Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor

Akinori Kato and Eduardo A. Groisman¹

Department of Molecular Microbiology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110, USA

A fundamental question in signal transduction is how an organism integrates multiple signals into a cellular response. Here we report the mechanism by which the *Salmonella* PmrA/PmrB two-component system responds to the signal controlling the PhoP/PhoQ two-component system. We establish that the PhoP-activated PmrD protein binds to the phosphorylated form of the response regulator PmrA, preventing both its intrinsic dephosphorylation and that promoted by its cognate sensor kinase PmrB. This results in PmrA-mediated transcription because phosphorylated PmrA exhibits higher affinity for its target promoters than unphosphorylated PmrA. A PmrD-independent form of the PmrA protein was resistant to PmrB-catalyzed dephosphorylation and promoted transcription of PmrA-activated genes in the absence of inducing signals. This is the first example of a protein that enables a two-component system to respond to the signal governing a different two-component system by protecting the phosphorylated form of a response regulator.

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The two-component system is the most prevalent form of signal transduction enabling bacteria to alter cellular behavior in response to environmental cues (Hoch and Silhavy 1995; Inouye and Dutta 2003). Typically, a two-component system consists of a sensor histidine kinase that responds to specific signals by altering the phosphorylated state of a cognate response regulator protein. Binding of a specific ligand promotes conformational changes in the sensor protein that can potentially alter three enzymatic activities: autophosphorylation from ATP, transfer of the phosphoryl group to its cognate regulator, and dephosphorylation of the phosphorylated cognate regulator (Stock et al. 2000). The vast majority of response regulators are DNA-binding transcription factors whose affinities for their target promoters are modulated by phosphorylation. Thus, by altering the phosphorylated state of a response regulator, a signal often modifies the gene expression profile of an organism.

The PmrA/PmrB two-component system of *Salmonella enterica* serovar Typhimurium governs inducible

resistance to the antibiotic polymyxin B (Groisman et al. 1997; Wösten et al. 2000). The PmrA/PmrB system independently responds to two signals: Fe³⁺, which is sensed by the cognate sensor PmrB (Wösten et al. 2000), and low Mg²⁺, which is sensed by the noncognate sensor PhoQ (Fig. 1; García Vescovi et al. 1996). The low Mg²⁺ activation not only requires the PhoQ protein but also its cognate response regulator PhoP, the PhoP-activated *pmrD* gene, as well as the PmrA and PmrB proteins (Kox et al. 2000). Expression of the *pmrD* gene from a heterologous promoter bypasses the requirement for the PhoP and PhoQ proteins and for the low Mg²⁺ signal, but not for the PmrA and PmrB proteins, indicating that the role of the PhoP/PhoQ system in the low Mg²⁺ activation of PmrA-regulated genes is solely to promote expression of the *pmrD* gene, and that the *pmrD* gene acts downstream of the PhoP/PhoQ system but upstream of (or at the same level as) the PmrA/PmrB system. The PmrD protein appears to control the PmrA/PmrB system at a posttranscriptional level, because expression of PmrA-activated genes was abolished in a *pmrD* mutant experiencing low Mg²⁺ even when the *pmrAB* genes were transcribed from the *lac* promoter (Kox et al. 2000).

In contrast to the low Mg²⁺ activation, Fe³⁺-promoted transcription of PmrA-regulated genes requires neither

¹Corresponding author.

E-MAIL groisman@borcim.wustl.edu; FAX (314) 747-8228.

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Figure 1. Model illustrating the regulatory interactions between the PhoP/PhoQ and PmrA/PmrB two-component systems and the PmrD protein. The PmrA protein promotes transcription of the *pbgP* gene during growth in low Mg^{2+} via the PhoP/PhoQ system, which turns on expression of the PhoP-activated PmrD protein, which, in turn, activates the PmrA/PmrB system at a posttranslational level. The PmrB protein responds to Fe^{3+} by activating the PmrA protein independently of the PhoP/PhoQ system and PmrD protein, which promotes transcription of the *pbgP* gene and represses transcription of the *pmrD* gene.

the PhoP/PhoQ system nor the *pmrD* gene, but it is dependent on the periplasmic Fe^{3+} -sensing domain of the PmrB protein (Wösten et al. 2000). The *pmrD* gene is intimately interwoven into the circuitry of the PhoP/PhoQ and PmrA/PmrB systems, because *pmrD* transcription is not only activated in low Mg^{2+} in a PhoP/PhoQ-dependent manner (Kox et al. 2000), but it is also subjected to negative feedback regulation by the PmrA/PmrB system in response to Fe^{3+} (Fig. 1; Kato et al. 2003). This indicates that *Salmonella* tightly regulates PmrD levels, possibly to prevent accumulation of activated PmrA protein.

In this study, we determined the mechanism by which the PmrD protein activates the PmrA protein, which expands the environments where *Salmonella* can express its polymyxin B resistance determinants. Using a series of in vitro and in vivo experiments, we establish that the PmrD protein specifically targets phosphorylated PmrA, protecting it from PmrB-promoted dephosphorylation. The PmrD protein provides the first example of a physiological connection between two-component regulatory systems resulting from protection of dephosphorylation of a response regulator.

Results

PmrD activates the PmrA/PmrB system at a posttranslational level

The PmrA/PmrB system is encoded by the *pmrCAB* operon (Roland et al. 1993) and transcribed from two promoters: a PmrA-activated promoter located upstream of the *pmrC* gene and a constitutive promoter present within the *pmrC* coding region (Fig. 2A; Gunn and Miller 1996; Soncini and Groisman 1996; Wösten and Groisman 1999; Lee et al. 2004). We reasoned that a *pmrD*

mutation might reduce the levels of the PmrA and PmrB proteins during growth in low Mg^{2+} because the PmrD protein activates the PmrA/PmrB system (Kox et al. 2000) and because activated PmrA positively regulates transcription of *pmrCAB* operon. Indeed, lower amounts of both PmrA and PmrB proteins were produced by a *pmrD* null mutant than by the isogenic wild-type strain grown in low Mg^{2+} (Fig. 2B). Although these results demonstrated that a *pmrD* mutation does affect the levels of the PmrA and PmrB proteins, they suggested that a potential posttranslational activity of PmrD on the PmrA

Figure 2. The PmrD protein activates the PmrA/PmrB system at a posttranslational level. (A) Genetic organization of the *pmrCAB* operon. The blue arrow indicates transcription from a promoter that is positively autoregulated by the PmrA/PmrB system; the black arrow denotes a constitutive promoter that produces basal levels of PmrA and PmrB proteins even under noninducing conditions. The red box indicates the location of the MudJ transposon insertion in strains EG9460 and EG11785. (B) Western blot analysis of extracts prepared from wild type (14028s), *pmrD* (EG11491), *pmrC::MudJ* (EG9460), *pmrC::MudJ pmrD* (EG11785), *pmrC::MudJ* (EG9460) harboring pUH-*pmrAB*, and *pmrC::MudJ pmrD* (EG11785) harboring pUH-*pmrAB* strains grown in N-minimal medium at pH 7.7 with 10 mM Mg^{2+} (H) or 10 μ M Mg^{2+} (L) detected with anti-PmrB_c (upper panel) or anti-PmrA (lower panel) antibodies. (C) β -galactosidase activity (Miller units) from a *pmrC-lac* transcriptional fusion expressed by bacteria grown in N-minimal medium at pH 7.7 with 10 mM Mg^{2+} (solid bar) or 10 μ M Mg^{2+} (open bar) was determined in *pmrC::MudJ* (EG9460), *pmrC::MudJ pmrD* (EG11785), *pmrC::MudJ* (EG9460) harboring pUH-*pmrAB*, and *pmrC::MudJ pmrD* (EG11785) harboring pUH-*pmrAB* strains. The data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are only shown if greater than the resolution of the figure).

and/or PmrB proteins might be masked by PmrA/PmrB autogenous regulation.

