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Two-Component Regulatory Systems Can Interact To Process Multiple Environmental Signals

FERNANDO C. SONCINI AND EDUARDO A. GROISMAN*

Department of Molecular Microbiology,
Washington University School of Medicine, St. Louis, Missouri 63110

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The PhoP/PhoQ two-component system of *Salmonella typhimurium* governs transcription of some 25 loci in response to the extracellular concentration of Mg^{2+}. We have now identified one of these loci as *pmrCAB*, which codes for a two-component system that mediates resistance to the antibiotic polymyxin B. Transcription of seven of 25 PhoP-activated loci was dependent on a functional PmrA protein, the response regulator of the PmrA/PmrB system. Expression of the PmrA-dependent loci was induced by either Mg^{2+} limitation or mild acidification, whereas transcription of a PmrA-independent gene was activated by Mg^{2+} limitation but not acid pH. Induction of PmrA-activated genes by Mg^{2+} limitation required the PhoP and PhoQ proteins. In contrast, the acid-mediated activation of PmrA-regulated genes occurred in strains that were missing either one of these proteins. Transcriptional regulation by a cascade of two-component systems allows pathogenic bacteria to express their virulence determinants in response to a broader spectrum of environmental cues.

Bacterial pathogens must modulate their gene expression repertoire in order to survive the hostile environments faced during the course of infection (10, 20). This adaptive response is often mediated by two-component regulatory systems, generally consisting of a sensor-kinase and a response regulator, that use reversible protein phosphorylation to regulate the adjustment to the new environments (1, 25, 26, 39). Sensors are generally integral membrane proteins that respond to specific environmental signals by phosphorylating their cognate regulators, which are often transcription factors whose affinity for DNA is modulated by phosphorylation. It has been estimated that enteric bacteria harbor some 40 different two-component systems that mediate the response to a variety of chemical and physical signals (38). However, the mechanism by which the various signals are integrated into a coordinated cellular response has remained largely unknown. In this article, we establish that a pair of two-component systems, PhoP/PhoQ and PmrA/PmrB, can interact to process multiple environmental cues.

PhoP/PhoQ is a two-component regulatory system that controls several pathogenic properties in the gram-negative bacterium *Salmonella typhimurium* (14, 16). We have recently identified extracellular Mg^{2+} as the signal that governs the PhoP/PhoQ system: transcription of PhoP-activated genes is induced in micromolar concentrations of Mg^{2+}, whereas growth in millimolar Mg^{2+} represses expression of PhoP-activated genes and attenuates the virulence properties of wild-type *Salmonella* species (15). The PhoQ protein is a Mg^{2+} sensor that changes conformation in the presence of periplasmic Mg^{2+} (15). PhoP is a regulatory protein that is necessary to transcribe some 25 different loci, many of which are essential for growth in low-Mg^{2+} conditions (36).

Apart from Mg^{2+}, additional signals can modulate expression of PhoP-regulated genes: for example, transcription of *psiD* is induced 50-fold when the pH of the growth medium is lowered from 7.5 to 6.1 (15). However, this induction is independent of PhoP and PhoQ and is in contrast to the activation that results from Mg^{2+} limitation, which requires both proteins. These experiments suggest that additional factors must mediate the response to mild acidification in PhoP-regulated loci such as *psiD*.

In this article, we demonstrate that the PhoP/PhoQ system controls expression of the PmrA/PmrB two-component system, we identify the targets of transcriptional control of the PmrA protein, and we establish that PmrA-regulated genes are controlled by an unusual regulatory cascade of two-component systems that integrates both pH and extracellular Mg^{2+} signals.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria broth (LB) (21) or in modified N-minimal medium containing 0.1% casamino acids and 38 mM glycerol (34) in which the 100 mM Tris-HCl was replaced by a mixture of 50 mM bis-Tris and 50 mM Tris adjusted to pH 8.5 to 5.8 with HCl. $MgCl_2$ was added to a final concentration of 10 μM or 10 mM. Ampicillin and kanamycin were used at a final concentration of 50 μg/ml, chloramphenicol was used at 25 μg/ml, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 70 μg/ml.

Genetic and molecular biology techniques. Phage P22-mediated transductions were performed as described previously (6). Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus as recommended by the manufacturer. Recombinant DNA techniques were performed according to standard protocols (30). Genomic libraries were constructed by ligating *Sal*I-digested chromosomal DNA to *Sal*I-cut plasmid pUC19. Clones harboring the junction fragments were selected as kanamycin (MudJ)- and ampicillin (pUC19)-resistant colonies. To determine the chromosomal DNA sequence adjacent to each MudJ insertion we used a primer complementary to the left end of MudJ and the plasmid clones harboring the junctions as templates as described previously (36).

Construction of a *pmrA* mutant. First, we cloned a PCR-generated fragment harboring the *pmrA* and *pmrB* coding regions between the *Bam*HI and *Hin*dIII sites of plasmid pUHE21-2*lacI*^{q} to form pEG9207. Then, the *Bsu*36I-*Spl*I fragment internal to pEG9207 was substituted by an equivalent fragment from pEG9070-1, a plasmid harboring a wild-type copy of the *pmrAB* genes, to form pEG9102. Analysis of pEG9102 revealed that the PCR-generated DNA had a wild-type sequence.

