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

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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 (García Véscovi 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...
the PmrD protein activates the PmrA/PmrB system at a posttranslational level

The PmrA/PmrB system is encoded by the \textit{pmrCAB} operon (Roland et al. 1993) and transcribed from two promoters: a PmrA-activated promoter located upstream of the \textit{pmrC} gene and a constitutive promoter present within the \textit{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 \textit{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 \textit{pmrCAB} operon. Indeed, lower amounts of both PmrA and PmrB proteins were produced by a \textit{pmrD} null mutant than by the isogenic wild-type strain grown in low Mg\(^{2+}\) [Fig. 2B]. Although these results demonstrated that a \textit{pmrD} mutation does affect the levels of the PmrA and PmrB proteins, they suggested that a potential posttranslational activity of PmrD on the PmrA
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 pbgP [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 Véscovi 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 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\(_c\), phospho-PmrB\(_c\), and Groisman 1999). The D51 residue is essential for PmrA phosphorylation because phosphotransfer from 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.)]

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 µM of regulator [PmrA and PmrAD51A] and 2.5 µM 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 µM, respectively. The small cationic proteins RNase A and cytochrome C were used as negative controls at a final concentration of 5 µM. (C) Phosphatase assay of phospho-PmrA protein was performed as follows: 32P-labeled phosphorylated GST-PmrB\(_c\)T156R 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\(_c\)T156R, and PmrD proteins were 2.5, 5, and 2.5 µM, respectively. The top band corresponds to phospho-PmrB\(_c\)T156R 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 µM, respectively. (E) Kinase/phosphatase assay of the PmrB\(_c\)T156R and PmrA proteins was performed in the presence (PmrD) or absence (−) of the PmrD protein. The concentrations of the PmrA, PmrB\(_c\)T156R, and PmrD proteins were 5, 2.5, and 5 µM, respectively. Samples were analyzed by 10% SDS-PAGE.
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 PmrBc 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 PmrBc [Fig. 3A], and/or by inhibiting a putative phosphatase activity of PmrBc 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 PmrBc (Fig. 3C, lanes 1–4), but that it was rapidly lost when PmrBc was present (Fig. 3C, lanes 8–10). Then, we established that the PmrD protein inhibited the PmrBc-promoted dephosphorylation of phospho-PmrA (Fig. 3C, lanes 11–13). On the other hand, PmrD affected neither the ability of PmrBc to autophosphorylate from ATP (Fig. 3D) nor the levels of phospho-PmrA in incubations carried out with PmrBcT156R as phosphodonor (Fig. 3E), a mutant PmrBc lacking phospho-PmrA phosphatase activity (Fig. 3C, lanes 5–7) that enabled us to examine the phosphotransfer reaction. Cumulatively, these results establish that PmrBc 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 PmrBc and/or by directly binding to phospho-PmrA, protecting it from PmrBc’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-PmrBc protein (Fig. 4A). PmrD appears to interact exclusively with the phosphorylated form of PmrA, because no precipitation was detected.
when ATP or PmrB\textsubscript{c} was omitted from the reaction [Fig. 4A]. This was confirmed by conducting PmrA phosphorylation with [γ\textsuperscript{32}P]ATP using either PmrB\textsubscript{c} or the phosphatase-defective PmrBT156R\textsubscript{c} and detecting the immunoprecipitated phospho-PmrA by autoradiography [Fig. 4B]. These results demonstrate that the PmrD protein specifically binds to the phosho-PmrA protein.

Consistent with the notion that PmrD specifically targets phosho-PmrA, we determined that the PmrD protein inhibited the spontaneous dephosphorylation of phosho-PmrA [i.e., in the absence of PmrB\textsubscript{c}; Fig. 5A] but had no effect on the dephosphorylation of phosho-YgiX (Fig. 5B). Because GST-PmrB\textsubscript{T156R} was used as phosphodonor for the PmrA protein in these experiments, it was possible that the PmrD protein was inhibiting a phosho-PmrA phosphatase activity mediated by trace amounts of GST-PmrB\textsubscript{T156R} 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\textsubscript{c}, which does not exhibit phosphatase activity towards phosho-PmrA (data not shown). The PmrD protein still inhibited the spontaneous dephosphorylation of phosho-PmrA under conditions in which there was no PmrB\textsubscript{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 phosho-PmrA protein, inhibiting its dephosphorylation.

