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A small protein that mediates the activation of a two-component system by another two-component system

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The PmrA–PmrB two-component system of *Salmonella enterica* controls resistance to the peptide antibiotic polymyxin B and to several antimicrobial proteins from human neutrophils. Transcription of PmrA-activated genes is induced by high iron, but can also be promoted by growth in low magnesium in a process that requires another two-component system, PhoP–PhoQ. Here, we define the genetic basis for the interaction between the PhoP–PhoQ and PmrA–PmrB systems. We have identified *pmrD* as a PhoP-activated gene that mediates the transcriptional activation of PmrA-regulated genes during growth in low magnesium. When transcription of *pmrD* is driven from a heterologous promoter, expression of PmrA-activated genes occurs even at repressing magnesium concentrations and becomes independent of the *phoP* and *phoQ* genes. The PmrD effect is specific for PmrA-regulated genes and requires functional PmrA and PmrB proteins. A *pmrD* mutant is sensitive to polymyxin if grown in low magnesium, but resistant if grown in high iron. The PmrD protein controls the activity of the PmrA–PmrB system at a post-transcriptional level.

Keywords: magnesium/PhoP–PhoQ/PmrA–PmrB/signal transduction/transcription

Introduction

Two-component regulatory systems are signal transduction machineries employed by eubacteria, archaebacteria, and the cell-wall-containing eukaryotes *Saccharomyces cerevisiae*, *Neurospora crassa* and *Arabidopsis thaliana* (Loomis *et al*., 1997; Perraud *et al*., 1999). These systems use reversible phosphorylation between two proteins, a sensor kinase and a response regulator, to mediate the adaptation to changing environmental conditions. Sensors are usually integral membrane proteins that respond to particular chemical or physical signals by modulating the phosphorylated state of their cognate regulators, which are often transcription factors whose conformation or affinity for their promoter sequences is controlled by phosphorylation (Stock *et al*., 1995). Eubacterial species harbor many two-component systems, each responding to distinct cues and controlling the expression of discrete sets of genes.

The PmrA–PmrB two-component system of *Salmonella enterica* serovar *typhimurium* is required for resistance to polymyxin B and to other antimicrobial compounds (Mäkelä *et al*., 1978; Shafer *et al*., 1984a,b). The PmrA–PmrB system controls the transcription of several loci including: *pbgP* and *ugd* (designated *pmrF* and *pmrE*, respectively, by Gunn *et al*., 1998), which mediate the modification of the lipopolysaccharide and are necessary for polymyxin resistance (Groisman *et al*., 1997; Gunn *et al*., 1998); *pmrG*, which encodes a protein of unknown function (Gunn *et al*., 1998); and the *pmrCAB* operon (Gunn and Miller, 1996; Soncini and Groisman, 1996), indicative that the PmrA–PmrB system is autogenously regulated (Figure 1). In addition, it has been hypothesized that the *pmrD* gene might be PmrA regulated because, when present in a medium-copy-number plasmid, it confers polymyxin resistance in a PmrA-dependent manner (Roland *et al*., 1994). The PmrA protein has been shown to bind and footprint the promoter region of the *pbgP*, *pmrC* and *pmrG* genes (Wösten and Groisman, 1999).

Transcription of PmrA-activated genes is promoted by either of two stimuli: (i) growth in low extracellular magnesium in a process that requires PhoP–PhoQ, a two-component system that responds to the magnesium levels in the environment (García Véscovi *et al*., 1996); and (ii) growth in the presence of high iron (M.M.S.M.Wösten and E.A.Groisman, unpublished results) or mild acid pH (Soncini and Groisman, 1996) in a process that does not require PhoP–PhoQ. The PmrB protein is necessary for iron sensing (M.M.S.M.Wösten and E.A.Groisman, unpublished results) and its cytoplasmic domain can phosphorylate the regulatory protein PmrA (Wösten and Groisman, 1999). The mechanism by which the PhoP–PhoQ system transduces the low magnesium signal to the PmrA–PmrB system remains unknown. However, it does not appear to involve cross-talk (i.e. phosphorylation of the regulatory protein PmrA by the sensor protein PhoQ) or a classical regulatory cascade (i.e. the PhoP–PhoQ system being solely responsible for transcription of the *pmrAB* genes) (Soncini and Groisman, 1996).

In this paper, we define the genetic basis for the interaction between the PhoP–PhoQ and PmrA–PmrB two-component systems. We show that *pmrD* is a PhoP-activated gene that mediates the transcriptional induction of PmrA-activated genes during growth in low magnesium, and demonstrate that *pmrD* expression does not depend on the PmrA–PmrB system. We establish that the PmrD protein acts specifically on PmrA-activated genes and that functional PmrA and PmrB proteins are required for the PmrD protein to exert its effect. Transcription of PmrA-activated genes during growth in high iron is independent of PmrD, which controls the activity of the PmrA–PmrB system at a post-transcriptional level.

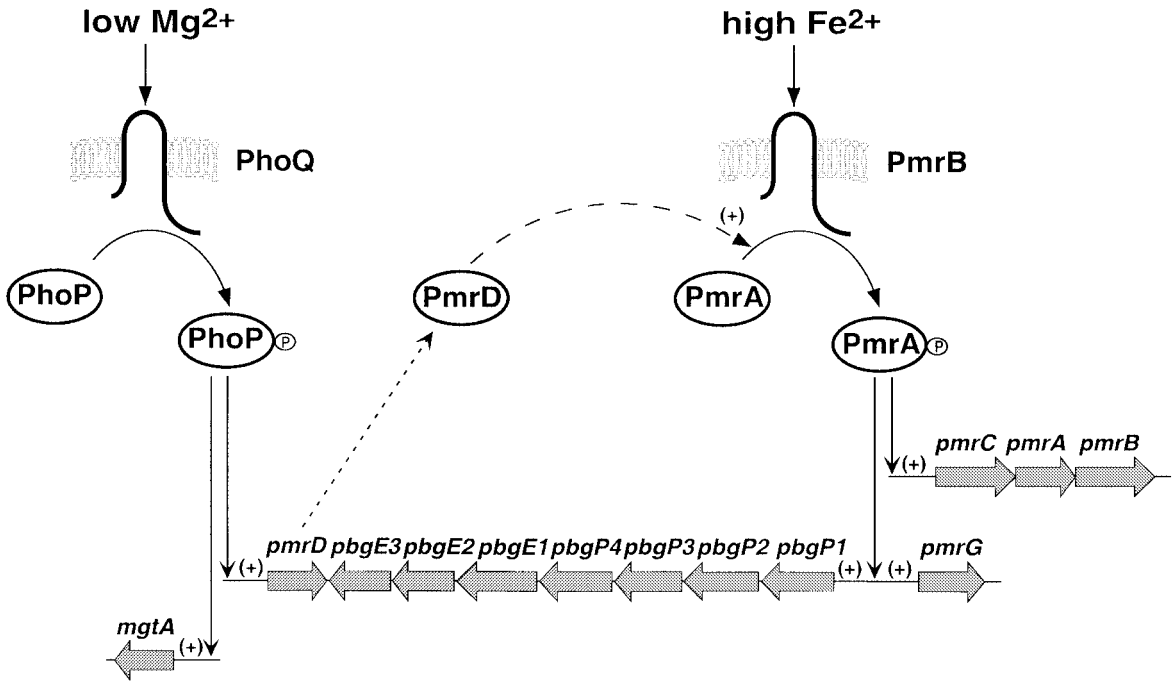

Fig. 1. Model for the activation of the PmrA–PmrB two-component system by the PhoP–PhoQ two-component system. Transcription of PmrA-activated genes can be induced by growth in high iron independently of the PhoP–PhoQ two-component system (right) or by growth in low magnesium in a PhoP–PhoQ-dependent manner (left). During growth in low magnesium, the PhoP–PhoQ system promotes expression of the *pmrD* gene. The PmrD protein controls the activity of the PmrA–PmrB system at a post-transcriptional level. The seven-gene *pbgP/E* operon has also been designated the *pmrF* locus (Gunn *et al.*, 1998).