To create an insertional mutation in the *pmrA* gene, we created a *Bgl*II site at position 292 in the *pmrA* open reading frame (position 2145 in reference 29). First, primers 351 (5′-AAGGATCCAGGAGACTAAGCG-3′) and 406 (5′-AACTGCAGATCTGCGCCGACATCC-3′) were used to amplify the 5′ region of the *pmrA* gene, which was cloned between the *Bam*HI and *Pst*I sites of pUC19 to form plasmid pEG7243. A second PCR product encompassing the 3′ region of

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8230, St. Louis, MO 63110. Phone: (314) 362-3692. Fax: (314) 362-1232. Electronic mail address: groisman@borcim.wustl.edu.

TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Description[a]	Reference or source
S. typhimurium		
14028s	Wild type	12
AA3007	*polA2 ara-9*	42
EG9395	*galE*::Tn*10* MuhP1-1 *proP*::Tn*10*dCam/pEG5005	This work
MS7953s	*phoP7953*::Tn*10*	11
MS5996s	*phoQ5996*::Tn*10*	11
EG9521	*mgtA9226*::MudJ	15
EG9523	*mgtA9226*::MudJ *phoP7953*::Tn*10*	15
EG9522	*mgtA9226*::MudJ *phoQ5996*::Tn*10*	15
EG9671	*mgtA9226*::MudJ *pmrA1*::*cat*	This work
EG9524	*ugd9228*::MudJ	15
EG9674	*ugd9228*::MudJ *pmrA1*::*cat*	This work
EG9230	*pbgE1*::MudJ	36
EG9676	*pbgE1*::MudJ *pmrA1*::*cat*	This work
EG9241	*pbgP1*::MudJ	36
EG9168	*pbgP1*::MudJ *phoP7953*::Tn*10*	36
EG9681	*pbgP1*::MudJ *pmrA1*::*cat*	This work
EG9279	*pmrC1*::MudJ	This work
EG9280	*pmrC1*::MudJ *phoP7953*::Tn*10*	This work
EG9687	*pmrC1*::MudJ *pmrA1*::*cat*	This work
E. coli JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/ e14$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17*	43
Plasmids		
pUHE21-2*lacI*^{q}	rep$_{pMB1}$ Ap^r *lacI*^{q}	35
pEG7243	rep$_{pMB1}$ *lacZa* Ap^r *lacI*^{q} *pmrA′*	This work
pEG7245	rep$_{pMB1}$ *lacZa* Ap^r *lacI*^{q} *pmrA* (*Bgl*II)	This work
pEG7246	rep$_{pMB1}$ *lacZa* Ap^r *lacI*^{q} *pmrA*::*cat*	This work
pEG9070-1	rep$_{pMB1}$ Km^r Mud5005::*pmrAB proP*::Tn*10*dCam	This work
pEG9102	rep$_{pMB1}$ Ap^r *lacI*^{q} *pmrAB*	This work
pKRP10	rep$_{pMB1}$ Ap^r Cm^r	27
pUC19	rep$_{pMB1}$ *lacZa* Ap^r	43

[a] Gene designations are as summarized by Sanderson et al. (31).

the *pmrA* gene was generated using primers 352 (5′-GGCAAGCTTAGCTTTC CTCAG-3′) and 407 (5′-GAAGATCTGGTTAAACCCTTC-3′) and cloned between the *Bgl*II and *Hin*dIII sites of pEG7243. The resulting plasmid, pEG7245, was used to disrupt the *pmrA* gene by introducing the *cat*-containing, 0.9-kb *Bam*HI fragment from pKRP10 into the single *Bgl*II site to form pEG7246. The *pmrA*::*cat* mutation in pEG7246 was transferred to the chromosome as described previously (17). The structure of the *pmrA* gene in the mutant strains was verified by Southern hybridization using both *pmrA*- and *cat*-specific probes (Fig. 1). The *pmrA* probe was a 0.7-kb fragment generated by the PCR using primers 351 (5′-AAGGATCCAGGAGACTAACGC-3′) and 352 (see above) corresponding to the coding region of the *pmrA* gene (29). The *cat* probe corresponds to the 0.9-kb *Bam*HI fragment from plasmid pKRP10 (27).

Determinations of β-galactosidase activity. β-Galactosidase activity was determined as described by Miller (21) on cells grown exponentially in LB or N-minimal medium. To determine expression in different Mg^{2+} concentrations and under different pH conditions, bacteria from overnight cultures grown in 10 mM $MgCl_2$–N-medium–bis-Tris–Tris-HCl (pH 7.7) were washed twice with N-minimal medium containing no Mg^{2+} and used to inoculated the appropriate fresh media. The bacterial cultures were incubated for 5 h at 37°C before the assay. For plasmid complementation assays, β-galactosidase activity was determined on cells grown overnight.