The PmrD protein targets the N-terminal domain of phosho-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\textsubscript{c} or YgiY\textsubscript{c}] in the

Figure 5. The PmrD protein specifically inhibits dephosphorylation of phosho-PmrA by targeting its N-terminal region. (A) Spontaneous dephosphorylation of phosho-PmrA protein in the presence (PmrD) or absence (−) of the PmrD protein. [\textsuperscript{32}P]-labeled phosphorylated GST-PmrB\textsubscript{T156R} 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 µM. (B) Spontaneous dephosphorylation of phosho-YgiX protein was performed in the presence (PmrD) or absence (−) of the PmrD protein. [\textsuperscript{32}P]-labeled phosphorylated GST-YgiY\textsubscript{c} beads were incubated with the YgiX protein for 1 h to produce phosho-YgiX protein and removed before the assay. The concentrations of the YgiX and PmrD proteins were 2.5 µM. (C) Spontaneous dephosphorylation of phosho-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. [\textsuperscript{32}P]-labeled phosphorylated GST-YgiY\textsubscript{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 µM. (D) Quantitative analysis of the phosphatase assay of phosho-PmrA protein. [\textsuperscript{32}P]-labeled phosphorylated GST-YgiY\textsubscript{c} beads were used as phosphodonor for the PmrA protein and removed before the assay. The concentrations of the PmrA, PmrB\textsubscript{c}, and PmrD proteins were 2.5, 5, and 2.5 µM, respectively. The small cationic proteins RNase A and cytochrome C were used as negative controls at a final concentration of 2.5 µM. (E) Quantitative analysis of the phosphatase assay of phosho-YgiX protein. [\textsuperscript{32}P]-labeled phosphorylated GST-PmrB\textsubscript{T156R} beads were incubated with the YgiX protein for 45 min to produce phosho-YgiX protein and removed before the assay. The concentrations of YgiX, YgiY\textsubscript{c}, and PmrD proteins were 2.5, 5, and 2.5 µM, respectively. (F) Quantitative analysis of the phosphatase assay of phosho-PmrA-YgiX protein. [\textsuperscript{32}P]-labeled phosphorylated GST-YgiY\textsubscript{c} beads were incubated with the PmrA-YgiX protein for 2 h to produce phosho-PmrA-YgiX protein and removed before the assay. The concentrations of the PmrA-YgiX, PmrB\textsubscript{c}, and PmrD proteins were 2.5, 5, and 2.5 µM, 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-PmrBc 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., Fe^{3+}), the PmrB protein dephosphorylates phospho-PmrA. The low Mg^{2+} signal activates the PhoP/PhoQ system [García Véscovi 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 PmrBc 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 Mg^{2+} but pmrD-independent when the inducing condition was Fe^{3+} [Fig. 6A,B]. In contrast, transcription of the pmrC gene was pmrD-independent under all conditions in bacteria expressing the phosphate-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’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 Mg^{2+} [solid bars], 10 µM Mg^{2+} [hatched bars], or 10 µM Mg^{2+} and 100 µM Fe^{3+} [open bars] was determined in a pmrB strain (EG14087) harboring pCCR9 (vector), pCCR9-pmrB (pmrB), or pCCR9-pmrBT156R (pmrB). (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 Mg^{2+} [solid bars], 10 µM Mg^{2+} [hatched bars], or 10 µM Mg^{2+} and 100 µM Fe^{3+} [open bars] was determined in the pmrB pmrD strain EG14090 harboring pCCR9 (vector), pCCR9-pmrB (pmrB), or pCCR9-pmrBT156R (pmrB). (C) β-galactosidase activity (Miller units) from a pbgP-lac transcriptional fusion expressed by bacteria