Results

PmrD is required for transcription of PmrA-activated genes during growth in low magnesium

To address the role of the PmrD protein in the PhoP–PhoQ-mediated activation of the PmrA–PmrB system, we examined whether transcription of PmrA-regulated genes required *pmrD*. This gene is located downstream of the PmrA-activated *pbgP/pmrF* locus (Gunn *et al.*, 1998) and has been implicated in polymyxin resistance (Roland *et al.*, 1994). When bacteria were grown in low-magnesium media, a condition that promotes expression of PmrA-activated genes in a PhoP- and PhoQ-dependent manner (Soncini and Groisman, 1996), transcription of the *pbgP*, *pmrC* and *ugd* genes was abolished in the *pmrD* mutant (Figure 2A). This effect was specific for PhoP-activated genes that are PmrA dependent because the PmrA-independent PhoP-activated genes *mgtA*, *mgtC* and *pcgL* were expressed at wild-type levels in a *pmrD* mutant strain (Figure 2B). As expected, a *phoP* null mutation abolished transcription of all six PhoP-activated genes, whereas a *pmrA* null allele prevented expression of the three PmrA-dependent loci (Figure 2A). These results show that PmrD is essential for the transcriptional induction of PmrA-activated genes that occurs during growth in low magnesium.

Transcription of the pmrD gene is PhoP dependent but PmrA independent

It has been hypothesized that *pmrD* might be a PmrA-regulated gene because a *pmrD*-containing plasmid conferred polymyxin resistance only in strains harboring a functional *pmrA* gene (Roland *et al.*, 1994). To explore the possibility that PmrA might control transcription of the *pmrD* gene, we carried out S1 nuclease experiments to map the *pmrD* promoter in wild-type, *pmrA* and *phoP* strains.

A transcription start site was identified 29 bp from the *pmrD* start codon with RNA harvested from wild-type bacteria grown in low magnesium (Figure 3A). Transcription of *pmrD* was dependent on a functional *phoP* gene, but did not require *pmrA* (Figure 3A). (Similar results were obtained in primer extension experiments; data not shown.) Consistent with these results, *pmrD* transcription was not detected in wild-type bacteria grown in high-magnesium media (Figure 3A), a condition that represses transcription of PhoP-activated genes (García Véscovi *et al.*, 1996). Furthermore, the *pmrD* promoter harbors a –10 region typical of σ70 promoters and a relatively weak –35 consensus, but also contains the sequence TGTTTA 23 bp upstream of the *pmrD* transcription start site (Figure 3B). This sequence matches the consensus (T/G)GTTTA, which is found as a direct repeat some 25 bp upstream of the transcription start sites of several PhoP-dependent promoters in *Escherichia coli* (Kato *et al.*, 1999). That *pmrD* transcription is not regulated by the PmrA protein is supported by the absence of a PmrA binding site (Wösten and Groisman, 1999) in the promoter region of *pmrD* (Figure 3B). Furthermore, growth of wild-type *Salmonella* in low magnesium with 100 μM iron, a condition that promotes expression of PmrA-activated genes in a PhoP- and PhoQ-independent manner (M.M.S.M.Wösten and E.A.Groisman, unpublished results), did not promote transcription of *pmrD* (actually, lower levels of *pmrD* transcription were observed; Figure 3A). These results demonstrate that *pmrD* is a PhoP-activated gene and that PmrA is not necessary for *pmrD* transcription.

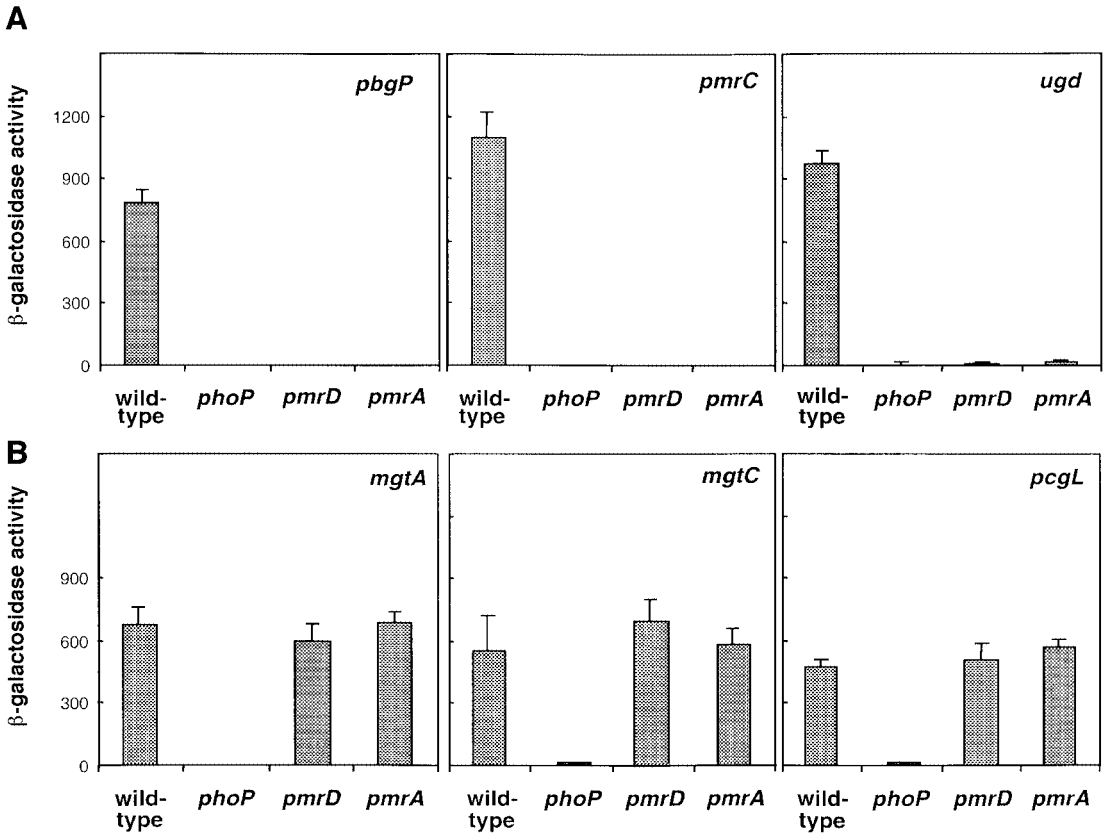

Fig. 2. The *pmrD* gene is necessary for transcription of PhoP-activated PmrA-dependent genes, but does not affect transcription of PhoP-activated PmrA-independent genes. β-galactosidase activities (Miller units) expressed by strains grown in N-minimal medium pH 7.7 with 10 μM Mg^{2+} were determined for mutants harboring a *lac* transcriptional fusion to the PhoP-activated, PmrA-dependent genes *pbgP*, *pmrC* and *ugd* (**A**) and the PhoP-activated, PmrA-independent genes *mgtA*, *mgtC* and *pcgL* (**B**). The transcriptional activity was investigated in four genetic backgrounds: wild type, *phoP*, *pmrD* and *pmrA*. Data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are only shown if greater than the resolution of the figure).

Role of the PhoP–PhoQ and PmrA–PmrB systems in transcription of the PmrA-activated gene pbgP

We examined the role of the PhoP–PhoQ and PmrA–PmrB two-component systems in transcription of *pbgP*, which was chosen as a prototypical PmrA-activated gene because the PmrA protein binds to the *pbgP* promoter (Wösten and Groisman, 1999). When bacteria were grown in low magnesium, *pbgP* transcription required functional *phoQ*, *phoP*, *pmrA* and *pmrB* genes (Figure 4A). On the other hand, when bacteria were grown in low-magnesium media containing 100 μM iron, *pbgP* transcription was observed in the *phoQ* and *phoP* mutants, but not in the *pmrA* or *pmrB* mutant strains (Figure 4B). The levels of *pbgP* transcription in the *phoP* and *phoQ* mutants grown in the presence of 100 μM iron were slightly reduced to 40–45% of those observed in the isogenic wild-type strain (Figure 4B).

In a *pmrD* mutant, transcription of *pbgP* was similar to that observed in the *phoP* or *phoQ* mutants: it was absent in bacteria grown in low magnesium (Figure 4A), but present in bacteria grown in the presence of 100 μM iron (Figure 4B). Plasmid pLK23, harboring a wild-type copy of the *pmrD* gene expressed from its own promoter, restored wild-type levels of *pbgP* expression to the *pmrD* mutant (Figure 4C), demonstrating that the lack of *pbgP* transcription is solely due to inactivation of *pmrD*. Cumulatively, these results establish that the *phoQ*, *phoP* and *pmrD* genes are essential for *pbgP* transcription during growth in low magnesium (mediating >100-fold activation in *pbgP* expression), but unnecessary (<5-fold effect) during growth in high iron.

