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The RstB Sensor Acts on the PhoQ Sensor to Control Expression of PhoP-Regulated Genes

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The PhoP response regulator and PhoQ sensor, which are encoded by the *phoPQ* operon, constitute the PhoP/PhoQ two-component system. Genome-wide transcription analysis revealed that heterologous expression of the RstB protein, a sensor of the RstA/RstB two-component system, leads to enhanced transcription of PhoP-activated genes in wild-type *Salmonella*. We determined that RstB-induction increases the levels of *phoP* mRNA as well as PhoP protein, while lack of the *phoPQ* genes abolishes RstB-promoted transcription of the PhoP-regulated genes. This regulatory function of RstB did not require its enzymatic activities, and thus the truncated RstB protein containing only periplasmic and transmembrane regions was able to promote PhoP-activated transcription. The RstB protein appeared to target the PhoQ sensor rather than the PhoP response regulator because RstB-induction failed to enhance transcription of the PhoP-regulated genes in a strain maintaining the normal PhoP function, even without PhoQ.

INTRODUCTION

Signal transduction pathways mediated by two-component regulatory systems enable bacterial cells to rapidly adapt to and survive various environmental conditions. A typical two-component system consists of a sensor protein for detection of environmental changes in the cytoplasmic membrane and its cognate response regulator in cytoplasm. Most response regulators are transcription factors. A sensor protein controls the phosphorylation state of a response regulator using two enzyme activities. In the absence of an environmental cue, a sensor protein functions as phosphatase to dephosphorylate a response regulator (Stock et al., 2000). However, a sensor protein is autophosphorylated on the conserved histidine residue in response to a specific signal using its kinase activity, and this phosphoryl group is subsequently transferred to the conserved aspartic acid of a response regulator (Stock et al., 2000). Once phosphorylated, response regulators bind to target promoters and activate (or repress) the transcription of genes (Stock et al., 2000).

The response regulator PhoP and its partner sensor PhoQ constitute the PhoP/PhoQ two-component system. This system has played a pivotal role in the control of virulence, Mg^{2+} homeostasis, and other physiological processes of *Salmonella enterica* (Groisman, 2001). It has also been shown that three different signals, low Mg^{2+}, acidic pH, and certain antimicrobial peptides can stimulate activity of the PhoP/PhoQ system (Bader et al., 2005; Garcia Vescovi et al., 1996; Prost et al., 2007). When *Salmonella* grows in the presence low levels of Mg^{2+}, the activated PhoQ sensor phosphorylates the PhoP response regulator (Garcia Vescovi et al., 1996), and phospho-PhoP then regulates the transcription of more than 100 genes by directly binding to the target promoters or via control of the expression and/or activity of other transcription factors (Zwir et al., 2005).

Expression of the RstA protein, a response regulator of the RstA/RstB two-component system, is positively regulated by the PhoP protein (Choi et al., 2009). To date, the Fe^{2+} transporter *feoB* gene has been reported to be the only gene in *Salmonella*, for which transcription is directly activated by the RstA protein (Jeon et al., 2008). In addition, we recently demonstrated that the PhoP protein activated by low Mg^{2+} or acidic pH induces RstA expression to similar levels, but that the RstA-dependent *feoB* transcription can only be promoted when *Salmonella* is grown at acidic pH (Choi et al., 2009). These results were due to the fact that acidic pH promotes not only the levels of RstA but also its activity, possibly via phosphorylation (Choi et al., 2009). Phosphorylation of a response regulator is primarily mediated by its cognate sensor protein (Laub and Goulian, 2007; Stock et al., 2000). However, the RstB sensor protein was not required for acidic-pH promoted RstA activity (Choi et al., 2009), implying that this protein can perform regulatory roles independently of the RstA protein.

In this study, we found that heterologous expression of the RstB protein enhanced transcription of PhoP-regulated genes in a process that required the PhoP/PhoQ system. We also revealed that the RstB domain lacking enzymatic activities is responsible for RstB-mediated upregulation of the PhoP-targets. Moreover, we provided evidence that the RstB sensor acts on PhoQ rather than PhoP to control expression of the PhoP-regulated genes.

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MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1 of the Supplementary Material. *Salmonella enterica* serovar Typhimurium strains were derived from strain 14028s. Bacteria were grown at 37°C in Luria-Bertani (LB) medium or N-minimal medium (Snavely et al., 1991) supplemented with 0.1% casamino acids, 38 mM glycerol, and different concentrations of $MgCl_2$ and adjusted to pH 7.7 or pH 5.7. Ampicillin, chloramphenicol, kanamycin, and isopropyl 1-thio-β-D-galato-side (IPTG) were used at 50 μg/ml, 25 μg/ml, 50 μg/ml and 0.5 mM, respectively.