RESULTS

PhoP controls expression of the PmrA/PmrB two-component system. While searching for PhoP-regulated *lac* gene fusions (36), we recovered seven in *pmrC*, the first gene of the *pmrCAB* operon. The *pmrA* and *pmrB* genes encode proteins homologous to the regulators and sensors, respectively, of the two-component family of signal transduction systems (29). (The *pmrC* gene encodes a putative membrane protein that has no homologs in the sequence databases.) We investigated the regulatory effect of the PhoP/PhoQ system by measuring the β-galactosidase activity originating from a *pmrC-lac* transcriptional fusion in wild-type, *phoP*, and *phoQ* strains: *pmrC* transcription was 9 and 90 times higher in the wild-type strain than in either mutant for bacteria grown in LB broth or N-minimal medium (10 μM Mg^{2+}), respectively. These results indicate that the PhoP and PhoQ proteins are necessary for expression of the PmrA/PmrB system and suggest that genes originally identified as controlled by PhoP may be regulated via the PmrA protein.

Construction of a *pmrA* mutant. To examine whether the PmrA protein was necessary for transcription of PhoP-regulated genes, we constructed strains having a chloramphenicol resistance cassette in the chromosomal copy of the *pmrA* gene. The 0.9-kb *Bam*HI fragment of plasmid pKRP10 harboring the *cat* gene was cloned into the single *Bgl*II site of plasmid pEG7245, which carries a mutant derivative of the *pmrA* gene. Then, the mutant copy of the *pmrA* gene in plasmid pEG7246 was used to replace the wild-type copy of the *pmrA* gene in the chromosome as described previously (17). The structure of the *pmrA* chromosomal region in the *pmrA* mutant was verified by

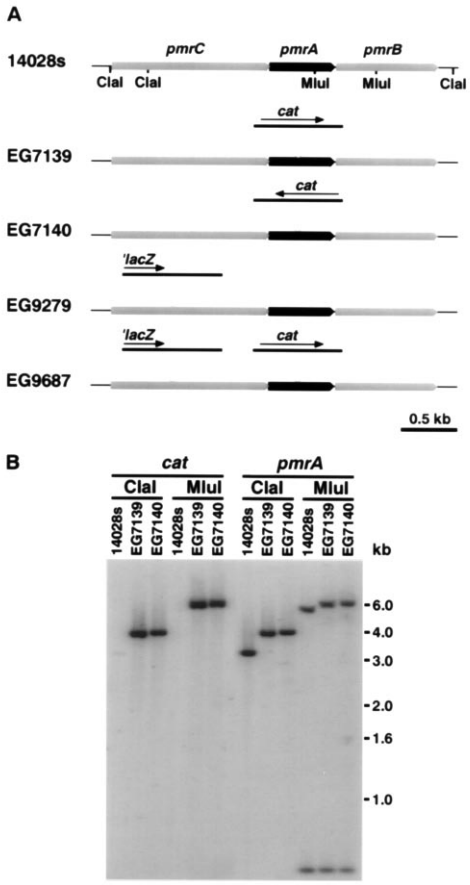

FIG. 1. Genetic and physical maps of the *pmrA* locus in *S. typhimurium* strains harboring mutations in the *pmrA* and *pmrC* genes. (A) Genetic organization of the *pmrCAB* operon in the wild-type strain 14028s, and location of MudJ and *cat* insertions in mutants EG7139, EG7140, EG9279 and EG9687. The MudJ transposon harboring the *'lacZ* gene is not drawn to scale. (B) Southern hybridization analysis of the *pmrA* gene in wild-type and *pmrA*::*cat* strains. Analysis was performed as described in Materials and Methods by using *cat*- and *pmrA*-specific probes.

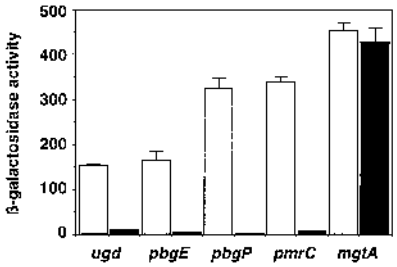

FIG. 2. PmrA governs expression of a subset of PhoP-regulated genes. β-Galactosidase activities (Miller units) expressed by strains grown in LB broth were determined for mutants harboring a *lac* transcriptional fusion to the PhoP-activated genes *ugd*, *pbgE*, *pbgP*, *pmrC*, and *mgtA*. Transcriptional activity was investigated in two different backgrounds: wild-type (shaded boxes) and strains lacking *pmrA* (solid box). The data correspond to mean values of at least three independent experiments done in duplicate.

Southern hybridization using both *pmrA*- and *cat*-specific probes (Fig. 1).

PmrA is essential for expression of a subset of PhoP-regulated genes. We investigated whether transcription of 25 PhoP-regulated *lac* gene fusions was PmrA dependent by streaking isogenic derivatives with and without *pmrA* onto LB X-Gal Agar plates. Mutation of the *pmrA* gene abolished transcription of seven PhoP-regulated *lac* gene fusions, *pbgE*, *pbgM*, *pbgP*, *pcgD*, *pcgP*, *pmrC*, and *ugd*, but had no effect on the expression of the remaining 18. Transcription of PmrA-dependent genes was 15 to 140 times higher in *pmrA*$^+$ than in the strains lacking *pmrA*, whereas expression of the PmrA-independent *mgtA* was not influenced by the *pmrA* allele (Fig. 2). This experiment defined two classes of PhoP-regulated genes: those which are PmrA dependent and those which are PmrA independent.

pH modulates transcription of PmrA-regulated genes. We hypothesized that PmrA-activated genes may be regulated by pH because the PmrA-regulated *psiD* gene had been previously shown to be induced by mild acid pH (15). Indeed, transcription of *pbgP*, *pbgE*, *pmrC*, and *ugd* was activated when the pH of the media was lowered from 7.7 to 5.8 (Fig. 3A). This activation required a functional PmrA protein because disruption of the *pmrA* gene abolished transcription from these loci.