PmrD acts downstream of PhoP–PhoQ

The results described above suggest the following model for the transcription of PmrA-activated genes during growth in low magnesium: the PhoQ protein activates the PhoP protein (presumably by promoting its phosphorylated state), which induces transcription of PhoP-activated genes including *pmrD*. The PmrD protein then specifically promotes transcription of PmrA-activated genes. We tested this model (i.e. that PmrD acts downstream of PhoP–PhoQ) by examining whether ectopic expression of *pmrD* could bypass the requirement for *phoP*, *phoQ* and growth in low magnesium. Plasmid pLK24, in which the *pmrD* gene is transcribed from the *lac* promoter in pUC19, restored *pbgP* transcription to *phoP* and *phoQ* mutants as well as to wild-type and *pmrD* cells, whether bacteria were grown under inducing (i.e. low magnesium) or repressing (i.e. high magnesium) conditions (Figure 5). In contrast, plasmid pLK23, in which the *pmrD* gene is expressed from its own promoter, could not restore *pbgP* expression to a *phoP* mutant (data not shown). These results suggest that the role of the

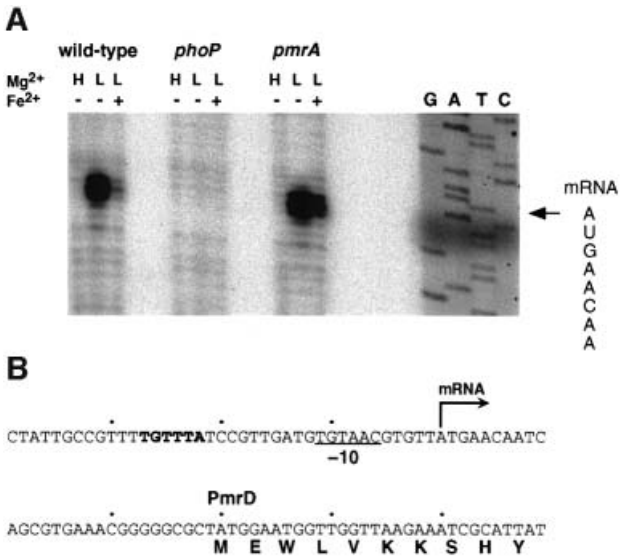

Fig. 3. Transcription of *pmrD* requires PhoP, but is PmrA independent. (**A**) S1 mapping of *pmrD* transcripts produced in wild-type (14028s), *phoP* (MS7953s) and *pmrA* (EG7139) bacteria grown in N-minimal medium pH 7.7, containing 10 mM Mg^{2+}, 10 μM Mg^{2+} or 10 μM Mg^{2+} and 100 μM Fe^{2+} and harvested during the logarithmic phase. The S1 protection assay was performed as described in Materials and methods. Lanes G, A, T and C correspond to dideoxy chain termination sequence reactions corresponding to this region. The sequence spanning the transcription start site is shown, and the transcription start site is marked with an arrow. (**B**) DNA sequence of the promoter region of the *pmrD* gene. The arrow corresponds to the +1 transcription start site, the underlined sequence is the predicted –10 region for the *pmrD* promoter and the sequence in bold matches the consensus found as a direct repeat in the promoter region of PhoP-activated genes in *E.coli* K-12 (Kato *et al.*, 1999).

PhoP–PhoQ system in activation of PmrA-activated genes is to promote expression of the *pmrD* gene.

PmrD acts upstream of PmrA–PmrB

We conducted epistasis experiments to examine whether the PmrD-mediated transcription of PmrA-activated loci required the *pmrA* and/or *pmrB* genes. Consistent with the notion that PmrD acts upstream or at the same level as PmrA, plasmid pLK24 (in which the *pmrD* gene is transcribed from the *lac* promoter in pUC19) could not restore *pbgP* expression to the *pmrA* null mutant: the levels of *pbgP* transcription were similar to those obtained in a strain harboring the pUC19 vector (Figure 5). Likewise, expression of *pmrD* from plasmid pLK24 in the *pmrB* mutant restored only 6% of the *pbgP* transcription levels found in the wild-type strain. These results establish that the PmrD protein requires a functional PmrA–PmrB system to exert its effect.

A pmrA constitutive mutant bypasses the requirement for pmrD in the low-magnesium activation of pbgP

Roland *et al*. (1993) described an allele of the *pmrA* gene, *pmrA505*, which confers heightened levels of polymyxin resistance to wild-type *Salmonella*. The *pmrA505* allele has a single nucleotide substitution, which results in an arginine to histidine substitution at amino acid 81 in the N-terminal response regulator domain of the PmrA protein (Roland *et al*., 1993).

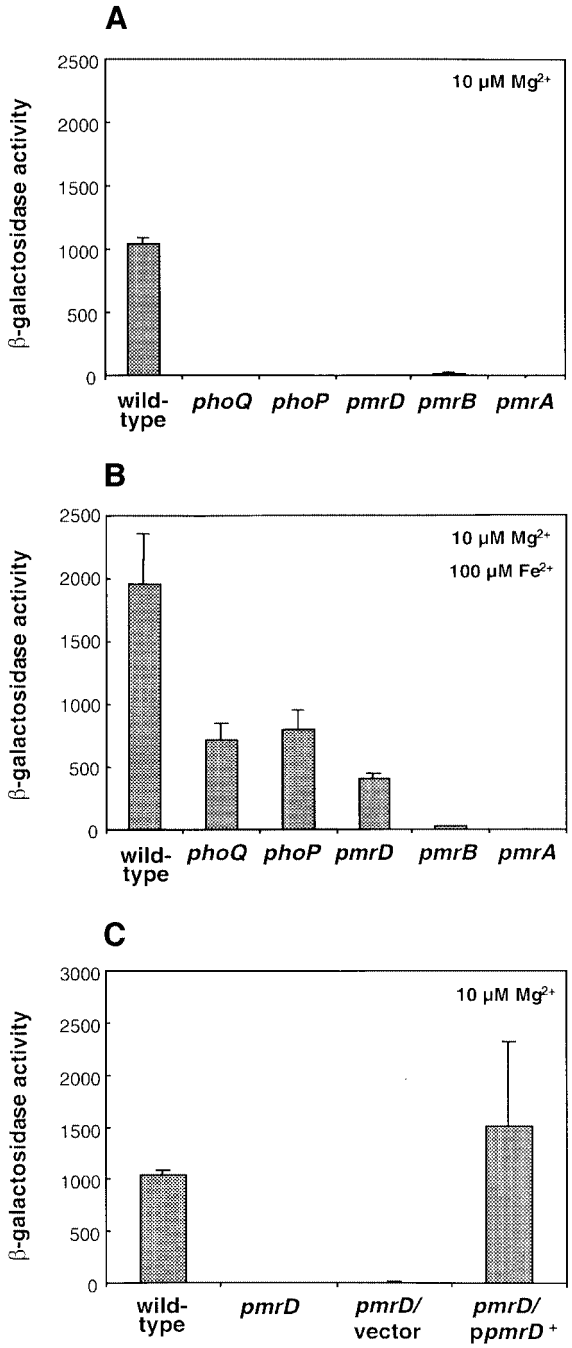

Fig. 4. PmrD is essential for transcription of the PmrA-activated gene *pbgP* during growth in low magnesium. Bacteria were grown in N-minimal medium pH 7.7 with 10 μM Mg^{2+} (A and C) or 10 μM Mg^{2+} and 100 μM Fe^{2+} (B). β-galactosidase activities (Miller units) were determined for mutants harboring a *lac* transcriptional fusion to *pbgP*. The transcriptional activity was determined in six genetic backgrounds: wild type, *phoQ*, *phoP*, *pmrD*, *pmrB* and *pmrA* (**A** and **B**) or wild-type and *pmrD* backgrounds for the complementation assay (**C**). The *pmrD*– strains used for this assay were transformed with a plasmid harboring the *pmrD* gene expressed from its own promoter (pLK23) or the cloning vector (pUC19). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are only shown if greater than the resolution of the figure).