To explore whether the PmrD protein affects the PmrA/PmrB system posttranslationally, we examined isogenic *pmrD* strains expressing constant low levels of the PmrA and PmrB proteins (Fig. 2B) due to the presence of a MudJ transposon insertion within the *pmrC* gene that blocks transcription from the PmrA-activated promoter upstream of the *pmrC* gene but does not affect the constitutive *pmrAB* promoter (Fig. 2A). Although inactivation of the *pmrD* gene had no effect on the levels of PmrA and PmrB proteins produced by the *pmrC::MudJ* strains (Fig. 2B), it abolished transcription of the PmrA-activated *pmrC* gene (Fig. 2C). Furthermore, even when the *pmrC::MudJ* strains produced constant high levels of PmrA and PmrB proteins due to the presence of a plasmid with the *pmrAB* genes under the control of the *lac* promoter (Fig. 2B), *pmrC* transcription was strictly dependent on the presence of a functional *pmrD* gene (Fig. 2C). These results indicate that the PmrD protein activates the PmrA/PmrB system at a posttranslational level.

PmrD-dependent and -independent pathways use the same promoters to transcribe *PmrA*-activated genes

We mapped the transcription start sites for the PmrA-activated *pbpG* (Supplementary Fig. S1A) and *pmrC* (Supplementary Fig. S1B) promoters in isogenic *pmrD* strains experiencing single activation signals (i.e., low Mg^{2+} and no Fe^{3+} or high Mg^{2+} and high Fe^{3+}). S1 products of the same size were obtained with RNA from wild-type bacteria, indicating that the same promoters are used in both activation conditions. As expected, these products were absent from the *pmrD* mutant when the inducing condition was low Mg^{2+} , and from both wild-type and *pmrD* bacteria grown in high Mg^{2+} (Supplementary Fig. S1A,B), a condition that activates neither the PhoP/PhoQ nor the PmrA/PmrB systems (García Vescovi et al. 1996; Wösten et al. 2000). These results indicate that PmrD-promoted transcription of PmrA-activated genes does not involve a change in promoter specificity.

The *PmrD* protein promotes phosphorylation of the *PmrA* protein

We hypothesized that the PmrD protein might promote the phosphorylated state of the PmrA protein because PmrA binds to its target promoters with higher affinity when phosphorylated (Wösten and Groisman 1999), and because PmrD-mediated activation is abolished upon mutation of the putative site of PmrA phosphorylation (i.e., D51; Kox et al. 2000). After the PmrA protein was mixed with the phosphorylated cytoplasmic domain of the PmrB protein (i.e., phospho-PmrB_c), rapid phosphotransfer to the PmrA protein was observed (Fig. 3A, lanes 1–6), in agreement with previous results (Wösten and Groisman 1999). The D51 residue is essential for PmrA phosphorylation because phosphotransfer from

Figure 3. The PmrD protein specifically promotes the phosphorylated state of the PmrA protein by inhibiting dephosphorylation of phospho-PmrA catalyzed by the PmrB protein. (A) Phosphotransfer assay from phospho-PmrB_c to the PmrA and PmrAD51A proteins was performed with 5 μ M of regulator (PmrA and PmrAD51A) and 2.5 μ M of PmrB_c proteins. (B) Kinase/phosphatase assay of the PmrB_c and PmrA proteins was performed in the presence (PmrD) or absence (–) of the PmrD protein. The concentrations of the PmrA, PmrB_c, and PmrD proteins were 5, 2.5, and 5 μ M, respectively. The small cationic proteins RNase A and cytochrome C were used as negative controls at a final concentration of 5 μ M. (C) Phosphatase assay of phospho-PmrA protein was performed as follows: ³²P-labeled phosphorylated GST-PmrB_cT156R beads were used as phospho-donor for the PmrA protein and removed before the phosphatase assay. The concentrations of the PmrA, histidine kinase (i.e., PmrB_c, PmrB_cT156R), and PmrD proteins were 2.5, 5, and 2.5 μ M, respectively. The top band corresponds to phospho-PmrB_cT156R or phospho-PmrB_c due to reverse-phosphorylation from phospho-PmrA. (D) Autokinase assay of the PmrB_c protein was performed in the presence (PmrD) or absence (–) of the PmrD protein. The concentrations of the PmrB_c and PmrD proteins were 2.5 and 5 μ M, respectively. (E) Kinase/phosphatase assay of the PmrB_cT156R and PmrA proteins was performed in the presence (PmrD) or absence (–) of the PmrD protein. The concentrations of the PmrA, PmrB_cT156R, and PmrD proteins were 5, 2.5, and 5 μ M, respectively. Samples were analyzed by 10% SDS-PAGE.

phospho-PmrB_c was abolished if the PmrA protein was replaced with the PmrAD51A protein (Fig. 3A, lanes 7–11). (We used the purified PmrB_c protein in our experiments because bacteria expressing PmrB_c recapitulate the PmrD-dependent activation of PmrA-regulated genes displayed by the full-length PmrB protein; Supplementary Fig. S2.)

The PmrD protein promoted higher levels of PmrA phosphorylation when incubated with PmrA, PmrB_c,

and ATP (Fig. 3B). Two types of controls indicate that PmrD specifically acts on the PmrA/PmrB system: First, the small (85 amino acid) and basic (pI, 9.5) PmrD could not be replaced by other small basic proteins, such as RNase A and cytochrome C, to promote the phosphorylated state of the PmrA protein (Fig. 3B). And second, PmrD had no effect on the phosphorylation of YgiX and YgiY (data not shown), which are the two-component system proteins exhibiting the highest identity to the *Salmonella* PmrA and PmrB proteins, respectively. The PmrD protein affects phosphorylation but not stability of the PmrA protein, because the same levels of PmrA protein were present when incubations were carried out in the presence/absence of PmrD (data not shown). These results demonstrate that the PmrD protein promotes the phosphorylated state of the PmrA protein, and that protein components other than PmrB_c are not required for the PmrD effect.

The PmrD protein inhibits PmrB-promoted dephosphorylation of phospho-PmrA

The PmrD protein may favor phosphorylation of the PmrA protein by promoting autokinase and/or PmrA phosphotransferase activities of PmrB_c (Fig. 3A), and/or by inhibiting a putative phosphatase activity of PmrB_c towards phospho-PmrA. To test the latter possibility, we first determined that the phosphoryl group in phospho-

PmrA was stable for at least 4 h in the absence of PmrB_c (Fig. 3C, lanes 1–4), but that it was rapidly lost when PmrB_c was present (Fig. 3C, lanes 8–10). Then, we established that the PmrD protein inhibited the PmrB_c-promoted dephosphorylation of phospho-PmrA (Fig. 3C, lanes 11–13). On the other hand, PmrD affected neither the ability of PmrB_c to autophosphorylate from ATP (Fig. 3D) nor the levels of phospho-PmrA in incubations carried out with PmrB_cT156R as phosphodonor (Fig. 3E), a mutant PmrB_c lacking phospho-PmrA phosphatase activity (Fig. 3C, lanes 5–7) that enabled us to examine the phosphotransfer reaction. Cumulatively, these results establish that PmrB_c has phospho-PmrA phosphatase activity and that PmrD can prevent the PmrB-promoted dephosphorylation of phospho-PmrA.