We further investigated the regulatory roles of pH and Mg^{2+} on the expression of the *pbgP* and *mgtA* genes, which were chosen as representative PmrA-dependent and -independent loci, respectively. Mild acid pH induced transcription of the *pbgP* gene at both millimolar and micromolar concentrations of Mg^{2+} (Fig. 3B). In contrast, transcription of *mgtA* changed less than fourfold in mild acid pH and was not affected by the presence of the *pmrA* gene (Fig. 4B). Magnesium, on the other hand, modulated expression of both PmrA-dependent and PmrA-independent genes (Fig. 4). These results demonstrate that transcription of PmrA-activated genes can be induced by two different environmental conditions: a decrease in pH from 7.7 to 5.8 or a decrease in the Mg^{2+} concentration from the millimolar to the micromolar range.

The PhoP and PhoQ proteins are not essential for the acid-mediated induction of PmrA-regulated genes. At alkaline pH, activation of the PmrA-regulated *pbgP* gene was dependent on PhoP/PhoQ and controlled by Mg^{2+} (Fig. 4). In contrast, the acid-mediated induction of *pbgP* transcription still occurred in strains lacking *phoP* (Fig. 4) and *phoQ* (data not shown). These results demonstrate that PmrA-regulated genes can be activated independently of the PhoP/PhoQ system. At 10 mM Mg^{2+}, pH 5.8, however, *pbgP* transcription was slightly reduced in the strains lacking *phoP* (Fig. 4) and *phoQ* (data not shown), which suggest that pH can influence the interaction of Mg^{2+} with the PhoQ protein.

Role of the PhoP/PhoQ system in the activation of PmrA-regulated genes. There are several possible mechanisms by which the PhoP/PhoQ system could promote transcription of PmrA-regulated genes. For example, PhoP/PhoQ may be necessary to transcribe the *pmrCAB* operon, leading to the production of the PmrA and PmrB proteins and the ensuing expression of PmrA-activated genes. This model predicts that transcription of *pmrAB* genes from a PhoP-independent promoter should restore expression of PmrA-dependent genes to *phoP* and *phoQ* mutants. However, a plasmid harboring the *pmrAB*$^+$ genes under the control of the *lac* promoter failed to activate *pbgP* transcription in either *phoP* (Fig. 5) or *phoQ* (data not shown) mutants. The *pmrAB*$^+$-containing plasmid was functional because it could restore *pbgP* transcription to a strain lacking *pmrA*. Moreover, this plasmid did not alter *pbgP* expression when introduced into the wild-type strain (Fig. 4). Taken together with the findings that the *pmrCAB* operon is autogenously regulated (see below), these results rule out a simple cascade model for activation of PmrA-regulated genes by the PhoP/PhoQ system.

The sensor proteins of certain two-component systems have been shown to activate noncognate response regulators (41). However, such a cross-talk mechanism does not appear to be responsible for the PhoP/PhoQ-mediated activation of *pbgP* transcription under Mg^{2+}-limiting conditions: a plasmid harboring the *phoQ* gene under the control of the *lac* promoter

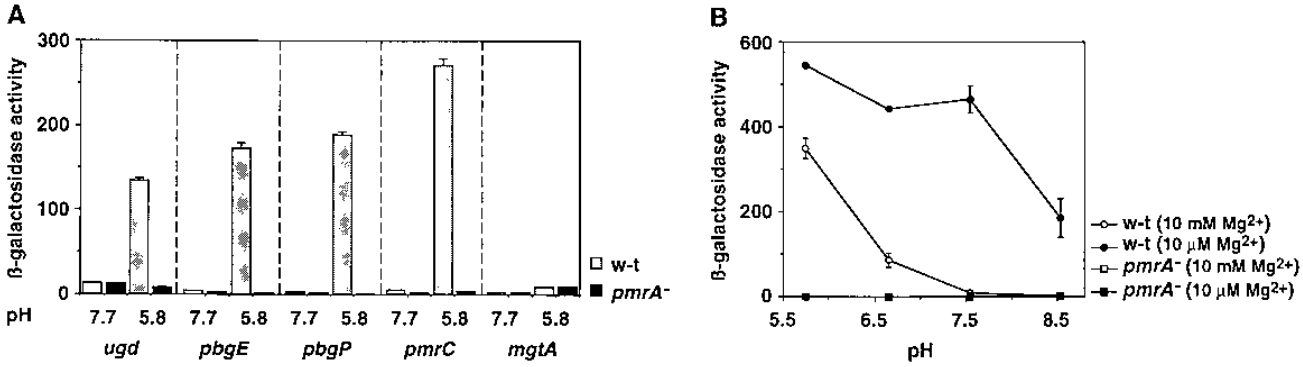

FIG. 3. pH modulates expression of PmrA-regulated genes. (A) Transcription of PmrA-regulated genes in strains grown in 10 mM $MgCl_2$–N-minimal medium at pH 7.7 or 5.8. (B) Transcription of the *pbgP* gene in strains grown in N-minimal medium (with 10 mM or 10 μM $MgCl_2$) at pH 8.55, 7.55, 6.60, or 5.75. Assays were performed as described in the legend to Fig. 2. w-t, wild type; *pmrA*$^-$, strain lacking *pmrA*.

