion of the LPS, suggesting that the PmrD protein may mediate polymyxin resistance by a mechanism that does not involve modification of the lipid A.

Polymyxin resistance and adaptation to low-Mg^{2+} environments. The *pbgE*, *pbgP*, and *ugd* loci are essential for both resistance to polymyxin (Table 2) and growth on low-Mg^{2+} solid media (49), suggesting that the PmrA-regulated LPS modifications associated with polymyxin resistance are part of a cellular response governing the adaptation to Mg^{2+}-limited environments. When exposed to a low-Mg^{2+} milieu, *Salmonella* upregulates the expression of several genes, including those encoding the high-affinity Mg^{2+} transporters MgtA and MgtB (13, 49). This promotes uptake of Mg^{2+} into the cytoplasm to maintain the Mg^{2+} concentration that is necessary for the various ATP-dependent reactions. But where is the Mg^{2+} coming from?

We suggest (i) that the LPS serves as a Mg^{2+} reservoir and is the main source of Mg^{2+} when bacteria face Mg^{2+}-limited environments and (ii) that the PmrA-controlled modifications of the LPS neutralize negative charges normally neutralized by Mg^{2+} and Ca^{2+} ions. *E. coli* K-12 cells grown in nutrient broth contain approximately 1 mol of Ca^{2+} and 2 to 3 mol of Mg^{2+} per mol of LPS (4). The Ca^{2+} content is similar in cells grown in M9 minimal medium, whereas the Mg^{2+} content increases some 30% to 4 mol of Mg^{2+} per mol of LPS (4). In *S. typhimurium*, the number of LPS molecules is 35×10^5 per cell (35) and there are 2×10^9 cells per mg of dry weight. If one uses the value of 4 mol of Mg^{2+} per LPS molecule determined for M9-grown *E. coli* cells (4), it can be estimated that the LPS contains 1.12 mg of Mg^{2+} per g of dry weight. Since the total Mg^{2+} content of *E. coli* is around 3.2 mg per g of dry weight (28), these calculations imply that more than one-third of the total Mg^{2+} is present in the LPS.

Our model may help explain the two phenotypes associated with mutations in the *pmrA* locus: resistance to cationic peptides and the inability to form colonies on low-Mg^{2+} solid media. Cationic antimicrobial peptides can easily displace Mg^{2+} and Ca^{2+} from the LPS because their affinities for divalent cation-binding sites in the LPS are several orders of magnitude higher than those of Mg^{2+} and Ca^{2+} ions (21). Then activation of the PmrA system may promote resistance to polymyxin by neutralizing the negative charges in the LPS through covalent modification, thereby converting the LPS into a form that is an effective barrier against cationic peptides. On the other hand, bacterial cells have a tendency to form intimate side-by-side alignments (46), and these interactions may be hindered in strains harboring mutations in either the *pmrA*, *pbgE*, *pbgP*, or *ugd* gene, which cannot form colonies on low-Mg^{2+} solid media but grow like the wild-type parent in Mg^{2+} liquid media (50). This growth defect may be due to a failure to modify the bacterial surface charge and compensate for the absence of Mg^{2+} ions, resulting in electrostatic repulsion between the negatively charged LPS molecules.

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