The PmrD protein binds to phospho-PmrA, inhibiting its dephosphorylation

The PmrD protein may inhibit dephosphorylation of phospho-PmrA by targeting PmrB_c and/or by directly binding to phospho-PmrA, protecting it from PmrB_c's phosphatase activity. We established that the PmrD protein specifically immunoprecipitated phospho-PmrA, as it could not precipitate the phospho-YgiX response regulator or the phospho-PmrB_c protein (Fig. 4A). PmrD appears to interact exclusively with the phosphorylated form of PmrA, because no precipitation was detected

Figure 4. The PmrD protein interacts with the N-terminal domain of phospho-PmrA in a phosphorylation-dependent manner. (A) Immunoprecipitation of PmrD-Flag protein complexed with phospho-PmrA. Regulator (PmrA and YgiX), sensor (PmrB_c and YgiY_c), and PmrD-Flag proteins were incubated with ATP for 16 h before immunoprecipitation (pre-IP). The PmrD-Flag protein was precipitated from the phosphorylation reaction mixture using anti-Flag beads (IP). (B) Immunoprecipitation of the PmrD-Flag protein complexed with phospho-PmrA after phosphorylation with [γ -³²P] ATP. PmrA, sensor (PmrB_c and PmrB_cT156R), and PmrD-Flag proteins were incubated with [γ -³²P] ATP for 4 h before immunoprecipitation (pre-IP). PmrD-Flag protein was precipitated from the phosphorylation reaction mixture using anti-Flag beads (IP). Coimmunoprecipitated PmrA protein retains phosphoryl group. (C) Immunoprecipitation of the PmrD-Flag protein complexed with phospho-PmrA-YgiX. Chimeric regulator (PmrA-YgiX and YgiX-PmrA), sensor (PmrB_c and YgiY_c), and PmrD-Flag proteins were incubated with ATP for 16 h before immunoprecipitation (pre-IP). The PmrD-Flag protein was precipitated from the phosphorylation reaction mixture using anti-Flag beads (IP).

when ATP or PmrB_c was omitted from the reaction (Fig. 4A). This was confirmed by conducting PmrA phosphorylation with [γ -³²P] ATP using either PmrB_c or the phosphatase-defective PmrBT156R_c and detecting the immunoprecipitated phospho-PmrA by autoradiography (Fig. 4B). These results demonstrate that the PmrD protein specifically binds to the phospho-PmrA protein.

Consistent with the notion that PmrD specifically targets phospho-PmrA, we determined that the PmrD protein inhibited the spontaneous dephosphorylation of phospho-PmrA (i.e., in the absence of PmrB_c; Fig. 5A) but had no effect on the dephosphorylation of phospho-YgiX (Fig. 5B). Because GST-PmrB_cT156R was used as phosphodonor for the PmrA protein in these experiments, it was possible that the PmrD protein was inhibiting a phospho-PmrA phosphatase activity mediated by trace amounts of GST-PmrB_cT156R protein (despite such putative activity being undetectable in our assays; Fig. 3C). To rule out this possibility, we repeated the experiment using PmrA protein that had been phosphorylated with the noncognate sensor GST-YgiY_c, which does not exhibit phosphatase activity towards phospho-PmrA (data not shown). The PmrD protein still inhibited the spontaneous dephosphorylation of phos-

pho-PmrA under conditions in which there was no PmrB_c protein and where none of the proteins used in the reaction had been in contact with PmrB (Fig. 5C). These experiments establish that the PmrD protein binds to the phospho-PmrA protein, inhibiting its dephosphorylation.

The PmrD protein targets the N-terminal domain of phospho-PmrA

To identify the domain of the PmrA protein targeted by PmrD, we investigated the ability of the PmrD protein to inhibit dephosphorylation of two chimeric proteins: one consisting of the N-terminal response regulator domain of the PmrA protein fused to the C-terminal DNA-binding domain of the YgiX protein, and the other harboring the N-terminal response regulator domain of the YgiX protein fused to the C-terminal DNA-binding domain of the PmrA protein. The PmrD protein inhibited dephosphorylation of the chimera harboring the N-terminal domain of the PmrA protein, but had no effect on the dephosphorylation of the chimera harboring the N-terminal domain of the YgiX protein, regardless of the presence of sensor proteins (i.e., PmrB_c or YgiY_c) in the

Figure 5. The PmrD protein specifically inhibits dephosphorylation of phospho-PmrA by targeting its N-terminal region. (A) Spontaneous dephosphorylation of phospho-PmrA protein in the presence (PmrD) or absence (–) of the PmrD protein: ³²P-labeled phosphorylated GST-PmrB_cT156R beads were used as phosphodonor for the PmrA protein and removed before the assay. The concentrations of the PmrA and PmrD proteins were 2.5 μ M. (B) Spontaneous dephosphorylation of phospho-YgiX protein was performed in the presence (PmrD) or absence (–) of the PmrD protein. ³²P-labeled phosphorylated GST-YgiY_c beads were incubated with the YgiX protein for 1 h to produce phospho-YgiX protein and removed before the assay. The concentrations of the YgiX and PmrD proteins were 2.5 μ M. (C) Spontaneous dephosphorylation of phospho-PmrA protein was performed in the presence (PmrD) or absence (–) of PmrD protein. All proteins used in this assay were not in contact with PmrB or derivatives, because they were independently overexpressed in *E. coli* strain EG13796, which lacks the entire *pmrAB* homolog *basRS*, and purified as described in Materials and Methods. ³²P-labeled phosphorylated GST-YgiY_c beads were

used as phosphodonor for the PmrA protein and removed before the assay. The concentration of the PmrA and PmrD proteins was 2.5 μ M. (D) Quantitative analysis of the phosphatase assay of phospho-PmrA protein. ³²P-labeled phosphorylated GST-YgiY_c beads were used as phosphodonor for the PmrA protein and removed before the assay. The concentrations of the PmrA, PmrB_c, and PmrD proteins were 2.5, 5, and 2.5 μ M, respectively. The small cationic proteins RNase A and cytochrome C were used as negative controls at a final concentration of 2.5 μ M. (E) Quantitative analysis of the phosphatase assay of phospho-YgiX protein. ³²P-labeled phosphorylated GST-PmrB_cT156R beads were incubated with the YgiX protein for 45 min to produce phospho-YgiX protein and removed before the assay. The concentrations of YgiX, YgiY_c, and PmrD proteins were 2.5, 5, and 2.5 μ M, respectively. (F) Quantitative analysis of the phosphatase assay of phospho-PmrA-YgiX protein. ³²P-labeled phosphorylated GST-YgiY_c beads were incubated with the PmrA-YgiX protein for 2 h to produce phospho-PmrA-YgiX protein and removed before the assay. The concentrations of the PmrA-YgiX, PmrB_c, and PmrD proteins were 2.5, 5, and 2.5 μ M, respectively. (G) Quantitative analysis of the phosphatase assay of phospho-YgiX-PmrA protein. ³²P-labeled phosphorylated GST-PmrB_cT156R beads were incubated with the YgiX-PmrA protein for 2 h to produce phospho-YgiX-PmrA protein and removed before the assay. The concentrations of the YgiX-PmrA, YgiY_c, and PmrD proteins were 2.5, 5 and 2.5 μ M, respectively. Samples were analyzed by 10% SDS-PAGE and quantified as described in Materials and Methods.

reaction (Fig. 5D–G). In agreement with these results, the PmrD protein immunoprecipitated phospho-PmrA-YgiX but not phospho-YgiX-PmrA (Fig. 4C). Cumulatively, these findings demonstrate that PmrD prevents dephosphorylation of phospho-PmrA by targeting its N-terminal region, which harbors the putative phosphorylation site (D51) that is required for PmrD-mediated activation of the PmrA protein in vivo (Kox et al. 2000) and for phosphotransfer from phospho-PmrB_c in vitro (Fig. 3A).