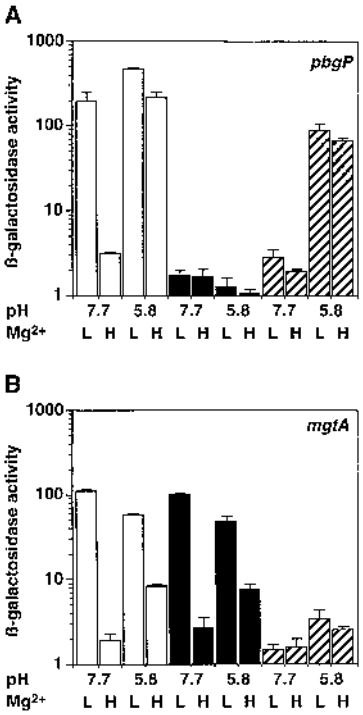

FIG. 4. Role of the PmrA and PhoP proteins in regulation of *pbgP* (A) and *mgtA* (B) transcription by pH and Mg^{2+}. Transcriptional activity was investigated in three different backgrounds: wild-type (EG9241 and EG9521) (shaded boxes) and strains lacking *pmrA* (EG9681 and EG9671) (solid boxes) and *phoP* (EG9168 and EG9523) (hatched boxes). Assays were performed as described in the legend to Fig. 2.

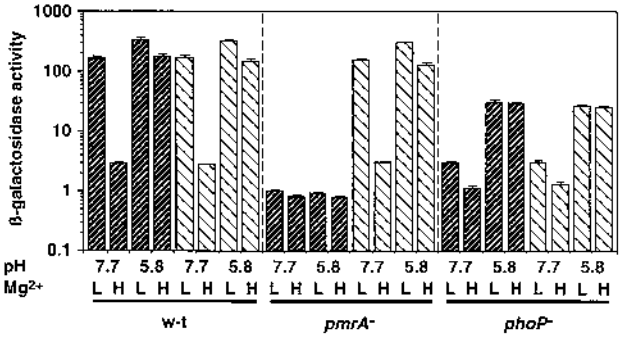

FIG. 5. Role of the PhoP protein in the Mg^{2+}-mediated activation of the *pbgP* gene. Transcriptional activity was investigated in three different backgrounds: wild-type (w-t) (EG9241) and strains lacking *pmrA* (EG9681) (*pmrA*$^-$) and *phoP* (EG9168) (*phoP*$^-$). β-Galactosidase activities (Miller units) were determined on overnight cultures of strains harboring either the *pmrAB*$^+$ plasmid pEG9102 (open hatched boxes) or the vector pUH21-2*lacI*^{q} (solid hatched boxes).

could not restore *pbgP* expression to a strain lacking *phoP* (data not shown). This indicates that activation of PmrA-regulated genes does not involve phosphorylation of PmrA by the PhoQ protein.

Autogenous control of the *pmrCAB* operon. We hypothesized that the PmrA protein may be required for expression of the *pmrCAB* operon because transcription of the *pmrC-lac* gene fusion was regulated in the same way as the PmrA-activated *pbgP* gene: it was induced in either micromolar Mg^{2+} concentrations or mild acid pH. Transcription of *pmrC* could not be activated in a strain harboring a chloramphenicol resistance cassette in the *pmrA* gene (Fig. 6), demonstrating that the *pmrCAB* operon is a PmrA-activated locus. The defect was solely due to the absence of the PmrA protein, because normal regulation was restored upon introduction of a plasmid harboring the *pmrA*$^+$ gene. These results indicate that a promoter within the *pmrC* open reading frame or in the MudJ transposon drives *pmrA* transcription in the *pmrC*::MudJ mutant.

PmrA governs growth on low Mg^{2+} solid media. We previously demonstrated that mutants defective in either the *phoP* or *phoQ* genes fail to form colonies on N-minimal medium–agarose plates containing <40 μM Mg^{2+} (15), and we identified six PhoP-activated loci that are required for growth on this medium (36). All six loci, *pbgE*, *pbgM*, *pbgP*, *pcgD*, *pcgP*, and *ugd*, are regulated by the PmrA protein, demonstrating that the PmrA/PmrB system governs the adaptation to low-Mg^{2+} solid media. And, as expected, the *pmrA* mutant could not grow on plates containing <40 μM Mg^{2+}. In contrast, mutants harboring MudJ transposon insertions in the *pmrC* gene grew in low-Mg^{2+} media like the wild-type strain. Because *pmrC* precedes the *pmrA* gene in the *pmrCAB* operon (Fig. 1A), these results suggest the presence of a second promoter within the *pmrC* gene or the MudJ transposon that is responsible for the production of PmrA protein in the *pmrC*::MudJ mutants.

DISCUSSION

We have identified a regulatory cascade in which a pair of two-component systems, PhoP/PhoQ and PmrA/PmrB, interact to control expression of a set of unlinked genes. Seven PhoP-activated loci required a functional PmrA protein for transcription, thereby defining two classes of PhoP-regulated loci that differ in their PmrA dependence and the signals that modulate their expression. Whereas all PhoP-activated genes are transcriptionally induced by Mg^{2+} limitation, those which are PmrA dependent, including the *pmrCAB* operon, are also induced by mild acidification. Being under the control of two distinct two-component systems allows PmrA-regulated genes to respond to a broader spectrum of environmental cues.