We established that *pmrA505* is a constitutive allele of *pmrA*: transcription of the *pbgP* gene was reduced <20% under repressing magnesium and iron concentrations

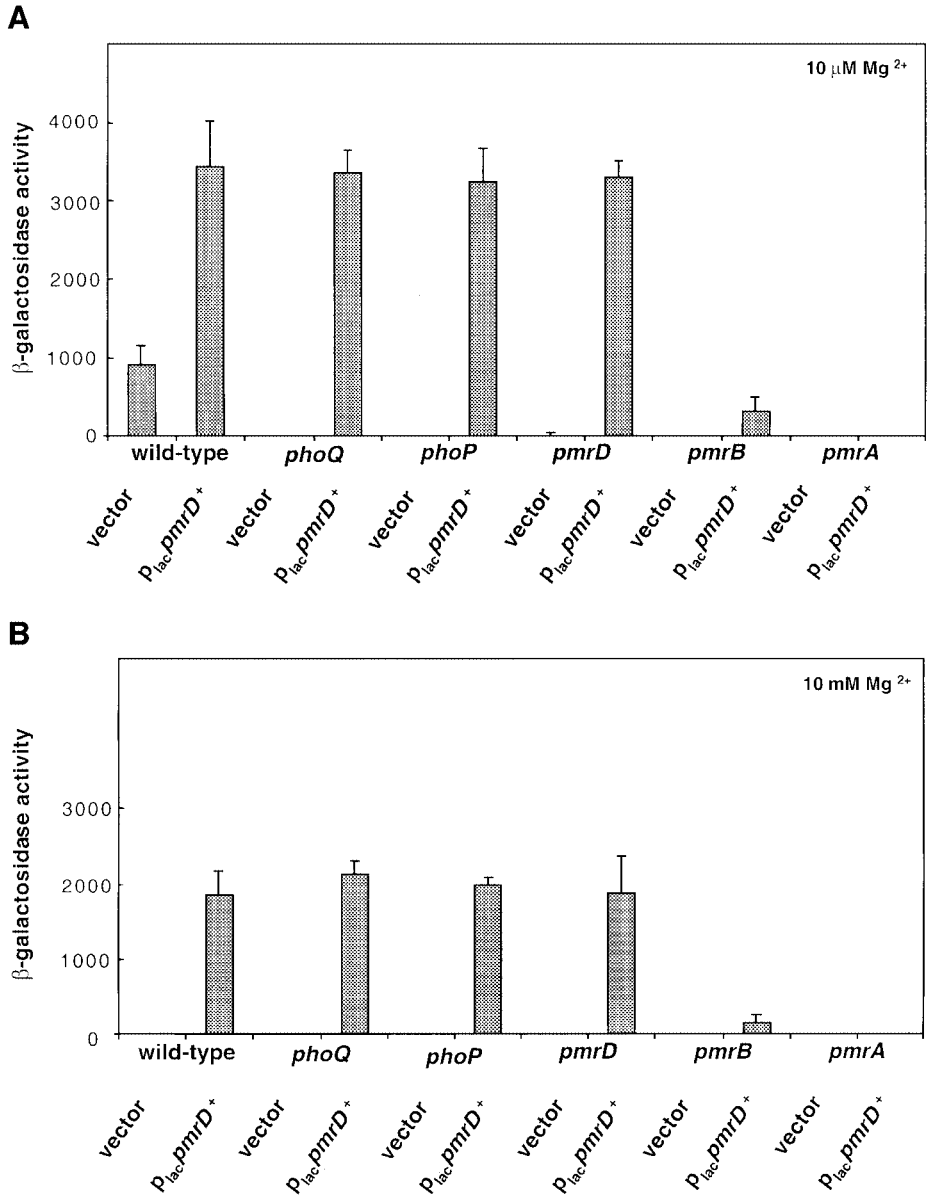

Fig. 5. PmrD acts downstream of the PhoP–PhoQ system and upstream of the PmrA–PmrB system. β-galactosidase activities (Miller units) expressed by transformants grown in N-minimal medium pH 7.7 with 10 μM Mg^{2+} (**A**) or 10 mM Mg^{2+} (**B**) were determined for mutants harboring a *lac* transcriptional fusion to *pbgP*. The transcriptional activity was determined in six genetic backgrounds: wild type, *phoQ*, *phoP*, *pmrD*, *pmrB* and *pmrA*. The strains were transformed with a plasmid harboring the *pmrD* gene expressed from the *lac* promoter (pLK24) or the cloning vector (pUC19). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are only shown if greater than the resolution of the figure).

(Figure 6), whereas identical growth conditions decreased *pbgP* expression >100-fold in an isogenic *pmrA*$^+$ strain (Figure 6). If the *pmrD* gene functions upstream of *pmrA*, *pbgP* transcription should be *pmrD* independent in a *pmrA505* mutant. Consistent with this notion, the *pmrD*::*cat* mutation hardly affected *pbgP* transcription in the *pmrA505* mutant regardless of the magnesium and iron concentrations (Figure 6).

PmrD controls the activity of the PmrA–PmrB system at a post-transcriptional level

The PmrD protein may function by regulating the transcription, stability and/or the activity of the PmrA and PmrB proteins. If the PmrD protein acts at a post-transcriptional level, a *pmrD* mutation should abolish transcription of PmrA-activated genes even if the *pmrAB* genes are transcribed from a heterologous promoter. We tested this hypothesis by examining *pbgP* transcription in a strain carrying a plasmid with the *pmrAB* genes under the control of a derivative of the *lac* promoter (this strain also harbored wild-type copies of the *pmrA* and *pmrB* genes at their normal chromosomal location). When grown in low magnesium, the *pmrAB*-expressing plasmid could not rescue the *pmrD* mutant: *pbgP* transcription was abolished as observed in the vector-carrying strain (Figure 7A). Control experiments demonstrated that the *pmrAB*-

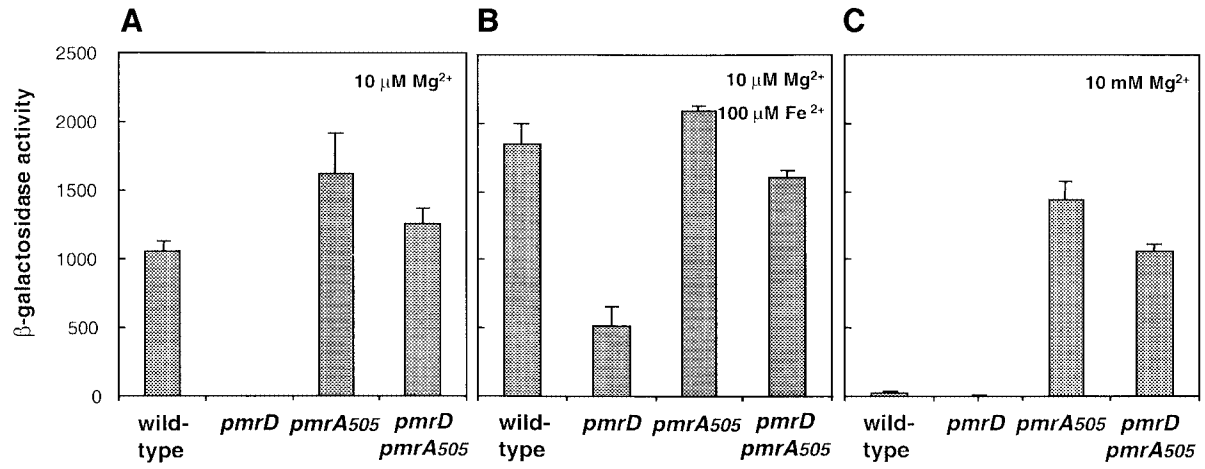

Fig. 6. Transcripton of *pbgP* in a strain grown in low magnesium expressing the constitutive *pmrA505* allele is independent of *pmrD*. β-galactosidase activities (Miller units) expressed by transformants grown in N-minimal medium pH 7.7 with 10 μM Mg^{2+} (**A**), 10 μM Mg^{2+} and 100 μM Fe^{2+} (**B**) or 10 mM Mg^{2+} (**C**) were determined for a mutant harboring a *lac* transcriptional fusion to *pbgP*. The transcriptional activity was investigated in four genetic backgrounds: wild type, *pmrD*, *pmrA505* and *pmrD pmrA505*. The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are only shown if greater than the resolution of the figure).

expressing plasmid could rescue the *pmrA* mutant, restoring wild-type levels of *pbgP* transcription (Figure 7A).