A model for PmrD action

The biochemical experiments described above suggest the following model for the PmrD-mediated activation of the PmrA/PmrB system: The PmrB protein continuously autophosphorylates from ATP and transfers the phosphoryl group to the PmrA protein (Fig. 3A). However, in the absence of its specific signal (i.e., Fe³⁺), the PmrB protein dephosphorylates phospho-PmrA. The low Mg²⁺ signal activates the PhoP/PhoQ system (García Vescovi et al. 1996), which promotes expression of the PmrD protein (Kox et al. 2000), which, in turn, binds phospho-PmrA (Fig. 4A,B) by targeting its N-terminal domain (Figs. 4C, 5D–G) and protects it from PmrB-promoted dephosphorylation (Figs. 3C, 5D,F). This promotes binding of phospho-PmrA to its target promoters to regulate gene transcription (Wösten and Groisman 1999). The model predicts that expression of PmrA-activated genes should still occur in the absence of the PmrD

protein in strains with either a defective PmrB protein lacking phosphatase activity or harboring a mutant PmrA that is resistant to PmrB-promoted dephosphorylation. Below, we describe genetic experiments that test these predictions.

A PmrB protein defective in phospho-PmrA phosphatase activity allows transcription of PmrA-activated genes independently of the PmrD protein

Because PmrB_c catalyzes the dephosphorylation of phospho-PmrA (Figs. 3C, 5D), it is likely that PmrD binding to phospho-PmrA is designed to prevent PmrB from dephosphorylating phospho-PmrA in vivo. Thus, we examined transcription of the PmrA-activated *pmrC* gene in isogenic *pmrD* strains deleted for the *pmrB* gene and harboring a plasmid expressing either wild-type PmrB or the mutant PmrBT156R, which, in vitro, lacks phospho-PmrA phosphatase activity (Fig. 3C). Consistent with our previous results (Kox et al. 2000), transcription of the *pmrC* gene was *pmrD*-dependent when the bacteria expressing the wild-type PmrB protein were grown in low Mg²⁺ but *pmrD*-independent when the inducing condition was Fe³⁺ (Fig. 6A,B). In contrast, transcription of the *pmrC* gene was *pmrD*-independent under all conditions in bacteria expressing the phosphatase-defective PmrBT156R protein (Fig. 6A,B). As expected, there was no *pmrC* expression when bacteria expressing wild-type PmrB were grown in the absence of inducing signals (i.e.,

Figure 6. Transcription of PmrA-activated genes is rendered *pmrD*-independent upon inactivation of the phosphatase activity of the PmrB protein or in a PmrA mutant protein resistant to PmrB_c's phosphatase activity. (A) β-galactosidase activity (Miller units) from a *pmrC-lac* transcriptional fusion expressed by bacteria grown in N-minimal medium at pH 7.7 with 10 mM Mg²⁺ (solid bars), 10 μM Mg²⁺ (hatched bars), or 10 μM Mg²⁺ and 100 μM Fe²⁺ (open bars) was determined in a *pmrB* strain (EG14087) harboring pCCR9 (vector), pCCR9-*pmrB* (*ppmrB*), or pCCR9-*pmrBT156R2* (*ppmrBT156R*). (B) β-galactosidase activity (Miller units) from a *pmrC-lac* transcriptional fusion expressed by bacteria grown in N-minimal medium at pH 7.7 with 10 mM Mg²⁺ (solid bars), 10 μM Mg²⁺ (hatched bars), or 10 μM Mg²⁺ and 100 μM Fe²⁺ (opened bars) was determined in the *pmrB pmrD* strain EG14090 harboring pCCR9 (vector), pCCR9-*pmrB* (*ppmrB*), or pCCR9-*pmrBT156R2* (*ppmrBT156R*). (C) β-galactosidase activity (Miller units) from a *phgP-lac* transcriptional fusion expressed by bacteria grown in N-minimal medium at pH 7.7 with 10 mM Mg²⁺ (solid bars) or 10 μM Mg²⁺ (opened bars) was determined in wild-type (EG9888), *pmrB* (EG13735), *pmrA505* (EG9868), or *pmrA505*

and *pmrB* (EG13641) strains. The data correspond to mean values of three independent experiments performed in duplicate. Error bars show standard deviation (and are only shown if greater than the resolution of the figure). (D) Quantitative analysis in the phosphatase assay of phospho-PmrA and phospho-PmrA505 proteins was performed as follows: ³²P-labeled phosphorylated GST-PmrB_cT156R beads were used as phosphodonor for the PmrA and PmrA505 proteins and removed before the assay. The concentrations of the PmrA, PmrA505, PmrB_c, and PmrD proteins were 2.5, 2.5, 5, and 2.5 μM, respectively. Samples were analyzed by 10% SDS-PAGE.

high Mg^{2+} and no Fe^{3+}) or in strains harboring the plasmid vector. These results support the notion that PmrD is required to antagonize PmrB-promoted dephosphorylation of phospho-PmrA.

A PmrD-independent allele of the PmrA protein is resistant to PmrB-promoted dephosphorylation

Salmonella strains with the PmrA505 protein (Roland et al. 1993) transcribe PmrA-activated genes even in a *pmrD* mutant and under repressing Mg^{2+} concentrations (Kox et al. 2000). This could be due to the PmrA505 protein adopting a conformation that mimics phospho-PmrA and thus no longer requiring phosphorylation for activation. Alternatively, the PmrA505 protein, which has the R81H substitution in the N-terminal response regulator domain, may depend on phosphorylation to activate transcription, but it may be resistant to PmrB-promoted dephosphorylation. To distinguish between these two possibilities, we examined transcription of the PmrA-activated *pbpP* gene in isogenic *pmrB* strains that expressed either wild-type PmrA or PmrA505 proteins. Transcription of the *pbpP* gene was dependent on a functional *pmrB* gene in the *pmrA505* strain as it is in a *pmrA*⁺ strain (Fig. 6C). These results indicate that the *pmrA505* allele is not truly constitutive because it requires the PmrB protein to promote *pbpP* transcription in vivo.

To investigate whether the PmrA505 protein is resistant to PmrB-promoted dephosphorylation, we purified this mutant protein, phosphorylated it with GST-PmrB_cT156R, and incubated it in the presence of PmrB_c. The phospho-PmrA505 protein displayed resistance to PmrB_c-promoted dephosphorylation (Fig. 6D), although its intrinsic dephosphorylation was similar to that of phospho-PmrA (Fig. 6D). Moreover, PmrD could still inhibit the dephosphorylation of phospho-PmrA505 (Fig. 6D). These results imply that the *pmrA505* strain expresses PmrA-activated genes under noninducing conditions (i.e., high Mg^{2+} and no Fe^{3+}) because it can resist PmrB's phosphatase activity.

Discussion

We have uncovered the mechanism by which the PmrA/PmrB two-component system can respond to the low Mg^{2+} signal controlling the PhoP/PhoQ two-component system. We established that the PhoP-activated PmrD protein promotes the phosphorylated state of the response regulator PmrA by binding phospho-PmrA at the N-terminal domain, which inhibits both its intrinsic dephosphorylation and that catalyzed by its cognate sensor PmrB. Because phospho-PmrA binds to its target promoters with higher affinity than unphosphorylated PmrA (Wösten and Groisman 1999), transcription of PmrA-activated genes is promoted in the presence of PmrD.