Activation of PmrA-regulated genes in low Mg^{2+}. The transcriptional induction of PmrA-activated genes in low Mg^{2+} required the PhoQ and PhoP proteins. Extracellular Mg^{2+} has been previously shown to govern the PhoP/PhoQ regulatory system: Mg^{2+} acts as a ligand that changes the conformation of the sensor protein PhoQ *in vitro* and regulates the transcrip-

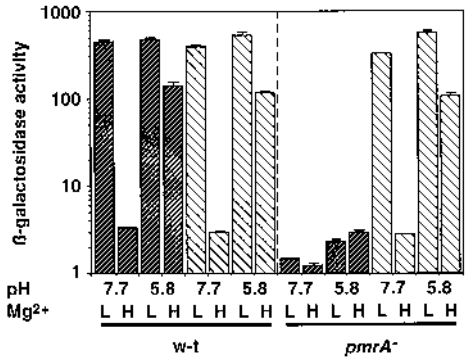

FIG. 6. The *pmrCAB* operon is transcriptionally autoregulated. Transcriptional activity was investigated in two different backgrounds: wild-type (EG9279) (w-t) and a strain lacking *pmrA* (EG9687) (*pmrA*$^-$). Assays were performed on strains harboring either the *pmrAB*$^+$ plasmid pEG9102 (open hatched boxes) or the vector pUH21-2*lacI*^{q} (solid hatched boxes) as described in the legend to Fig. 5.

tional activity of the PhoP protein *in vivo* (15). Regulatory cascades in which an activator protein is necessary for transcription of a gene encoding another activator protein have been described for several bacterial species, including *Vibrio cholerae* (8) and *Shigella flexneri* (40). However, this model cannot explain the PhoP/PhoQ-mediated activation of PmrA-regulated genes because (i) transcription of the *pmrAB* genes from a heterologous promoter could not restore expression of PmrA-regulated genes to strains lacking either *phoP* or *phoQ* (Fig. 5) and (ii) the *pmrCAB* operon is transcriptionally autoregulated (Fig. 6). Then, how does the PhoP/PhoQ system activate transcription of PmrA-regulated genes?

The PhoP/PhoQ system could activate the PmrA protein by using a mechanism similar to the phosphorelay that phosphorylates the Spo0A transcription factor to initiate sporulation in *Bacillus subtilis* (5). In this phosphorelay, signal-sensing kinases phosphorylate the Spo0F response regulator, and the phospho-Spo0F transfers the phosphate to the Spo0A protein with Spo0B being an intermediate in this process. In low-Mg^{2+} media the PhoQ protein would phosphorylate the PhoP protein and the phospho-PhoP would transfer the phosphate to the PmrA protein either directly or by first phosphorylating the PmrB protein. Consistent with this model, which predicts phosphotransfer between noncognate regulators (i.e., PhoP) and sensors (i.e., PmrB), the cytoplasmic domain of the *Escherichia coli* PmrB could phosphorylate the OmpR protein *in vivo* and *in vitro* (24). Alternatively, activation of PmrA-regulated genes in low-Mg^{2+} media may involve binding of both phospho-PhoP and PmrA proteins to the promoter region of PmrA-regulated genes. Purification of the proteins that comprise the PhoP/PhoQ and PmrA/PmrB systems will allow us to examine whether these or, potentially, other mechanisms are responsible for the activation of PmrA-regulated genes in low-Mg^{2+} media.

Activation of PmrA-regulated genes in mild acid pH. In addition to Mg^{2+} limitation, PmrA-regulated genes were transcriptionally induced by mild acidification. This activation was independent of the PhoP and PhoQ proteins and specific to PmrA-regulated genes because transcription of the PmrA-independent *mgtA* gene was not significantly induced in mild acid pH. However, *pbgP* transcription was slightly lower in the strain lacking *phoP* in media containing repressing concentrations of Mg^{2+} (Fig. 4). Cumulatively, these results indicate that the PhoP/PhoQ system is not required to sense pH and that acidification influences the interaction of Mg^{2+} with the PhoQ protein.

The PmrB protein might be involved in sensing pH (or a ligand whose availability changes with pH), since it is the likely cognate sensor for the PmrA protein, given the genetic organization of the *pmrA* and *pmrB* genes, which is typical of two-component systems. Moreover, the PmrB protein features a periplasmic domain rich in charged residues, including three histidines which have a pK_a in the pH range that activates PmrA-regulated genes. However, the role of the PmrB protein in sensing pH will remain unclear until the construction and evaluation of strains lacking the *pmrB* gene.