grown in N-minimal medium at pH 7.7 with 10 mM Mg^{2+} [solid bars], 10 µM Mg^{2+} [hatched bars], or 10 µM Mg^{2+} and 100 µM Fe^{3+} [open bars] was determined in the pmrB pmrD strain EG14090 harboring pCCR9 (vector), pCCR9-pmrB (pmrB), or pCCR9-pmrBT156R (pmrB). (D) Quantitative analysis in the phosphatase assay of phospho-PmrA and phospho-PmrAS05 proteins was performed as follows: 32P-labeled phosphorylated GST-PmrBT156R beads were used as phosphodonor for the PmrA and PmrAS05 proteins and removed before the assay. The concentrations of the PmrA, PmrAS05, PmrBc, and PmrD proteins were 2.5, 2.5, 5, and 2.5 µM, respectively. Samples were analyzed by 10% SDS-PAGE.](image-url)
high Mg\textsuperscript{2+} and no Fe\textsuperscript{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\textsuperscript{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 *pbgP* gene in isogenic *pmrB* strains that expressed either wild-type PmrA or PmrA505 proteins. Transcription of the *pbgP* gene was dependent on a functional PmrB gene in the PmrA505 strain as it is in a PmrA\textsuperscript{+} strain (Fig. 6C). These results indicate that the PmrA505 allele is not truly constitutive because it requires the PmrB protein to promote *pbgP* 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\textsubscript{c},T156R, and incubated it in the presence of PmrB<sub>c</sub>. The phospho-PmrA505 protein displayed resistance to PmrB<sub>c</sub>-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 *pmrA*505 strain expresses PmrA-activated genes under noninducing conditions [i.e., high Mg\textsuperscript{2+} and no Fe\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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., Kipl 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, Kipl, 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.
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 pmrB region derived from plasmid pCCR9-[pmrB]T156R (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 TCCATATGAAAATCTGGGT-3'] and 2545 [5'-GGCCAG CTTAACAGGACGACCATCAG-3'] and pUH-pmrAB [Soncini and Groisman 1996] as template. Another PCR fragment was amplified using primers 1121 [5'-CTGATGTCGGCTGGCTTAAGGCTTTC-3'] and 2454 [5'-TCAACAGAAAGTTGGTGCTTCCTCCATGTTGGAAC-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 Ndel and HindIII and ligated to pT7-7 plasmid DNA that had been digested with Ndel 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 Ndel 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 Ndel 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 Ndel and Sall sites of pT7-7 plasmid a PCR fragment generated with primers 2395 [5'-GAGGA TCCATATGCAAAGTTGGTGCTTCCTCCATGTTGGAAC-3'] and 2452 [5'-GGCCAG CTTAACAGGACGACCATCAG-3'] and pUH-pmrAB [Soncini and Groisman 1996] as template. Another PCR fragment was ampliﬁed using primers 2339 [5'-CTGATGTCGGCTGGCTTAAGGCTTTC-3'] and 2432 [5'-TCAACAGAAAGTTGGTGCTTCCTCCATGTTGGAAC-3'] and pUH-ygiXY as template, and digested with Ndel 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 2430 [5'-GGCCAGAATTGCTACTGGCTGGCTTTATTATGGCGCGCCACG-3'] and pUH-pmrAB [Soncini and Groisman 1996] as template. Another PCR fragment was ampliﬁed using primers 2339 [5'-CTGATGTCGGCTGGCTTAAGGCTTTC-3'] and 2432 [5'-TCAACAGAAAGTTGGTGCTTCCTCCATGTTGGAAC-3'] and pUH-ygiXY as template. After digestion with DpnI to eliminate the original plasmid DNA, both fragments were annealed to each other and ampliﬁed using primers 2453 and 2432. The resulting PCR fragment was digested with Ndel and XhoI and ligated to pT7-7 plasmid DNA that had been digested with Ndel and Sall.