Mutation of the putative site of PmrA phosphorylation abolishes PmrD-mediated activation of pbgP

Response regulators are normally activated by phosphorylation, typically from their cognate sensor kinases. The PmrA protein is phosphorylated by the cytoplasmic domain of the PmrB protein (Wösten and Groisman, 1999) and mutation of the putative site of PmrA phosphorylation abolished the high-iron (PmrD-independent) activation of the *pbgP* gene (data not shown). Consistent with the notion that the low-magnesium (i.e. PmrD-mediated) activation of PmrA-regulated genes is a phosphorylation-dependent mechanism, a strain with the chromosomal copy of the *pmrA* gene inactivated and harboring a plasmid expressing a PmrA protein mutated in the putative phosphorylation site (i.e. D51A) failed to activate *pbgP* transcription whether bacteria were grown in low-magnesium (Figure 7B), high-magnesium or high-iron conditions (data not shown). In contrast, the isogenic $pmrA^+$-plasmid-containing strain exhibited a normal profile of *pbgP* transcription: high expression during growth in low magnesium (Figure 7B) or high iron and no expression in high-magnesium media (data not shown).

The pmrD gene is necessary for polymyxin resistance during growth in low magnesium but expendable during growth in high iron

It was originally reported that the *pmrD* gene confers polymyxin resistance when present in a medium-copy plasmid, but that a strain with a *tet* resistance cassette in the chromosomal copy of the *pmrD* gene displays wild-type levels of polymyxin resistance (Roland *et al*., 1994). We hypothesized that the inability to detect differences between wild-type and *pmrD Salmonella* might have been due to not growing the microorganism under conditions promoting expression of PmrA-activated genes (which were not known at that time). Thus, we performed polymyxin sensitivity assays with bacteria grown in defined media with pH 5.8 and 10 μM Mg^{2+}, a condition that promotes transcription of PmrA-activated genes and the phenotypic display of polymyxin resistance in wild-type *Salmonella* (Groisman *et al*., 1997). The *pmrD* mutant was >5000-fold more sensitive to polymyxin than the wild-type strain; this level of sensitivity is similar to that displayed by a *pmrA* null mutant and much higher than that exhibited by strains harboring mutations in the PmrA-activated genes *pbgP* and *ugd* (Figure 8A). The polymyxin sensitivity of the *pmrD* mutant was solely due to inactivation of *pmrD* because wild-type levels of polymyxin resistance could be restored to the *pmrD* mutant by plasmid pLK23, which harbors a wild-type copy of the *pmrD* gene (Figure 8C). These data demonstrate that *pmrD* is necessary for polymyxin resistance during growth in low magnesium.

Because growth in 100 μM iron restored *pbgP* transcription to the *pmrD* mutant (Figure 4B), we hypothesized that the *pmrD* mutant might become resistant to polymyxin if grown in the presence of 100 μM iron. Consistent with this notion, the *pmrD* mutant exhibited wild-type levels of polymyxin resistance when grown in N-minimal medium pH 5.8 and 10 μM Mg^{2+} supplemented with 100 μM iron (Figure 8B). In contrast, the *pmrA* mutant remained >5000-fold more sensitive to polymyxin than the wild-type strain (Figure 8B). Likewise, growth in 100 μM iron could not rescue polymyxin resistance in the *pbgP* and *ugd* mutants (Figure 8B). These results argue that the PmrD protein mediates polymyxin resistance only indirectly, by promoting transcription of PmrA-regulated genes during growth in low magnesium.

Discussion

Two-component systems are signal transduction machineries that promote changes in gene expression or bacterial behavior in response to distinct physical or chemical cues. Eubacterial species typically harbor several dozen two-

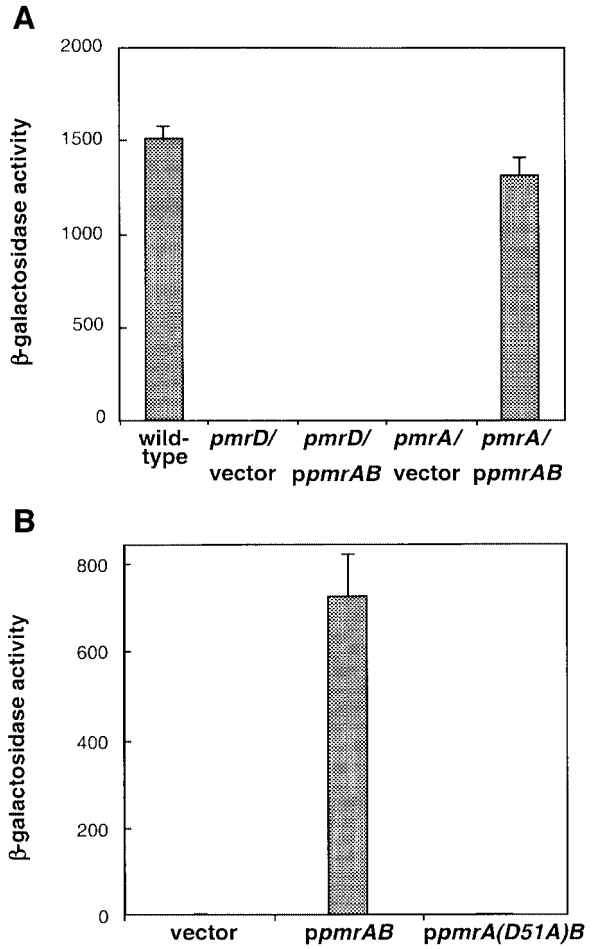

Fig. 7. PmrD activates the PmrA–PmrB system by a post-transcriptional mechanism. (**A**) Expression of the *pmrAB* genes from a heterologous promoter does not rescue *pbgP* transcripton in a *pmrD* mutant. β-galactosidase activities (Miller units) expressed by bacteria harboring a *lac* transcriptional fusion to the *pbgP* gene grown in N-minimal medium pH 7.7 with 10 μM Mg^{2+}. The transcriptional activity was investigated in three genetic backgrounds: wild type, *pmrD* and *pmrA*, the latter carrying either a plasmid with the *pmrAB* genes under the control of a derivative of the *lac* promoter or the plasmid vector. The data correspond to mean values of four independent experiments performed in duplicate. (**B**) *pmrD*-mediated activation is dependent on the putative site of PmrA phosphorylation. β-galactosidase activities (Miller units) expressed by bacteria harboring a *lac* transcriptional fusion to the *pbgP* gene grown in N-minimal medium pH 7.7 with 10 μM Mg^{2+}. The transcriptional activity was investigated in three genetic backgrounds: a *pmrA* null mutant harboring plasmid pEG9102 with the *pmrAB* genes under the control of a derivative of the *lac* promoter, plasmid pLK39 with the *pmrA*(*D51A*)*B* genes under the control of a derivative of the *lac* promoter or the plasmid vector. Similar results were obtained with plamids harboring only the *pmrA* gene under the control of a derivative of the *lac* promoter: the D51A mutation abolished *pbgP* transcription. The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are only shown if greater than the resolution of the figure).

component systems that exhibit exquisite specificity so that a sensor kinase only modulates the activity of its cognate response regulator. Yet, certain two-component systems appear to communicate with one another, allowing their regulated genes to respond to more than one cue (Wanner, 1992; Birkey *et al*., 1998). In this paper, we have described a novel type of interaction between a pair of two-component systems, PhoP–PhoQ and PmrA–PmrB, which allows *Salmonella* to regulate expression of antimicrobial resistance determinants in response to two different conditions: high iron and low magnesium.

Our results suggest the following model (Figure 1) for the transcription of PmrA-regulated genes: high extracellular iron is detected by the sensor protein PmrB (M.M.S.M.Wösten and E.A.Groisman, unpublished results), which promotes phosphorylation of the regulatory protein PmrA; phosphorylated PmrA binds to the promoter region of the *pbgP* and *pmrC* genes (Wösten and Groisman, 1999), thereby activating their transcription and resulting in the phenotypic display of polymyxin resistance. Mutants lacking functional *pmrA* or *pmrB* genes fail to express PmrA-activated genes regardless of the iron or magnesium concentrations (Figure 4) and are hypersensitive to polymyxin (Figure 8). The PhoP–PhoQ system is not necessary for the iron-mediated activation of the *pbgP* and *pmrC* genes (Figure 4), allowing for transcription of PmrA-regulated genes to occur independently of the PhoP–PhoQ system.