Transcriptional autoregulation of the *pmrCAB* operon. Expression of a *pmrC-lac* gene fusion was induced in micromolar Mg^{2+} concentrations or mild acid pH and required a functional PmrA protein (Fig. 2, 3, and 6), which demonstrates that the *pmrCAB* operon is positively autoregulated. Inactivation of *pmrC* did not eliminate expression of PmrA-dependent phenotypes such as growth on low-Mg^{2+} solid media, which suggests that *pmrA* is transcribed from two promoters: an environmentally sensitive, PmrA-dependent promoter preceding *pmrC* and a second promoter within the *pmrC* open reading frame. Similar regulation has been described for the *phoPQ* operon, where two different promoters were identified: one that is induced in low Mg^{2+} and requires PhoP and PhoQ for activity and another that is independent of these proteins and does not respond to the levels of Mg^{2+} (35). However, our results do not rule out the possibilities that a single promoter transcribes *pmrCAB* in a wild-type strain and that a promoter with the MudJ transposon is responsible for *pmrA* transcription in the *pmrC*::MudJ mutant.

Function of PmrA-regulated determinants. The *pmrA* locus was originally defined by mutations that conferred resistance to the membrane-damaging antibiotic polymyxin B (19). Polymyxin-resistant mutants exhibit cross-resistance to several other cationic antimicrobial polypeptides, including protamine and the neutrophil-derived bactericidal/permeability-increasing protein (i.e., CAP57) (33, 37) and azurocidin (i.e., CAP37) (32). These mutants have a modified lipopolysaccharide (LPS) that contains larger amounts of 4-aminoarabinose and ethanolamine in the lipid A portion (18), and these modifications are presumably responsible for the decreased binding of polymyxin to resistant organisms. The polymyxin-resistant mutants harbor a single amino acid substitution in the N-terminal domain of PmrA (29) that renders the protein hyperactive and results in increased expression of its target genes.

The PmrA-regulated loci identified in this study encode proteins that could mediate the chemical modifications in the LPS that confer polymyxin resistance. Mutations in six of these loci prevented growth on low-Mg^{2+} solid media but had no effect on growth in low-Mg^{2+} liquid media (36), which suggests that these determinants mediate changes in the surface of the microbial cell. To reach the bacterial membrane, cationic peptides must displace the Mg^{2+} ions that stabilize the LPS. Thus, the LPS modifications allowing growth in Mg^{2+}-limiting conditions may also confer resistance to polymyxin and could be mediated by the same set of genes. The PmrA-regulated *ugd* gene is essential on low-Mg^{2+} solid media and has been mapped to one of the gene clusters involved in LPS biosynthesis in *S. typhimurium*, *E. coli*, and *S. flexneri* (3, 4, 23). It encodes a protein that is homologous to the UDP-glucose dehydrogenase of *Streptococcus pneumoniae* (7, 9), in which it is necessary for capsule biosynthesis.

Roland and coworkers have identified a gene, *pmrD*, that appears to be regulated by the PmrA/PmrB system because it requires a functional PmrA to confer resistance to polymyxin B (28). This gene encodes an 85-amino-acid polypeptide that exhibits similarity to a protease from Rous sarcoma virus. PmrD-mediated polymyxin resistance is unlikely to result from modifications in the LPS, because the PmrD protein does not confer resistance to the bactericidal/permeability-increasing protein (i.e., CAP57), which also targets the lipid A portion of the LPS.

Expression of PmrA-regulated genes in mammalian host cells. The Miller and Finlay laboratories have demonstrated that PhoP-activated genes are expressed in mammalian cells (2, 13). The PhoP-activated *pagA*, *pagB*, and *pagC* genes are transcribed in macrophages but not in epithelial cells (2). In contrast, the *mgtCB* locus, which is also regulated by the PhoP/PhoQ system, is activated in epithelial cells (13). One possible interpretation of these discordant results is that *mgtCB* belongs to one class of PhoP-regulated loci, whereas *pagA*, *pagB*, and *pagC* belong to another. The *mgtCB* locus does not require PmrA for expression and may respond to Mg^{2+} limitation in epithelial cells (*mgtCB* expression in macrophages has not been reported). On the other hand, the *pag* genes are known to be activated by acidification *in vitro* (2), which suggests they belong to the PmrA-dependent class of PhoP-regulated loci.

The *pagA* gene could be allelic with *ugd*, since both map to the 42′ region of the *S. typhimurium* chromosome (3, 4, 22). It is presently unclear whether *pagB* and *pagC* correspond to any of the PmrA-regulated loci described in this study. In sum, control by a pair of two-component systems may ensure the proper temporal and spacial expression of the different classes of PhoP-regulated genes.