The PmrB protein appears to phosphorylate the PmrA protein independently of the absence/presence of signals, because even when the ability of the PmrB protein to

dephosphorylate phospho-PmrA is blocked, either by the T156R mutation in PmrB or the R81H mutation in PmrA, significant transcription of the PmrA-activated genes is observed in high Mg^{2+} (Fig. 6A,C). This PmrB activity is responsible for generating phospho-PmrA, which constitutes the target of the PmrD protein (Fig. 4), and explains the requirement for the PmrB protein in the PmrD-mediated activation of the PmrA protein that takes place in low Mg^{2+} (Fig. 6C; Kox et al. 2000). Moreover, by interacting with phospho-PmrA (and not with unphosphorylated PmrA), the PmrD protein specifically inhibits the dephosphorylation of phospho-PmrA catalyzed by PmrB without affecting the phosphotransfer reaction from phospho-PmrB to PmrA.

The PmrD-mediated protection of a phosphorylated response regulator from dephosphorylation by its cognate sensor kinase constitutes a unique regulatory strategy in two-component signal transduction. By targeting phospho-PmrA, the PmrD protein expands the environments where PmrA-activated genes (including those mediating resistance to polymyxin B) are expressed without interfering with PmrB's autokinase activity (Fig. 3D) or PmrB's phosphotransferase activity to PmrA (Fig. 3E). This is in contrast to previously described activities affecting two-component system proteins. For example, the decision of *Bacillus subtilis* to sporulate is modulated by two families of phosphatases (i.e., Rap and Spo0E) acting on the response regulators Spo0F and Spo0A (Perego et al. 1994; Perego and Hoch 1996; Perego 1998) and by inhibitors of the sensor kinase KinA (i.e., KipI and Sda; Wang et al. 1997; Burkholder et al. 2001; Rowland et al. 2004), which altogether provide multiple checkpoints for the sporulation phosphorelay. Likewise, when *Escherichia coli* experiences high cytoplasmic levels of glutamine, the PII protein interacts with the histidine kinase NRII, which decreases NRII's autokinase activity and promotes its phosphatase activity towards its cognate regulator NRI. This results in lower levels of phospho-NRI and leads to reduced transcription of the glutamine synthetase *glnA* gene (Atkinson et al. 1994; Jiang et al. 1998; Jiang and Ninfa 1999; Pioszak et al. 2000). Thus, in contrast to the Rap, Spo0E, KipI, Sda, and PII proteins, which integrate signals by functioning as Boolean NOT operators, the PmrD protein expands the conditions in which a two-component system operates by functioning as an OR operator (Bijlsma and Groisman 2003).

The protective activity of the PmrD protein is reminiscent of that displayed by certain members of the 14–3–3 protein family in eukaryotes in that both modify cellular behavior by protecting the phosphorylated state of certain proteins (Tzivion and Avruch 2002). However, whereas 14–3–3 proteins recognize particular phosphoserine- and phosphothreonine-containing domains (Yaffe et al. 1997; Obsil et al. 2001; Yaffe and Smerdon 2001), the PmrD protein specifically targets the N-terminal domain of PmrA, which harbors the aspartate residue (D51) that is the predicted site of phosphorylation (Fig. 3A). Basic residues in the high-pI PmrD protein may participate in the recognition of the phosphoaspartate in PmrA

Regulatory networks, pathways, and proteins themselves are frequently constructed in a cassette-like fashion using domains that mediate molecular interactions or have distinct enzymatic activities [Pawson and Nash 2003]. Signal transduction in eukaryotes usually involves kinases phosphorylating tyrosine, serine, or threonine residues, which are recognized in certain amino acid contexts by interaction modules such as SH2, PTB, FHA, WW domains and 14-3-3 proteins that have been used repeatedly to regulate different aspects of cellular organization [Yaffe and Smerdon 2001; Pawson and Nash 2003]. Two-component signal transduction, which is present in all domains of life and is the dominant form that bacteria use to respond to environmental stress, relies on sensor kinases that autophosphorylate on a histidine residue and modify the activity of regulatory proteins by phosphorylation of an aspartate residue. The activity of the PmrD protein uncovered in this study demonstrates that bacteria have the means of interconnecting signal transduction pathways in a phosphorylation-dependent manner.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella enterica* serovar Typhimurium strains are derived from wild-type strain 14028s. Phage P22-mediated transductions were performed as described (Davis et al. 1980). Bacteria were grown at 37°C in Luria-Bertani broth (LB; Sambrook et al. 1989) or in N-minimal medium at pH 7.7 (Snavelly et al. 1991) supplemented with 0.1% Casamino Acids, 38 mM glycerol, 10 µM or 10 mM MgCl₂, and 100 µM FeSO₄ or 100 µM FeCl₃. Ampicillin and kanamycin were used at 50 µg/mL, tetracycline at 12.5 µg/mL, and chloramphenicol at 20 µg/mL.

Plasmid constructions

Plasmid pTYB11-PmrD-Flag encoding the PmrD protein fused to a self-cleavable Intein tag at the N terminus and a Flag epitope tag at the C terminus was constructed by cloning between the Sapl and PstI sites of plasmid pTYB11 (New England Biolabs) a PCR fragment containing the *pmrD* coding region and a Flag epitope tag generated with primers 1706 (5'-GGTGGT GCTCTTCCAACATGGAATGGTTGGTTA-3') and 1752 (5'-AACTGCAGTCACTGTGCATCGTCGTCCTTGTAGTCTG ATGGCTTGC GCGTCAAC-3') and pLK4 as template, and digested with Sapl and PstI.

Plasmid pUH-*ygiXY*, containing the coding regions of the YgiX and YgiY proteins, was constructed by cloning between the BamHI and SalI sites of plasmid pUHE21-2*lacI^q* a PCR fragment generated with primers 1575 (5'-GAGGATCCGATGC GAATTTTACTG-3') and 1606 (5'-AAGCTCGAGTTACCAA CTTACTACG-3') and 14028s genomic DNA as template, and digested with BamHI and XhoI.

Plasmid pCCR9-*pmrBT156R2* was constructed by cloning between the BamHI and HindIII sites of plasmid pCCR9 a 1.8-kb BamHI–HindIII fragment containing the *pmrBT156R* coding region derived from plasmid pCCR9-*pmrBT156R* (Kato et al. 2003).

Plasmid pT7-7-PmrAD51A-His6 is a pT7-7-PmrA-His6 derivative (Wösten and Groisman 1999) that encodes a mutant PmrA protein harboring the D51A substitution in the putative phosphorylation site of the PmrA protein. The plasmid encoding this mutant PmrA protein was constructed as follows: A PCR fragment was amplified using primers 2453 (5'-GAGGA TCCATATGAAGATACTGATTG-3') and 2545 (5'-GGGCAG CCCTAACGCCAGCACCATCAG-3') and pUH-*pmrAB* (Soncini and Groisman 1996) as template. Another PCR fragment was amplified using primers 1121 (5'-CTGATGGTGCTGGC GTTAGGGCTGCCC-3') and 2454 (5'-TCCAAGCTTAGTGG TGGTGGTGGTGGTGGCTTTCCTCAGTGGCAACC-3') and pUH-*pmrAB* as template. After digestion with DpnI to eliminate the original pUH-*pmrAB* DNA, these two fragments were annealed to each other and amplified using primers 2453 and 2454. The resulting PCR fragment was digested with NdeI and HindIII and ligated to pT7-7 plasmid DNA that had been digested with NdeI and HindIII.