The *pbgP* and *ugd* genes are essential for growth in low-magnesium solid media (Groisman *et al*., 1997) and, not surprisingly, PmrA-regulated genes are transcriptionally induced also during growth in low magnesium in a PhoP–PhoQ-dependent manner (Figure 2). We suggest that this occurs by the PhoQ protein serving as a magnesium sensor (Waldburger and Sauer, 1996; García Véscovi *et al*., 1997) that modulates the ability of the PhoP protein to activate transcription of the *pmrD* gene (Figure 3), which harbors a sequence matching the consensus found as a direct repeat in the promoter region of several PhoP-activated genes in *E.coli* K-12 (Kato *et al*., 1999). Consistent with this notion (Figure 1), transcription of the *pmrD* gene from a heterologous promoter bypassed the requirement for low magnesium and functional *phoP* and *phoQ* genes in transcription of the *pbgP* gene (Figure 5). Moreover, it ruled out models involving activation via the PhoP protein binding to the *pbgP* promoter, the PhoQ protein serving as a phosphate donor for the PmrA protein, or the PhoP protein serving as a phosphate donor for the PmrB protein.

On the other hand, transcription of the *pmrD* gene from a heterologous promoter did not rescue *pbgP* transcription in *pmrA* or *pmrB* mutants (Figure 5), which argues against the PmrD protein being a transcription factor acting on the *pbgP* promoter independently of the PmrA protein. Moreover, cells harboring a constitutive allele of the *pmrA* gene (Roland *et al*., 1993) allowed *pbgP* transcription in a *pmrD*-independent manner (Figure 6), indicating that *pmrD* functions upstream of *pmrA*. This raised the possibility of the PhoP–PhoQ system controlling transcription of the *pmrAB* genes via the PmrD protein. For example, when phosphate levels are limiting, the PhoP–PhoR two-component system of *Bacillus subtilis* (which is unrelated to the *Salmonella* PhoP–PhoQ system) activates the ResD–ResE two-component system by the PhoP protein binding to the promoter region and activating transcription of the *resABCDE* operon (Birkey *et al*., 1998). While a role for PmrD in controlling *pmrAB* transcription can not be ruled out, our data show that the PmrD protein does have an effect at the post-transcriptional level: inactivation of *pmrD* prevented the

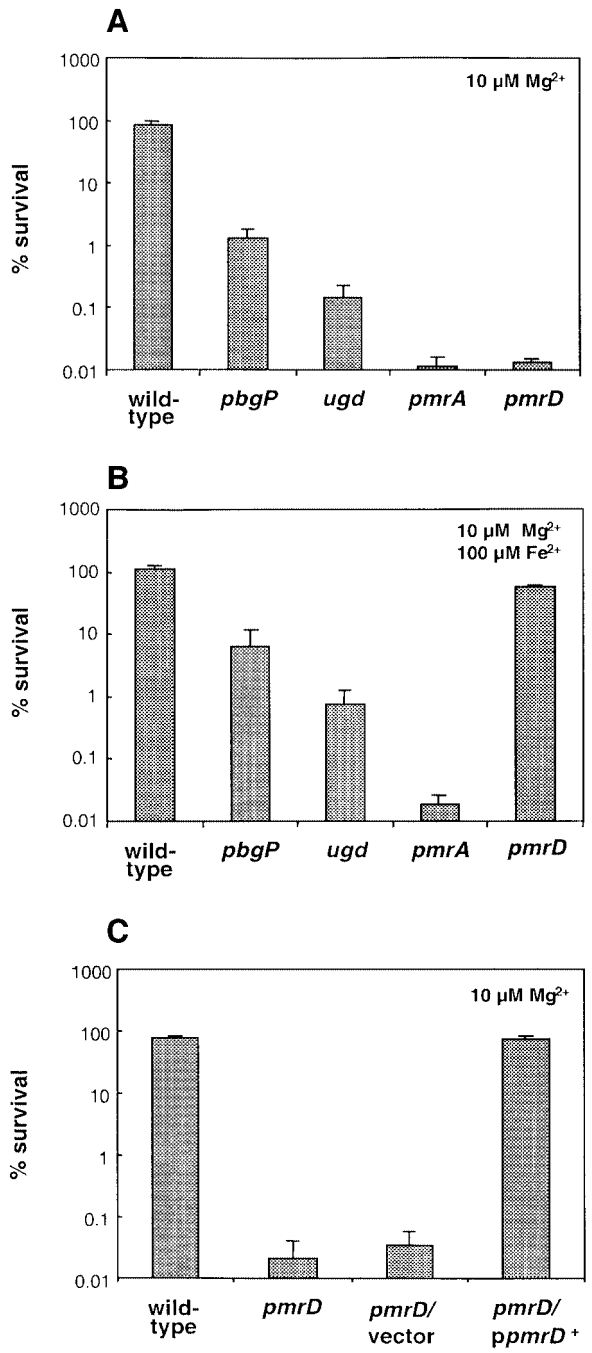

Fig. 8. The *pmrD* gene is required for polymyxin resistance during growth in low magnesium. Wild-type (14028s), *pbgP* (EG9241), *ugd* (EG9524), *pmrA* (EG7139) and *pmrD* (EG11491) bacteria were grown to logarithmic phase in N-minimal medium pH 5.8 with 10 μM Mg^{2+} (**A** and **C**) or 10 μM Mg^{2+} and 100 μM Fe^{2+} (**B**), washed, and incubated in the presence of polymyxin (a final concentration of 2.5 μg/ml) for 1 h at 37°C. Samples were diluted in phosphate-buffered saline and plated on LB-agar plates to assess bacterial viability. Survival values are relative to the original inoculum. Complementation experiments were carried out with the *pmrD* mutant transformed with a plasmid harboring the *pmrD* gene expressed from its own promoter (pLK23) or the cloning vector (pUC19) (C). Data correspond to mean values from three independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are only shown if bigger than the resolution of the figure).

low-magnesium activation of *pbgP* when the *pmrAB* genes were transcribed from a heterologous promoter (Figure 7A).

PmrD-mediated activation appears to be a phosphorylation-dependent mechanism because mutation of the putative site of PmrA phosphorylation abolished *pbgP* transcription (Figure 7B). Thus, the PmrD protein may function by increasing the levels of phosphorylated PmrA protein, which binds the promoters of the *pmrC* and *pbgP* genes with higher affinity than unphosphorylated PmrA (Wösten and Groisman, 1999). This could be achieved by promoting the phosphorylation of the PmrA protein and/or by inhibiting its dephosphorylation. Increased PmrA phosphorylation may result from the PmrB protein adopting an active conformation during growth in high iron or in the presence of the PmrD protein, conditions that promote *pbgP* expression in a PmrB-dependent manner (Figure 4; M.M.S.M.Wösten and E.A.Groisman, unpublished results). An active PmrB conformation may have higher autokinase activity and/or be a better phosphate donor for the PmrA protein. Alternatively, or in addition, PmrD might inhibit an autophosphatase activity of PmrA or a PmrA phosphatase activity of PmrB. For example, certain phosphorylated response regulators exhibit autophosphatase activity (e.g. P-CheY, P-CheB, P-NR_I, P-PhoB; Stock *et al*., 1989), whereas others are dephosphorylated by their cognate sensor kinases. For example, the sensor EnvZ of *E.coli* catalyzes both phosphorylation of the response regulator OmpR and dephosphorylation of phosphorylated OmpR (Igo *et al*., 1989). The PmrD protein could also antagonize the activity of a yet to be described PmrA phosphatase, such as PrpA, which *in vivo* exhibits histidine and/or aspartyl phosphatase activity towards the CpxA–CpxR two-component system of *E.coli* (Missiakas and Raina, 1997), or SixA, which *in vitro* displays histidine phosphatase activity towards the histidine-containing phosphotransfer domain of the ArcB sensor of *E.coli* (Ogino *et al*., 1998).

The 85 amino acid PmrD protein has no homologs in the sequence databases, and thus it does not resemble the kinases or the phosphatase inhibitors controlling the two-component phosphorelay that governs sporulation in *B.subtilis* (Fabret *et al*., 1999). Yet, the phosphatase inhibitor NH_4VO_3 could rescue *pbgP* transcription in *phoP*, *phoQ* and *pmrD* mutants (to 25% of wild-type levels), but not in *pmrA* or *pmrB* mutants (our unpublished results). This effect appears to be specific to PmrA-activated genes because NH_4VO_3 could not rescue transcription of PhoP-activated genes, which are PmrA independent. While this result is consistent with PmrD being a phosphatase inhibitor, we can not exclude other possible roles for NH_4VO_3, such as affecting the cellular ATP pools, thereby impacting on the ability of the PmrB protein to become phosphorylated.