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REFERENCES

1. **Alex, L. A., and M. I. Simon.** 1994. Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. Trends Genet. **10:**133–138.
2. **Alpuche-Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller.** 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. USA **89:**10079–10083.
3. **Bastin, D. A., G. Stevenson, P. K. Brown, A. Haase, and P. R. Reeves.** 1993. Repeat unit polysaccharide of bacteria: a model for polymerization resembling that of ribosomes and fatty acid synthetase, with a novel mechanism for determining chain length. Mol. Microbiol. **7:**725–734.
4. **Batchelor, R. A., P. Alifano, E. Biffali, S. I. Hull, and R. A. Hull.** 1992. Nucleotide sequences of the genes regulating O-polysaccharide antigen chain length (*rol*) from *Escherichia coli* and *Salmonella typhimurium*: protein homology and functional complementation. J. Bacteriol. **174:**5228–5236.
5. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545–552.
6. **Davis, R. W., D. Bolstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Dillard, J. P., M. W. Vandersea, and J. Yother.** 1995. Characterization of the cassette containing genes for type 3 capsular polysaccharide biosynthesis in *Streptococcus pneumoniae*. J. Exp. Med. **181:**973–983.
8. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88:**5403–5407.
9. **Dougherty, B. A., and I. van de Rijn.** 1993. Molecular characterization of *hasB* from an operon required for hyaluronic acid synthesis in group A streptococci. J. Biol. Chem. **268:**7118–7124.
10. **Dziejman, M., and J. J. Mekalanos.** 1995. Two-component signal transduction and its role in the expression of bacterial virulence factors, p. 305–317. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
11. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
12. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
13. **García del Portillo, F., J. W. Foster, M. E. Maguire, and B. B. Finlay.** 1992. Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. Mol. Microbiol. **6:**3289–3297.
14. **García Véscovi, E., F. C. Soncini, and E. A. Groisman.** 1994. The role of the PhoP/PhoQ regulon in *Salmonella* virulence. Res. Microbiol. **145:**473–480.
15. **García Véscovi, E., F. C. Soncini, and E. A. Groisman.** 1996. Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell **84:**165–174.
16. **Groisman, E. A., and F. Heffron.** 1995. Regulation of *Salmonella* virulence by two-component regulatory systems, p. 319–332. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
17. **Groisman, E. A., M. A. Sturmoski, F. R. Solomon, R. Lin, and H. Ochman.** 1993. Molecular, functional, and evolutionary analysis of sequences specific to *Salmonella*. Proc. Natl. Acad. Sci. USA **90:**1033–1037.
18. **Helander, I. M., I. Kilpeläinen, and M. Vaara.** 1994. Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a ^{31}P-NMR study. Mol. Microbiol. **11:**481–487.
19. **Mäkelä, P. H., M. Sarvas, S. Calcagno, and K. Lounatmaa.** 1978. Isolation and characterization of polymyxin-resistant mutants of *Salmonella*. FEMS Microbiol. **3:**323–326.
20. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
23. **Morona, R., L. Van Den Bosch, and P. A. Manning.** 1995. Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. J. Bacteriol. **177:**1059–1068.
24. **Nagasawa, S., K. Ishige, and T. Mizuno.** 1993. Novel members of the two-component signal transduction genes in *Escherichia coli*. J. Biochem. **114:** 350–357.
25. **Parkinson, J. S.** 1993. Signal transduction schemes of bacteria. Cell **73:**857–871.
26. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
27. **Reece, K. S., and G. J. Phillips.** 1995. New plasmids carrying antibiotic-resistance cassettes. Gene **165:**141–142.
28. **Roland, K. L., C. R. Esther, and J. K. Spitznagel.** 1994. Isolation and characterization of a gene, *pmrD*, from *Salmonella typhimurium* that confers resistance to polymyxin when expressed in multiple copies. J. Bacteriol. **176:**3589–3597.
29. **Roland, K. L., L. E. Martin, C. R. Esther, and J. K. Spitznagel.** 1993. Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. J. Bacteriol. **175:**4154–4164.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sanderson, K. E., A. Hessel, and K. E. Rudd.** 1995. Genetic map of *Salmonella typhimurium*, edition VIII. Microbiol. Rev. **59:**241–303.
32. **Shafer, W. M., S. G. Casey, and J. K. Spitznagel.** 1984. Lipid A and resistance of *Salmonella typhimurium* to antimicrobial granule proteins of human neutrophils. Infect. Immun. **43:**834–838.
33. **Shafer, W. M., L. E. Martin, and J. K. Spitznagel.** 1984. Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. Infect. Immun. **45:**29–35.
34. **Snavely, M. D., S. A. Gravina, T.-B. T. Cheung, C. G. Miller, and M. E. Maguire.** 1991. Magnesium transport in *Salmonella typhimurium*: regulation of *mgtA* and *mgtB* expression. J. Biol. Chem. **266:**824–829.
35. **Soncini, F. C., E. García Véscovi, and E. A. Groisman.** 1995. Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. J. Bacteriol. **177:**4364–4371.
36. **Soncini, F. C., E. García Véscovi, F. Solomon, and E. A. Groisman.** 1996. Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. J. Bacteriol. **178:**5092–5099.
37. **Stinavage, P., L. E. Martin, and J. K. Spitznagel.** 1989. O-antigen and lipid A phosphoryl groups in resistance of *Salmonella typhimurium* LT-2 to non-oxidative killing in human polymorphonuclear cells. Infect. Immun. **57:**3894–3900.
38. **Stock, J. B., M. G. Surette, M. Levit, and P. Park.** 1995. Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis, p. 25–51. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
39. **Swanson, R. V., L. A. Alex, and M. I. Simon.** 1994. Histidine and aspartate phosphorylation: two-component systems and the limits of homology. Trends Biochem. Sci. **19:**485–490.
40. **Tobe, T., S. Nagai, N. Okada, B. Adler, M. Yoshikawa, and C. Sasakawa.** 1991. Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. Mol. Microbiol. **5:**887–893.
41. **Wanner, B. L.** 1992. Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? J. Bacteriol. **174:** 2053–2058.
42. **Whitfield, H. J., and G. Levine.** 1973. Isolation and characterization of a mutant of *Salmonella typhimurium* deficient in a major deoxyribonucleic acid polymerase activity. J. Bacteriol. **116:**54.
43. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.