Plasmid pT7-7-AX-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-PmrA-His6 using primers 2454, 2431 [5'-GACGTACGCCGGCCACGCCAGGCCTACG-3'] and 14028s genomic DNA as template, and digested with BamHI and XhoI.

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 (Snayel et al. 1991) supplemented with 0.1% Casamino Acids, 38 mM glyceral, 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 SapI 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'-GGGGTGGTT GCTTCTCAACTGGAATGTTGGTTA-3'] and 1752 [5'-AACGCTGACTATTCTCGCTGCTCTTTACTGTT-3'] and 14028s genomic DNA as template, and digested with BamHI and XhoI.

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 pUHE21-2lacβ a PCR fragment generated with primers 1575 [5'-GAGGATCCGGATCC GAAATTCTAG-3'] and 1605 [5'-AAGCTGATTACCTACCA AACCTTACGC-3'] and 14028s genomic DNA as template, and digested with BamHI and XhoI.
Table 1. Bacterial strains and plasmids used

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| S. enterica serovar Typhimurium | | |
| 14028s | Wild type | Fields et al. 1986 |
| EG10056 | ΔpmrB::Cm<sup>R</sup> | Kox et al. 2000 |
| EG11491 | pmrD::Cm<sup>R</sup> | Kox et al. 2000 |
| EG10065 | pbgP1::MudJ ΔpmrB::Cm<sup>R</sup> | Kox et al. 2000 |
| EG12060 | pbgP1::MudJ pmrD::Cm<sup>R</sup> ΔpmrB::Cm<sup>R</sup> | Kox et al. 2000 |
| EG9492 | pmrA505 zid::Tn10d-Cam | Groisman et al. 1997 |
| EG9888 | pbgP1::MudJ zid::Tn10d-Cam | Groisman et al. 1997 |
| EG13732 | ΔpmrB::Km<sup>R</sup> | This work |
| EG13733 | ΔpmrB::Km<sup>R</sup> zid::Tn10d-Cam | This work |
| EG13734 | ΔpmrB zid::Tn10d-Cam | This work |
| EG13735 | pbgP1::MudJ ΔpmrB zid::Tn10d-Cam | This work |
| EG9868 | pbgP1::MudJ pmrA505 zid::Tn10d-Cam | Groisman et al. 1997 |
| EG13831 | pmrA505 ΔpmrB zid::Tn10d-Cam | This work |
| EG13641 | pbgP1::MudJ pmrA505 ΔpmrB zid::Tn10d-Cam | This work |
| EG9460 | pmrC::MudJ | Kox et al. 2000 |
| EG11785 | pmrC::MudJ pmrD::Cm<sup>R</sup> | Kox et al. 2000 |
| EG14087 | pmrC::MudJ ΔpmrB::Cm<sup>R</sup> | This work |
| EG14089 | ΔpmrD | This work |
| EG14090 | pmrC::MudJ ΔpmrB::Cm<sup>R</sup> ΔpmrD | This work |

**E. coli**

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| DH5α | F<sup>−</sup> supE44 ΔlacU169 [ΔgalXΔM15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | Hanahan 1983 |
| ER2566 | hfla2 [lon] ompT lacZ::T7 gene1 gal sulA11 Δ(mcrA-mrr)14::IS10 10 R[mcr-7::miniT10-TetR<sup>R</sup>]2 R[<sup>80</sup>d-Cam-210::Tn10-Tet<sup>R</sup>]<sup>10</sup> endA1 [dcm] | New England Biolabs |
| EG13796 | ER2566 ΔbasR::Cm<sup>R</sup> | This work |