Plasmid pT7-7-PmrA505-His6 is a pT7-7-PmrA-His6 derivative (Wösten and Groisman 1999) that harbors the *pmrA505* allele, which encodes a PmrA protein with the R81H substitution that confers high levels of expression of PmrA-activated genes in LB (Roland et al. 1993) and in N-minimal medium at pH 7.7 with 10 mM Mg²⁺ (Kox et al. 2000) conditions. It was constructed by cloning between the NdeI and HindIII sites of pT7-7 a PCR fragment containing the *pmrA505* coding region amplified using primers 2453 and 2454 and EG9492 (Groisman et al. 1997) genomic DNA as template, and digested with NdeI and HindIII.

Plasmid pT7-7-YgiX-His6 encodes a YgiX protein with His6 tag at the C-terminal end. It was constructed by cloning between the NdeI and SalI sites of pT7-7 plasmid a PCR fragment generated with primers 2395 (5'-GAGGATCCATATGCGAA TTTTACTGGTAGAAGATG-3') and 2432 (5'-GCGCTCGAG TCAGTGGTGGTGGTGGTGGTGTGCGTCACCCAGGGTG TA-3') and pUH-ygiXY as template, and digested with NdeI and XhoI.

Plasmid pT7-7-AX-His6 encodes a chimeric protein whose N-terminal receiver domain and C-terminal DNA-binding domain are derived from the PmrA and YgiX proteins, respectively. This protein also harbors a C-terminal His6 tag. The plasmid encoding this chimera was constructed as follows: A PCR fragment was amplified using primers 2453 and 2340 (5'-GCGCAATTCGCTACTGGCCTGGTTATTATGGCGGCGCAGC-3') and pUH-*pmrAB* (Soncini and Groisman 1996) as template. Another PCR fragment was amplified using primers 2339 (5'-CTGCGCCGCCATAATAACGAGGCCAGTAGCGAATTGCGCC-3') and 2432 and pUH-*ygiXY* as template. After digestion with DpnI to eliminate the original plasmid DNAs, both fragments were annealed to each other and amplified using primers 2453 and 2432. The resulting PCR fragment was digested with NdeI and XhoI and ligated to pT7-7 plasmid DNA that had been digested with NdeI and SalI.

Plasmid pT7-7-XA-His6 encodes a chimeric protein whose N-terminal receiver domain and C-terminal DNA-binding domain are derived from the YgiX and PmrA proteins, respectively. This protein also harbors a C-terminal His6 tag. The plasmid encoding this chimera was constructed in a manner analogous to that used to make plasmid pT7-7-AX-His6 using primers 2454, 2341 [5'-GGTACGCCGCGCCAGCGGTCAGG GTGAAAGTGAAGTACG-3'], 2342 [5'-CGTCAGTTCACT

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Reference or source
<i>S. enterica</i> serovar Typhimurium		
14028s	Wild type	Fields et al. 1986
EG10056	$\Delta pmrB::Cm^R$	Kox et al. 2000
EG11491	$pmrD::Cm^R$	Kox et al. 2000
EG10065	$pbpP1::MudJ \Delta pmrB::Cm^R$	Kox et al. 2000
EG12060	$pbpP1::MudJ pmrD::Cm^R \Delta pmrB::Cm^R$	Kox et al. 2000
EG9492	$pmrA505 zjd::Tn10d-Cam$	Groisman et al. 1997
EG9888	$pbpP1::MudJ zjd::Tn10d-Cam$	Groisman et al. 1997
EG13732	$\Delta pmrB::Km^R$	This work
EG13733	$\Delta pmrB::Km^R zjd::Tn10d-Cam$	This work
EG13734	$\Delta pmrB zjd::Tn10d-Cam$	This work
EG13735	$pbpP1::MudJ \Delta pmrB zjd::Tn10d-Cam$	This work
EG9868	$pbpP1::MudJ pmrA505 zjd::Tn10d-Cam$	Groisman et al. 1997
EG13831	$pmrA505 \Delta pmrB zjd::Tn10d-Cam$	This work
EG13641	$pbpP1::MudJ pmrA505 \Delta pmrB zjd::Tn10d-Cam$	This work
EG9460	$pmrC::MudJ$	Kox et al. 2000
EG11785	$pmrC::MudJ pmrD::Cm^R$	Kox et al. 2000
EG14087	$pmrC::MudJ \Delta pmrB::Cm^R$	This work
EG14089	$\Delta pmrD$	This work
EG14090	$pmrC::MudJ \Delta pmrB::Cm^R \Delta pmrD$	This work
<i>E. coli</i>		
DH5 α	F ⁻ <i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan 1983
ER2566	<i>fhuA2</i> [<i>lon</i>] <i>ompT</i> <i>lacZ::T7 gene1 gal</i> <i>sulA11</i> Δ (<i>mcrC-mrr</i>)114::IS10 R(<i>mcr-73::miniTn10-Tet^S</i>) 2 R(<i>zgb-210::Tn10-Tet^S</i>) <i>endA1</i> [<i>dcm</i>]	New England Biolabs
EG13796	ER2566 Δ <i>basRS::Cm^R</i>	This work
Plasmid		
pKD3	rep _{R6Kγ} Ap ^R FRT Cm ^R FRT	Datsenko and Wanner 2000
pKD46	rep _{pSC101} ts Ap ^R P _{araBAD} γ β exo	Datsenko and Wanner 2000
pCP20	rep _{pSC101} ts Ap ^R Cm ^R <i>cl857</i> λ P _R <i>flp</i>	Cherepanov and Wackernagel 1995
pUHE21-2 <i>locI^q</i>	rep _{pMB1} Ap ^R <i>lacI^q</i>	Soncini et al. 1995
pUH- <i>pmrAB</i>	rep _{pMB1} Ap ^R <i>lacI^q</i> <i>pmrAB</i> (pEG9102)	Soncini and Groisman 1996
pUH- <i>pmrB</i>	rep _{pMB1} Ap ^R <i>lacI^q</i> <i>pmrB</i>	Wösten et al. 2000
pUH- <i>pmrB_c</i>	rep _{pMB1} Ap ^R <i>lacI^q</i> <i>pmrB_c</i>	Wösten et al. 2000
pLK39	rep _{pMB1} Ap ^R <i>lacI^q</i> <i>pmrA</i> (D51A) B	Kox et al. 2000
pUH-ygiXY	rep _{pMB1} Ap ^R <i>lacI^q</i> <i>ygiXY</i>	This work
pLK4	rep _{pMB1} Ap ^R <i>pbpP/E pmrD</i>	Kox et al. 2000
pCCR9	rep _{pSC101} Tc ^R	Randegger et al. 2000
pCCR9- <i>pmrB</i>	rep _{pSC101} Tc ^R <i>pmrB</i>	Kato et al. 2003
pCCR9- <i>pmrBT156R</i>	rep _{pSC101} Tc ^R <i>pmrBT156R</i>	Kato et al. 2003
pCCR9- <i>pmrBT156R2</i>	rep _{pSC101} Tc ^R <i>pmrBT156R</i>	This work
pTYB11	rep _{pMB1} Ap ^R <i>lacI^q</i> T7 Intein	New England Biolabs
pTYB11-PmrD-Flag	rep _{pMB1} Ap ^R <i>lacI^q</i> T7 Intein- <i>pmrD</i> -Flag	This work
pT7-7	rep _{pMB1} Ap ^R p _{T7}	Tabor and Richardson 1985
pT7-7-PmrA-His6	rep _{pMB1} Ap ^R p _{T7} <i>pmrA</i> -His6	Wösten and Groisman 1999
pT7-7-PmrAD51A-His6	rep _{pMB1} Ap ^R p _{T7} <i>pmrAD51A</i> -His6	This work
pT7-7-PmrA505-His6	rep _{pMB1} Ap ^R p _{T7} <i>pmrA505</i> -His6	This work
pT7-7-YgiX-His6	rep _{pMB1} Ap ^R p _{T7} <i>ygiX</i> -His6	This work
pT7-7-AX-His6	rep _{pMB1} Ap ^R p _{T7} <i>pmrA-ygiX</i> -His6	This work
pT7-7-XA-His6	rep _{pMB1} Ap ^R p _{T7} <i>ygiX-pmrA</i> -His6	This work
pT7-7-His6-PmrB _c	rep _{pMB1} Ap ^R p _{T7} His6- <i>pmrB_c</i>	Wösten and Groisman 1999
pT7-7-His6-PmrB _c T156R	rep _{pMB1} Ap ^R p _{T7} His6- <i>pmrB_c</i> T156R	This work
pT7-7-His6-YgiY _c	rep _{pMB1} Ap ^R p _{T7} His6- <i>ygiY_c</i>	This work
pGEX-2T	rep _{pMB1} Ap ^R p _{tac} GST	Amersham Biosciences
pGEX-PmrB _c T156R	rep _{pMB1} Ap ^R p _{tac} GST- <i>pmrB_c</i> T156R	Kato et al. 2003
pGEX-YgiY _c	rep _{pMB1} Ap ^R p _{tac} GST- <i>ygiY_c</i>	This work