PmrD is the first example of a protein mediating the activation of a two-component system by another two-component system at a post-transcriptional level. Thus, PmrD-mediated activation is different from classical cascades in which a two-component system activates a second two-component system by the response regulator of the first system binding to the promoter and promoting transcription of the genes encoding the second two-component system (Birkey *et al*., 1998; Lee *et al*., 2000). Moreover, unlike the well characterized kinases and phosphatases controlling the onset of sporulation in *B.subtilis*, which channel different inputs into a single

phosphorelay system (Perego and Hoch, 1996), PmrD connects two distinct two-component systems (PhoP–PhoQ and PmrA–PmrB) that can be activated independently of one another.

It was originally reported that a medium-copy-number plasmid harboring the *pmrD* gene conferred polymyxin resistance in a *pmrA*-dependent manner, but that a *Salmonella pmrD* mutant retained wild-type levels of polymyxin resistance (Roland *et al*., 1994). Our experiments provide a molecular interpretation for these seemingly contradictory results: when bacteria are grown in low-magnesium media (to promote expression of PmrA-activated genes) the *pmrD* mutant is >5000-fold more sensitive to polymyxin than wild-type *Salmonella* (Figure 8A). Roland *et al*. (1994) grew the bacteria under non-inducing conditions, and thus could only detect differences in polymyxin resistance when the *pmrD* gene was expressed from a plasmid vector. Likewise, the *pmrA* dependence of *pmrD*-mediated polymyxin resistance (Roland *et al*., 1994) reflects the fact that the PmrD

Table I. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
S.enterica serovar *typhimurium*		
14028s	wild type	Fields *et al.* (1986)
AA3007	*polA2 ara-9*	Whitfield and Levine (1973)
MS7953s	*phoP7953*::Tn*10*	Fields *et al.* (1989)
MS5996s	*phoQ5996*::Tn*10*	Fields *et al.* (1989)
EG11491	*pmrD1*::*cat*	this work
EG7139	*pmrA1* ::*cat*	Soncini and Groisman (1996)
EG10056	Δ*pmrB*::*cat*	this work
EG9241	*pbgP1*::MudJ	Soncini *et al.* (1996)
EG9168	*pbgP1*::MudJ *phoP7953*::Tn*10*	Soncini *et al.* (1996)
EG10236	*pbgP1*::MudJ *phoQ5996*::Tn*10*	this work
EG11775	*pbgP1*::MudJ *pmrD*::*cat*	this work
EG9813	*pbgP1*::MudJ *pmrA1*::*cat*	this work
EG10065	*pbgP1*::MudJ Δ*pmrB*::*cat*	this work
EG9524	*ugd* (*pbgC*)*9228*::MudJ	García Véscovi *et al.* (1996)
EG9526	*ugd9228*::MudJ *phoP7953*::Tn*10*	García Véscovi *et al.* (1996)
EG11773	*ugd9228*::MudJ *pmrD1*::*cat*	this work
EG9811	*ugd9228*::MudJ *pmrA1*::*cat*	this work
EG9521	*mgtA9226*::MudJ	García Véscovi *et al.* (1996)
EG9523	*mgtA9226*::MudJ *phoP7953*::Tn*10*	García Véscovi *et al.* (1996)
EG11777	*mgtA9226*::MudJ *pmrD1*::*cat*	this work
EG11779	*mgtA9226*::MudJ *pmrA1*::*cat*	this work
EG9527	*mgtC9232*::MudJ	García Véscovi *et al.* (1996)
EG9529	*mgtC9232*::MudJ *phoP7953*::Tn*10*	García Véscovi *et al.* (1996)
EG11781	*mgtC9232*::MudJ *pmrD1*::*cat*	this work
EG11783	*mgtC9232*::MudJ *pmrA1*::*cat*	this work
EG10627	*pcgL9331*::MudJ	Soncini *et al.* (1996)
EG10745	*pcgL9331*::MudJ *phoP7953*::Tn*10*	Soncini *et al.* (1996)
EG11789	*pcgL9331*::MudJ *pmrD1*::*cat*	this work
EG11791	*pcgL9331*::MudJ *pmrA1*::*cat*	this work
EG9460	*psiD9065*(*pmrC*)::MudJ	García Véscovi *et al.* (1996)
EG9280	*psiD9065*(*pmrC*)::MudJ *phoP7953*::Tn*10*	García Véscovi *et al.* (1996)
EG11785	*psiD9065*(*pmrC*)::MudJ *pmrD1*::*cat*	this work
EG11787	*psiD9065*(*pmrC*)::MudJ *pmrA1*::*cat*	this work
EG9888	*pbgP1*::MudJ *zjd*::Tn*10d*-Cam	Groisman *et al.* (1997)
EG9868	*pbgP1*::MudJ *pmrA505 zjd*::Tn*10d*-Cam	Groisman *et al.* (1997)
EG12115	*pbgP1*::MudJ *pmrD*::*cat zjd*::Tn*10d*-Cam	this work
EG12117	*pbgP1*::MudJ *pmrA505 pmrD*::*cat* zjd::Tn*10d*-Cam	this work
E.coli		
DH5α	*sup*E44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Hanahan (1983)
Plasmids		
pKRP10	rep_{pMB1} Ap^r Cm^r	Reece and Phillips (1995)
pUC19	rep_{pMB1} *lacZa* Ap^R $lacI^q$	Yanisch-Perron *et al.* (1985)
pLK4	rep_{pMB1} Ap^r *pbgP/E pmrD*	this work
PLK7	rep_{pMB1} Ap^r *pbgP/EΔpbgP3 pmrD*	this work
PLK10	rep_{pMB1} Ap^r *pbgP/EΔpbgP3 pmrD*::*cat*	this work
pLK38	rep_{pMB1} *lacZa*- Ap^R *pmrA(D51A)B*	this work
pLK23	rep_{pMB1} Ap^r *pmrD*	this work
pLK24	rep_{pMB1} *lacZa* Ap^r $laqI^q$ *pmrD*	this work
pUHE21-2laqI^q	rep_{pMB1} Ap^r $lacI^q$	Soncini *et al.* (1995)
pEG9102	rep_{pMB1} Ap^r $lacI^q$ *pmrAB*	Soncini and Groisman (1996)
pLK39	rep_{pMB1} Ap^R $lacI^q$ *pmrA(D51A)B*	this work

[a]Gene designations are as described (Sanderson *et al*., 1995).

protein acts upstream of the PmrA–PmrB system (Figures 5 and 6). Finally, our data show that the *pmrD* mutant exhibits wild-type levels of polymyxin resistance when grown in the presence of iron (Figure 8B). This indicates that the PmrD protein mediates polymyxin resistance only indirectly, by promoting transcription of PmrA-regulated genes during growth in low magnesium.

While the experiments described in this paper were carried out in *Salmonella*, the PmrD activation mechanism is likely to operate in other species because homologs of the *phoP*, *phoQ*, *pmrA*, *pmrB* and *pmrD* genes have been detected in at least five Gram-negative species (Groisman *et al*., 1989; our unpublished results). Moreover, the *pmrD* gene of *E.coli* K-12 fully rescued a *pmrD* mutant of *Salmonella*, indicating that the *E.coli pmrD* gene is functional and has a similar role in both enteric species (our unpublished results).

What is the physiological significance of our findings? When enteric bacteria experience low-magnesium environments, the PhoP–PhoQ system becomes activated, resulting in the expression of several proteins including the MgtA and MgtB Mg^{2+} transporters (Soncini *et al*., 1996). PhoP also promotes PmrD expression, which activates the PmrA–PmrB system and results in the synthesis of PmrA-regulated genes, some of which are necessary for growth in low-magnesium media (Groisman *et al*., 1997). However, the PmrA–PmrB system can also be activated by high iron in a mechanism that is PhoP-, PhoQ- and PmrD-independent (M.M.S.M.Wösten and E.A.Groisman, unpublished results), consistent with PmrA-activated genes being required for growth in high iron (M.M.S.M.Wösten and E.A.Groisman, unpublished results). This physiological plasticity allows the independent activation of two distinct two-component regulatory systems in response to a broader spectrum of environmental cues.

Materials and methods

Bacterial strains, plasmids, recombinant molecular techniques and growth conditions

Bacterial strains and plasmids used in this study are listed in Table I. Mutants were constructed by phage P22-mediated transductions as described elsewhere (Davis *et al*., 1980). Construction of the Δ*pmrB*::*cat* mutant strain will be described elsewhere. Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al*., 1989). Bacteria were grown at 37°C in Luria broth (LB) or in a modified N-minimal medium containing 0.1% casamino acids and 38 mM glycerol (Snavely *et al*., 1991) 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. $FeSO_4$ was used at a final concentration of 0.1 mM from a freshly prepared 0.1 M stock solution. Kanamycin was used at a final concentration of 50 μg/ml, chloramphenicol at 25 μg/ml, tetracycline at 10 μg/ml, ampicillin at 50 μg/ml and polymyxin B (6000 U/mg) (Sigma) at 2.5 μg/ml.