Plasmid construction

To construct plasmid pRstB in which the RstB protein was expressed from the *lac* promoter, the *rstB* gene was PCR-amplified using primers rstB-F/rstB-R and chromosomal DNA from strain 14028s as the template. The resulting products were then digested with *Bam*HI and *Pst*I and introduced into pUHE21 (Soncini et al., 1995) that had been treated with the same restriction enzymes. Plasmids pRstB (H221A) and pRstB (T225R) are derivatives of pRstB that expressed variants of RstB with H221A and T225R substitutions, respectively. These two plasmids were constructed using the QuikChange II Site-directed Mutagenesis Kit (Stratagene), primers rstB (H221A)-F/rstB (H221A)-R and rstB (T225R)-F/rstB (T225R)-R, and pRstB as the DNA template. Plasmids $pRstB^{1-167}$ and $pRstB^{168-433}$ produce N-terminal 167 amino acids and C-terminal 266 amino acids of the RstB protein from the *lac* promoter, respectively. The N-terminal and C-terminal regions of RstB were amplified from the chromosomal DNA by PCR using primers rstB-F/N-rstB-R and C-rstB-F/rstB-R. After digestion with *Bam*HI and *Pst*I, the PCR products were introduced into the corresponding restriction sites on pUHE21. Plasmids pEnvZ and pPmrB express the EnvZ and PmrB proteins, respectively, from the *lac* promoter. For construction of these plasmids, the *envZ* and *pmrB* genes were amplified from the chromosomal DNA using primers B-envZ-F/B-envZ-R and B-pmrB-F/B-pmrB-R, respectively, which were introduced between the BamHI and PstI restriction sites on pUHE21. Sequences of the cloned regions on the recombinant plasmids were confirmed by nucleotide sequencing. In addition to the same expression vector (i.e., pUHE21) being used for construction of the plasmids above, all vectors were designed to share the nucleotide sequences between the *lac* promoter and the start codon so that different levels of expression of each protein could be minimized. The sequences of primers used in plasmid construction are listed on Supplementary Table S2.

DNA microarray analysis

Wild-type *Salmonella* harboring plasmid pRstB was grown in LB medium. When the OD_{600} of the culture reached 0.5, IPTG (0.5 mM) was added to the medium to induce RstB expression. Immediately before and 30 min after the addition of IPTG, 0.5 ml of the bacterial culture was removed and mixed with 1 ml of RNAprotect™ Bacteria Reagent (Qiagen). The total RNA was then isolated using a Rneasy Mini Kit (Qiagen), and the RNA sample was treated further with RNase-free DNase (Ambion). The DNA chip (CombiMatrix) used contains oligonucleotides specific to the open reading frames of the *Salmonella* Typhimurium strain LT2 genome. cDNA synthesis/modification, hybridization, labeling with Cy™5 dye on the DNA chip, and data processing was conducted as previously described (Jeon et al., 2008). Microarray experiments were conducted on the six RNA samples obtained from three independent cultures.

Quantitative real-time PCR (qRT-PCR) analysis

Isolation and DNase treatment of RNA was performed as described above. cDNA was synthesized using Omnitranscript Reverse Transcription reagents (Qiagen) and random primers (Invitrogen) and quantified using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI7300 Sequence Detection System (Applied Biosystems). The cDNA concentrations were determined using a standard curve obtained from PCR on serially diluted genomic DNA as templates. mRNA levels of a gene of interest were normalized against the *gyrB* mRNA levels, which were constant throughout the experiments. The sequences of the primers used are shown in Supplementary Table S3.

Western blot analysis

Salmonella strain carrying the *phoP*-HA gene was grown in 20 ml of LB medium until the OD_{600} of the culture reached 0.5. Bacterial cells were then washed once with phosphate-buffered saline (PBS) and suspended in 0.5 ml of PBS. Next, the bacterial cell lysate was obtained using sonication and cleared by centrifugation. Cell lysates containing 25 μg of total proteins were resolved on 12% SDS polyacrylamide gel, transferred onto a nitrocellulose membrane, and then analyzed using monoclonal anti-HA (Santa Cruz Biotechnology) or anti-DnaK antibody (Stressgen). Blots were developed using anti-mouse IgG horseradish peroxidase-linked antibody (GE Healthcare) and the ECL detection system (GE Healthcare).