TTCACCCTGACCGCTGGCGCGGCTACC-3'), and 2395 instead of 2453, 2340, 2339, and 2432. The resulting PCR fragment was digested with NdeI and HindIII and ligated to pT7-7 plasmid DNA that had been digested with NdeI and HindIII.

Plasmid pT7-7-His6-PmrB_cT156R encodes a mutant PmrB cytoplasmic domain with the T156R substitution, which is crucial for phosphatase activity in the sensor EnvZ of *E. coli* (Dutta et al. 2000). It was constructed by cloning between the NdeI and

HindIII sites of the pT7-7 plasmid a PCR fragment containing the *pmrB_c* T156R coding region amplified using primers 988 (5'-AGATATACATATGCACCACCACCACCACCACCGGC GTATTACCGTCCGCTC-3') and 1232 (5'-ACGAAGCTTAT GCCTTTTTCA-3') and pCCR9-*pmrBT*156R as template, and digested with NdeI and HindIII.

Plasmid pGEX-YgiY_c, encoding an N-terminal GST fusion of the YgiY cytoplasmic domain, was constructed by cloning between the BamHI and EcoRI sites of pGEX-2T a PCR fragment containing the *ygiY_c* coding region amplified using primers 2543 (5'-CGGGATCCACCGCGAGCTCAGACCATT-3') and 2544 (5'-GGAATTCTTACCAACTTACTACGGCCT-3') and pUH-*ygiXY* as template, and digested with BamHI and EcoRI.

All PCR reactions were performed using Platinum Taq DNA polymerase high fidelity (Invitrogen), which minimizes the production of DNA fragments with heterogeneous ends because of its 3'-to-5' exonuclease activity. All plasmids constructed using PCR reactions were analyzed by DNA sequencing, and we confirmed that the DNA regions generated by PCR had the predicted sequences and no unexpected substitutions.

Construction of chromosomal gene deletion mutants

Strains EG13732 and EG13831, which have a deletion in the *pmrB* gene, were constructed by the one-step gene inactivation method (Datsenko and Wanner 2000). A Km^R cassette was amplified using primers 2124 (5'-CTACATGCTGGTTGCCACT GAGGAAAGCTAAGTGAGCCTGGTGTAGGCTGGAGCT GCTTC-3') and 1583 (5'-TTTATCTATGTGTGGGTACGA CGTATTAAACGCCTGTTACATATGAATATCCTCCTTAG-3') and pKD4 as template and recombined into the *pmrB* region in strains 14028s and EG9492, respectively. The Km^R cassette was removed by using plasmid pCP20 as described (Datsenko and Wanner 2000).

Strain EG14089, which has a deletion of the *pmrD* gene, was constructed as follows: A Cm^R cassette was amplified using primers 1485 (5'-GTGTAACGTGTTATGAACAATCAGCGT GAAACGGGGGCGCTGTAGGCTGGAGCTGCTTCG-3') and 1486 (5'-TTATGGCGGGGTAATGCTGATTTTTCTGCC CCGAGATCACATATGAATATCCTCCTTAG-3') and pKD3 as template, and recombined into the *pmrD* region in the strain 14028s. The Cm^R cassette was removed using plasmid pCP20.

E. coli strain EG13796, which has a deletion in the *pmrAB* orthologs *basRS* genes, was constructed by one-step gene inactivation method. A Cm^R cassette was amplified using primers 2116 (5'-CTGCGGATGATATTCTGCAAACTTGCAGGAGA GTGAGTGATGTAGGCTGGAGCTGCTTCG-3') and 2115 (5'-CTACCGTGTTACAGCGTGCTGGTGGTCAGCAGCTTT CTTTACATATGAATATCCTCCTTAG-3') and pKD3 as template, and recombined into the *basRS* region of strain ER2566.

Overproduction and purification of proteins

The PmrA, PmrAD51A, PmrA505, PmrB_c, PmrB_cT156R, YgiX, YgiY_c, and the chimeric proteins PmrA-YgiX and YgiX-PmrA were overproduced in *E. coli* EG13796 harboring pT7-7-PmrA-His6, pT7-7-PmrAD51A-His6, pT7-7-PmrA505-His6, pT7-7-His6-PmrB_c, pT7-7-His6-PmrB_cT156R, pT7-7-YgiX-His6, pT7-7-His6-YgiY_c, pT7-7-AX-His6, and pT7-7-XA-His6, respectively. The conditions for expression of His6-tagged proteins were incubation with IPTG (final concentration 1 mM) for 3 h at 30°C. Protein purification was performed as described (Kato et al. 2003) with the modifications described below. After purification, the buffer of the eluate was exchanged with 10 mM Tris-HCl (pH 7.5)/138 mM NaCl/2.7 mM KCl (TBS) and concen-

trated using Amicon Ultra-15 (MW 10,000; Millipore). Glycerol was added to the protein solution to a 50% final concentration, and it was stored at -20°C.

The purification of the glutathione Sepharose beads bound to the GST-YgiY_c and GST-PmrB_cT156R proteins was performed as described for GST-PmrB_cT156R (Kato et al. 2003) with the modifications described below. The GST-YgiY_c and GST-PmrB_cT156R proteins were overproduced in *E. coli* strain EG13796 harboring pGEX-YgiY_c and pGEX-PmrB_cT156R, respectively. The glutathione Sepharose beads-bound GST-YgiY_c and GST-PmrB_cT156R proteins were stored in 1/2 TBS/50% glycerol at -20°C.

The Intein-PmrD-Flag protein was overproduced in *E. coli* strains ER2566 or EG13796 harboring pTYB11-PmrD-Flag. The conditions for expression of Intein-PmrD-Flag protein were induction with IPTG (final concentration 300 μM) in 4 L of LB for 20 h at 15°C. Autocleavage and purification of the PmrD-Flag protein was performed following the manufacturer's procedure (New England Biolabs). The conditions for autocleavage were incubation with 20 mM Na-phosphate buffer (pH 8.0)/300 mM NaCl/50 mM DTT for 2 d at 4°C followed by 1 d at room temperature. After purification, the buffer of the eluate was exchanged with 20 mM Na-phosphate buffer (pH 8.0)/300 mM NaCl and concentrated using Amicon Centriprep YM-3 (MW 3000; Millipore). Glycerol was added to the protein solution at 50% final concentration, and it was stored at -20°C.