Construction of plasmids

Plasmid pLK4, containing the complete *pbgP/E* and *pmrD* loci, was constructed by simultaneous ligation of a 4.5 kb *Sph*I–*Kpn*I fragment from *pbgP/E* plasmid pML3 and a 4.2 kb *Kpn*I–*Pvu*II fragment from *pbgP/E* plasmid pML1 into the *Pvu*II-digested plasmid pUC19 (Yanisch-Perron *et al*., 1985). Plasmids pML1 and pML3 were identified by screening a Mud5005-generated library of *Salmonella* for clones hybridizing to PCR-generated probes corresponding to sequences immediately adjacent to the MudJ insertions in the *pbgP1*::MudJ and *pbgE1*::MudJ mutants (Soncini *et al*., 1996). Plasmid pLK7 was constructed by deleting the third open reading frame in the *pbgP/E* operon (*pbgP3*) in plasmid pLK4 by digesting with *Age*I and *Hin*dIII, filling in the 5′ overhanging ends with T4 DNA polymerase (Gibco-BRL) and ligating to the pUC19 vector. This plasmid was designated pLK7. The nucleotide sequence of the *Age*I–*Hin*dIII junction was checked by sequence analysis on a ABI Prism® 310 Genetic Analyzer (Perkin Elmer) using the ABI Prism® Big Dye®Terminator Cycle Sequencing Ready Reaction Kit. Plasmid pLK10 was constructed by inserting the 0.8 kb *cat* *Eco*RI fragment from plasmid pKRP10 (Reece and Phillips, 1995) into the unique *Eco*RI site of plasmid pLK7 within the *pmrD* open reading frame. Digestion of the plasmid with *Sca*I showed that the *cat* gene was present in the opposite orientation of the *pmrD* gene.

Plasmid pLK23 contains the *pmrD* gene expressed from its own promoter and was constructed by digesting plasmid pLK15 DNA with *Nde*I and *Bam*HI, repairing the overhanging ends with T4 DNA polymerase and ligation. Plasmid pLK15 is a derivative of plasmid pLK7 in which a *Bam*HI site was created between *pmrD* and the seventh open reading frame of the *pbgP/E* operon (*pbgE3*). To construct pLK15, we simultaneously ligated four fragments: the 0.8 kb *cat* fragment from plasmid pKRP10, a PCR fragment generated with primers 647 (5′-TGGCTGGGGTTACTGCCTGG-3′) and 1028 (5′-CGCGGATCCTCATGATCTGGCGGGCAG-3′) and plasmid pLK4 DNA as template and digested with *Apa*I and *Bam*HI, a second PCR product generated with primers 1029 (5′-CCCGGATCCTCATGATGGCTTCGCCGTCA-3′) and 641 and plasmid pLK4 DNA as template and digested with *Eco*RI and *Bam*HI, and plasmid pLK7 digested with *Apa*I–*Eco*RI. Digestion of pLK15 with *Sca*I showed that the *cat* gene is inserted with the same orientation as the *pmrD* gene. Nucleotide sequence analysis showed that the PCR-generated DNAs in pLK15 had a wild-type sequence.

Plasmid pLK24 was constructed by cloning between the *Xba*I and *Kpn*I sites of pUC19 a PCR fragment containing the *pmrD*-coding region generated with primers 1090 (5′-GCTCTAGAAACGGGGGCGCTATGG-3′) and 1089 (5′-GGGGTACCAGATCATGATGGCTTGC-3′) and *Salmonella* chromosomal DNA as template, and digested with *Xba*I and *Kpn*I. Nucleotide sequence analysis showed that the PCR-generated DNAs in pLK24 had a wild-type sequence.

Plasmid pLK39, a pEG9102 derivative containing a mutation in the putative phosphorylation site of PmrA (D51A), was constructed as follows. First, we performed an inverse PCR with primers 1185 (5′-CTAGCTAGCTTTAGGGCTGCCCGATGAG-3′) and 1186 (5′-CTAGCTAGCACCATCAGACTGTAATG-3′) and a pUC19 derivative harboring the *pmrAB* genes as template. After digestion of the PCR product with *Nhe*I and self-ligation, the ligation mixture was used to transform *E.coli* DH5α cells. Ampicillin-resistant transformants were screened by colony PCR with primers 351 (5′-AAGGATCCAGGAGACTAAGCG-3′) and 352 (5′-GGCAAGCTTAGCTTTCCTCAG-3′) and digestion of the generated PCR product with *Nhe*I. One of the plasmids that could be digested with *Nhe*I was designated pLK38. Nucleotide sequencing confirmed the presence of wild-type *pmrAB* sequence except in the predicted mutated codon. A *Bam*HI–*Bsu*36I fragment from plasmid pLK38 harboring the mutant portion of the *pmrA* gene was ligated to pEG9102 DNA that had been digested with *Bam*HI and *Bsu*36I. After transformation of *E.coli* DH5α cells, transformants were screened for the presence of the mutation by colony PCR with primers 351 and 352. One of the plasmids that could be digested with *Nhe*I was designated pLK39. Western blot analysis with anti-PmrA antibodies showed similar steady-state levels of $PmrA^+$ and PmrA(D51A) proteins.

Construction of chromosomal pmrD mutant

The *pmrD*::*cat* mutation in pLK10 was transferred to the chromosome of *S.enterica* serovar *typhimurium* strain 14028s as described previously (Groisman *et al*., 1993). One of eight transductants analyzed harbored a wild-type *pbgP3* gene as tested by colony PCR using primers 655 (5′-GCCGTGCGCCGCTAAACTGG-3′) and 587 (5′-CCGCGATATCGACGCTACGC-3′). The structure of the disrupted *pmrD* gene in this transductant, designated EG11491, was confirmed by colony PCR using primers 1001 (5′-CTGTCTAGACTGCCCGCCAGATCATGA-3′) and 641, and further verified by Southern hybridization of chromosomal DNA digested with *Nsi*I, *Hph*I and *Hph*I–*Apa*I double digests using *pmrD*- and *cat*-specific probes. The *pmrD* probe was the *Kpn*I–*Xba*I fragment from plasmid pLK24. The *cat* probe corresponds to the 0.8 kb *Eco*RI fragment from pKRP10.

Polymyxin resistance and β-galactosidase assays

One-hour polymyxin sensitivity assays were performed as described previously (Groisman *et al*., 1997). β-galactosidase activity was determined as described elsewhere (Miller, 1972).

S1 nuclease assay

The S1 nuclease protection assay was performed as described previously (García Véscovi *et al*., 1996) with RNA harvested from mid-exponential phase cultures (OD_{600} 0.4–0.6) grown in 50 ml N-minimal medium pH 7.7, containing either 10 mM $MgCl_2$, 10 μM $MgCl_2$ or 10 μM $MgCl_2$ and 0.1 mM $FeSO_4$. Total RNA was isolated with Trizol (Gibco-BRL) according to the manufacturer's specifications. A PCR product generated with primers 1064 (5′-GCCCTCTTTTTGACATAATG-3′) and 641 (5′-CGTGCCGGTAGAAGATAAAG-3′) and *Salmonella* chromosomal DNA as template was used as probe. This probe was labeled at the 5′ end by phosphorylation with [γ-^{32}P]ATP using T4 polynucleotide kinase (Gibco-BRL). In brief, total RNA (50 μg) and the labeled DNA probe were mixed in 50 μl of hybridization buffer (80% formamide, 20 mM HEPES pH 6.5, 0.4 M NaCl). The mixture was incubated at 75°C for 10 min and then left to cool down in an incubator at 37°C overnight. After adding 220 μl of H_2O, 30 μl of 10× S1 nuclease buffer (0.3 M sodium acetate pH 4.5, 0.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol), the mixture was treated with 10 U of S1 nuclease (Promega) for 30 min. The reaction was stopped by the addition of 300 μl of phenol–chloroform, and the aqueous phase was precipitated with ethanol. The precipitate was dissolved in sequence loading buffer and electrophoresed on a 6% acrylamide–7 M urea gel together with a sequence ladder initiated with primer 1064 using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham).

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