**Plasmid**

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| pKD3 | rep<sub>RBK</sub>, Ap<sup>R</sup> FRT Cm<sup>R</sup> FRT | Datsenko and Wanner 2000 |
| pKD46 | rep<sub>PSC101</sub>, Ap<sup>R</sup> p<sub>BAD</sub> γ β exo | Datsenko and Wanner 2000 |
| pCP20 | rep<sub>PSC101</sub>, Ap<sup>R</sup> Cm<sup>R</sup> cI857 X<sub>B</sub> flp | Cherepanov and Wackernagel 1995 |
| pUHE21-2loc<sup>i</sup> | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> | Soncini et al. 1995 |
| pUH-pmrAB | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> pmrAB (pEG9102) | Soncini and Groisman 1996 |
| pUH-pmrB | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> pmrB | Wösthen et al. 2000 |
| pUH-pmrB<sub>e</sub> | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> pmrB<sub>e</sub> | Wösthen et al. 2000 |
| pLX59 | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> pmrA505<sub>J</sub>B | Wösthen et al. 2000 |
| pUH-ygiXY | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> ygiXY | Wösthen et al. 2000 |
| pLK4 | rep<sub>PM1</sub>, Ap<sup>R</sup> pbgP1/E pmrD | Wösthen et al. 2000 |
| pCCR9 | rep<sub>PSC101</sub>, Tc<sup>R</sup> | Randegger et al. 2000 |
| pCCR9-<wbr/>pmrB | rep<sub>PSC101</sub>, Tc<sup>R</sup> pmrB | Kato et al. 2003 |
| pCCR9-<wbr/>pmrBT156R | rep<sub>PSC101</sub>, Tc<sup>R</sup> pmrBT156R | Kato et al. 2003 |
| pCCR9-<wbr/>pmrBT156R2 | rep<sub>PSC101</sub>, Tc<sup>R</sup> pmrBT156R | Kato et al. 2003 |
| pTBY11 | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> T7 Intein | New England Biolabs |
| pTBY11-PmrD-Flag | rep<sub>PM1</sub>, Ap<sup>R</sup> lacI<sup>q</sup> T7 Intein-PmrD-Flag | This work |
| pT7-7 | rep<sub>PM1</sub>, Ap<sup>R</sup> T<sub>7</sub> | Tabor and Richardson 1985 |
| pT7-7-Pmra-His6 | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> pmrA-His6 | Wösthen and Groisman 1999 |
| pT7-7-PmraDS1A-His6 | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> pmrADS1A-His6 | This work |
| pT7-7-Pmra505-His6 | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> pmrA505-His6 | This work |
| pT7-7-YgiX-His6 | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> ygiX-His6 | This work |
| pT7-7-AH6 | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> pmrA-ygiX-His6 | This work |
| pT7-7-XA-His6 | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> ygiX-pmrA-His6 | This work |
| pT7-7-His6-PmrB<sub>e</sub> | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> His6-pmrB<sub>e</sub> | Wösthen and Groisman 1999 |
| pT7-7-His6-YgiY<sub>e</sub> | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>T7</sub> His6-ygiY<sub>e</sub> | This work |
| pGEX-2T | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>mac</sub> GST | Amersham Biosciences |
| pGEX-PmrB<sub>e</sub> T156R | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>mac</sub> GST-pmrB<sub>e</sub> T156R | Kato et al. 2003 |
| pGEX-YgiY<sub>e</sub> | rep<sub>PM1</sub>, Ap<sup>R</sup> P<sub>mac</sub> GST-ygiY<sub>e</sub> | This work |

**Plasmid**

pT7-7-His6-pmrB<sub>e</sub> T156R 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.

TTCACCCGTTACC GCCGCTGCGCGCGGCTACG-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.
Constructing 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 cassette was amplified using primers 2124 (5'-CTACATGCTGTTGGACCCTTGGGAATGTTATGAACAATCAGCGTATTAAAGCCTGTTACATATGAATATCCTCCTTAGG-3') and 2116 (5'-CTGTTAACGTGTTATGAACTACAATCGGCTGACTGACGTCCTTCG-3') and 1583 (5'-TTTATCTATTGTGGTGCGACGGTATTAAACCGCCTTATGATATCCTCTTCTAG-3') and pKD4 as template and recombined into the pmrB region in strains 14028s and EG9492, respectively. The Km 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 Km cassette was amplified using primers 1485 (5'-GCTATACGCTGTTAGACCACCGTGGTAGGCTGACTTATGAAAGCTATATGAAACGCTGATTAAAGCCTGTTACATATGAATATCCTCCTTAGG-3') and 1486 (5'-TATATCATTGTGGTGCGACGGTATTAAACCGCCTTATGATATCCTCTTCTAG-3') and pKD4 as template and recombined into the pmrB region in strain 14028s. The Km cassette was removed by using plasmid pCP20 as described (Datsenko and Wanner 2000).

Overproduction and purification of proteins

The PmrA, PmrAD51A, PmrA505, PmrB, PmrB-T156R, YgiX, YgiYc, and the chimeric proteins PmrA-YgiX and YgiX-PmrA were overproduced in E. coli strain EG13796 harboring pT7-7-PmrA-His6, pT7-7-PmrAD51A-His6, pT7-7-PmrA505-His6, pT7-7-His6-PmrB, pT7-7-His6-PmrB-T156R, pT7-7-YgiX-His6, pT7-7-His6-YgiYc, pT7-7-AX-His6, and pT7-7-XA-His6, respectively. The conditions for expression of Hsf6-tagged proteins were incubation with IPTG (final concentration 0.1 mM) for 5 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 phosphorylation reaction. Thirty microliters of phosphorylated-protein mixture 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-YgiYc, and GST-PmrB-T156R, proteins was performed as described for GST-PmrB-T156R (Kato et al. 2003) with the modifications described below. The GST-YgiYc and GST-PmrB-T156R proteins were stored in 1/2 TBS/50% glycerol at −20°C.