Autokinase assay

The His6-PmrB_c (2.5 μM) and PmrD-Flag (5 μM) proteins were incubated with 3.75 μCi [γ-³²P] ATP (3000 Ci/mmol) in 30 μL of TBS/1 mM MgCl₂/1 mM DTT at room temperature. The reaction was started with addition of the histidine kinase to the mixture. A 10-μL aliquot was then mixed with 4× SDS sample buffer to stop the reaction at different time points. Samples were kept on ice until the performance of SDS-PAGE. After electrophoresis, the gel was autoradiographed.

Phosphotransfer assay

To obtain phospho-His6-PmrB_c protein, the His6-PmrB_c protein (5 μM) was preincubated with 20 μCi [γ-³²P] ATP in 80 μL of TBS/1 mM MgCl₂/1 mM DTT for 1 h at room temperature. After chilling the reaction on ice, 5 μL was mixed with 4× SDS sample buffer and kept as a reference sample for the phospho-PmrB_c protein before the phosphotransfer reaction. Thirty microliters of phospho-His6-PmrB_c protein mixture was added into 30 μL of TBS/1 mM MgCl₂/1 mM DTT containing 10 μM response regulator proteins (i.e., PmrA-His6 and PmrAD51A-His6) to initiate the phosphotransfer reaction and incubated at room temperature. A 10-μL aliquot was then mixed with 4× SDS sample buffer to stop the reaction at different time points. Samples were kept on ice until SDS-PAGE. After electrophoresis, the gel was autoradiographed.

Kinase/phosphatase assay

Five micromolar response regulator proteins (i.e., PmrA-His6 and YgiX-His6), 2.5 μM histidine kinase protein (i.e., His6-PmrB_c, His6-YgiY_c and His6-PmrB_cT156R), and 5 μM PmrD-Flag protein were incubated with 3.75 μCi [γ-³²P] ATP in 30 μL of TBS/1 mM MgCl₂/1 mM DTT at room temperature. The reaction was started with addition of histidine kinase to the mixture. A 10-μL aliquot was then mixed with 4× SDS sample buffer to stop the reaction at different time points. Samples

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were kept on ice until SDS-PAGE. After electrophoresis, the gel was autoradiographed.

Phosphatase assay

To generate substrates for the phosphatase assay, phosphorylated response regulator proteins (i.e., phospho-PmrA-His6, phospho-PmrA505-His6, phospho-YgiX-His6, phospho-PmrA-YgiX-His6, and phospho-YgiX-PmrA-His6) were prepared as follows: First, 0.3 nmole of GST-PmrB_cT156R or GST-YgiY_c beads were incubated with 5 μ Ci [γ -³²P] ATP in 60 μ L of TBS/1 mM MgCl₂/1 mM DTT for 16 h at room temperature, and then washed with TBS three times to remove free ATP. Three nanomoles of response regulator protein was incubated with phosphorylated GST-PmrB_cT156R or GST-YgiY_c beads in 50 μ L of TBS/1 mM MgCl₂/1 mM DTT for 4 h at room temperature. After the incubation, GST-fusion protein beads were spun down and removed. Possible trace amounts of ATP contaminant were removed from phosphorylated response regulator fraction using a Micro Bio-Spin 6 Chromatography Column (Bio-Rad) which had been pre-equilibrated with TBS.

Phosphorylated response regulator protein (2.5 μ M), histidine kinase protein (His6-PmrB_c, His6-PmrB_cT156R, and His6-YgiY_c, 5 μ M), and PmrD-Flag (2.5 μ M) protein were incubated in 35 μ L of TBS/1 mM MgCl₂/1 mM DTT at room temperature. The reaction was started by addition of phosphorylated response regulator to the mixture. A 10- μ L aliquot was then mixed with 4 $\times$ SDS sample buffer to stop the reaction at the indicated time points. Samples were kept on ice until SDS-PAGE. After electrophoresis, the gel was autoradiographed.

Quantitative analysis of ³²P-labeled phosphorylated response regulator was carried out using a BAS-MS 3543 imaging plate and an FLA-5000 imaging system (Fuji Film).

Immunoprecipitation analysis

Two micromolar response regulator proteins (i.e., PmrA-His6, YgiX-His6, PmrA-YgiX-His6, and YgiX-PmrA-His6), 2 μ M histidine kinase protein (i.e., His6-PmrB_c, His6-YgiY_c), and 1 μ M PmrD-Flag protein were incubated in 120 μ L of TBS/0.2 mM ATP/1 mM MgCl₂/1 mM DTT for 16 h at room temperature. After the reaction, the mixture was chilled on ice, and 20 μ L aliquot was removed and kept as a preimmunoprecipitation (pre-IP) sample; 900 μ L of 10 mM Tris-HCl (pH 7.5)/1 M NaCl/2.7 mM KCl/1% Triton X-100 (IP-washing buffer) and 10 μ L of 50% EZview Red anti-Flag M2 Affinity Gel (Sigma), which had been pre-equilibrated with IP-washing buffer, were added and agitated gently for 1 h at 4°C. Unbound proteins were removed by a centrifugation for 30 sec at 8200 relative centrifugal force. The beads that bound proteins were extensively washed with 500 μ L of IP-washing buffer four times and with 500 μ L of TBS/1% Triton X-100 twice, and then incubated with 20 μ L of TBS/1% Triton X-100/Flag peptide (200 μ g/ μ L) for 30 min at 4°C to elute specifically bound proteins. The beads were spun down and removed. A 10- μ L aliquot was mixed with 4 $\times$ SDS sample buffer and kept on ice until SDS-PAGE. After electrophoresis, the gel was silver-stained.

To visualize phosphorylated proteins, 12 μ Ci [γ -³²P] ATP was used for the phosphorylation reaction instead of cold ATP and incubated for 4 h at room temperature before immunoprecipitation analysis. After electrophoresis, the gel was autoradiographed.

S1 nuclease and β -galactosidase assays

The S1 nuclease protection assay was performed as described (Kato et al. 2003) with RNA from early-exponential (O.D.₆₀₀:

0.2–0.3)-phase cultures grown in 25 mL N-minimal medium at pH 7.7, with 10 mM MgCl₂, 10 μ M MgCl₂, 10 mM and 100 μ M FeCl₃, or 10 μ M MgCl₂ and 100 μ M FeCl₃. β -galactosidase assays were carried out in triplicate, and the activity was determined as described (Miller 1972). Bacteria from overnight cultures grown in N-minimal medium at pH 7.7 with 10 mM MgCl₂ were washed three times with N-minimal medium containing no Mg²⁺, and added into the appropriate fresh media with 50 $\times$ dilution. The bacterial cultures were shaken for 4 h at 37°C before the assay. Data correspond to mean values of three independent experiments performed in duplicate.

Western blot analysis

Bacteria were grown as described in β -galactosidase assays. Bacterial cells were collected from 3- mL cultures and resuspended in an appropriate volume of B-PER (Pierce). Whole-cell lysate (20 μ g of protein) was run on a Bis-Tris 10% gel (Invitrogen) with MOPS SDS Running buffer, transferred to nitrocellulose membrane, and analyzed by Western blot using anti-PmrA or anti-PmrB_c polyclonal antibodies. Western blots were developed by using anti-rabbit IgG horseradish peroxidase-linked antibodies (Amersham Biosciences) and Supersignal west femto (Pierce).

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Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor

Akinori Kato and Eduardo A. Groisman

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