The Intein-PmrD-Flag protein was overproduced in E. coli strains ER2566 and EG13796 harboring pTBY111-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 30000, Millipore). Glycerol was added to the protein solution at 50% final concentration, and it was stored at −20°C.

Auto kinase assay

To obtain phospho-His6-PmrB protein, the His6-PmrB protein [5 µM] was preincubated with 20 µCi [γ-32P] ATP [3000 Ci/m mole] 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 protein, the His6-PmrB 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 protein before the phosphotransfer reaction. Thirty microliters of phospho-His6-PmrB 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, His6-YgiYc and His6-PmrB-T156R), 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 were kept on ice until SDS-PAGE. After electrophoresis, the gel was autoradiographed.

Preventing dephosphorylation of response regulator

The purification of the glutathione Sepharose beads bound to the GST-YgiYc, and GST-PmrB-T156R, proteins was performed as described for GST-PmrB-T156R (Kato et al. 2003) with the modifications described below. The GST-YgiYc and GST-PmrB-T156R proteins were stored in 1/2 TBS/50% glycerol at −20°C.

The Intein-PmrD-Flag protein was overproduced in E. coli strains ER2566 and EG13796 harboring pTBY111-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.
were incubated 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-PmrA-YgiX-His6, and phospho-PmrA-YgiX-PmrA-His6] were prepared as follows: First, 0.3 nmole of GST-PmrBc-T156R or GST-YgiYc beads were incubated with 5 µCi [γ-32P] ATP in 60 µL 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 µM), histidine kinase protein (His6-PmrBc, His6-PmrBcT156R, and His6-YgiYc, 5 µM), and PmrD-Flag protein were incubated in 50 µL of TBS/1 mM MgCl2/1 mM DTT for 4 h at room temperature. After the incubation, the mixture was chilled on ice, and 20 µL of TBS/1 mM MgCl2/1 mM DTT was added to stop the reaction at the indicated time points. Samples were kept on ice until SDS-PAGE. After electrophoresis, the gel was autoradiographed.

Phosphatase activity analysis

Two micromolar response regulator proteins (i.e., PmrA-His6, YgiX-His6, PmrA-YgiX-His6, and YgiX-PmrA-His6), 2 µM histidine kinase protein (His6-PmrBc, His6-PmrBcT156R, and His6-YgiYc), and 1 µM PmrD-Flag protein were incubated in 120 µL of TBS/0.2 mM ATP/1 mM MgCl2/1 mM DTT for 16 h at room temperature. The reaction was started by addition of phosphorylated response regulator to the mixture. A 10-µL aliquot was then mixed with 4× SDS sample buffer to stop the reaction at the indicated temperature. Samples were kept on ice until SDS-PAGE. After electrophoresis, the gel was autoradiographed.

Immunoprecipitation analysis

Two micromolar response regulator proteins [i.e., PmrA-His6, YgiX-His6, PmrA-YgiX-His6, and YgiX-PmrA-His6], 2 µM histidine kinase protein [i.e., His6-PmrBc, His6-PmrBcT156R, and His6-YgiYc], and 1 µM PmrD-Flag protein were incubated in 20 µL of TBS/0.2 mM ATP/100 µM MgCl2 for 4 h at room temperature. After the reaction, the mixture was chilled on ice, and 20 µL aliquot was removed and kept as a preimmunoprecipitation (pre-IP) sample. 900 µL of 10 mM Tris-Cl (pH 7.5)/1 M NaCl/2.7 mM KCl/1% Triton X-100 (IP-washing buffer) and 10 µL of 50% E2/3red 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 centrifugation for 30 sec at 8200 relative centrifugal force. The beads that bound proteins were extensively washed with 500 µL of IP-washing buffer four times and with 500 µL of TBS/1% Triton X-100 twice, and then incubated with 20 µL of TBS/1% Triton X-100/Flag peptide (200 µg/µL) for 30 min at 4°C to elute specifically bound proteins. The beads were spun down and removed. A 10-µL aliquot was mixed with 4× SDS sample buffer and kept on ice until SDS-PAGE. After electrophoresis, the gel was silver-stained.

To visualize phosphorylated proteins, 12 µCi [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 µM MgCl2, 10 mM and 100 µM FeCl3, or 10 µM MgCl2 and 100 µM 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 50x 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 lysates (20 µg of protein) were 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, 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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