RESULTS

The *rstB* gene is not induced with the *rstA* gene upon activation of the PhoP/PhoQ system

In many two-component systems, genes encoding a response regulator and its cognate sensor are linked to constitute a bicistronic operon structure. However, between the *rstA* and *rstB* genes, which encode the RstA response regulator and RstB sensor, respectively, the *STM1474*, *ompN*, and *STM1472* genes are located on the *Salmonella* chromosome (Fig. 1A). We recently reported that, when activated by acidic pH or low Mg^{2+} signal, the PhoP/PhoQ system promotes expression of the *rstA* gene (Fig. 1B) (Choi et al., 2009). Conversely, when *Salmonella* was grown in medium with an acidic pH or low Mg^{2+}, *rstB* transcription did not increase, but was maintained at low levels regardless of *phoPQ* deletion (Fig. 1B). These results indicate that *rstB* expression is unresponsive for activation of the PhoP/PhoQ system. Moreover, considering that the *rstB* gene is likely to carry its own promoter sequences (Fig. 1A), these data suggest that expression of the *rstB* gene might be controlled independently of *rstA* expression.

Heterologous expression of the RstB protein enhances expression of the PhoP-activated genes via the PhoP/PhoQ system

We assumed that the RstB sensor might play a regulatory role independently of RstA because (i) the *rstB* gene was not co-induced with the *rstA* gene (Fig. 1B); (ii) the RstB protein was not responsible for the acidic pH-promoted RstA activity (Choi et al., 2009); and (iii) some sensor proteins can control activity of a non-cognate response regulator (Laub and Goulian, 2007). To investigate RstB-mediated gene regulation, we employed *Salmonella* strains that produced the RstB sensor from the plasmid-linked *lac* promoter because no environmental cues promoting expression and activity of the RstB protein have

Table 1. Expression of the PhoP-regulated genes in DNA microarray analysis

Gene	Protein	Fold expression[a]	*p*-value[b]	References
pagK	PagK, unknown function	8.1	0.02	Zwir et al. (2005)
yrbL	YrbL, putative cytoplasmic protein	7.3	0.01	Zwir et al. (2005)
pmrD	PmrD, polymyxin B resistance protein	6.3	0.01	Kato et al. (2003)
STM2585	PagK-like protein	5.1	0.03	Zhao et al. (2008)
mgtC	MgtC, Mg^{2+}-transport protein	5.0	0.01	Soncini et al. (1996)
phoN	PhoN, non-specific acid phosphatase	3.5	0.01	Bader et al. (2005)
virK	VirK, virulence protein	2.7	0.01	Zhao et al. (2008)
mgtB	MgtB, Mg^{2+} transporter	2.5	0.03	Soncini et al. (1996)
pagC	PagC, virulence membrane protein precursor	2.3	0.02	Zwir et al. (2005)

[a]Fold expression indicates the gene expression ratio in the wild-type strain harboring the RstB-expression plasmid pRstB that was grown in the absence or presence of 0.5 mM IPTG.
[b]Student's *t*-test

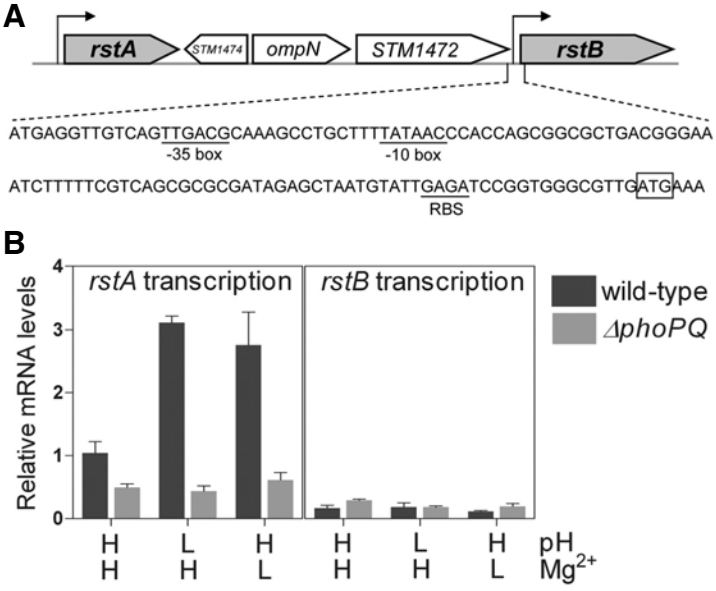

Fig. 1. Transcription of the *rstB* gene is not induced upon activation of the PhoP/PhoQ system. (A) Schematic description for arrangement of the *rstA* and *rstB* genes in *Salmonella enterica*. The nucleotide sequences indicated are the predicted promoter region of the *rstB* gene. The conserved sequences of the promoter (i.e., -10 and -35 boxes) and the ribosome-binding site (RBS) are underlined, and the start codon is boxed. (B) The *rstA* and *rstB* mRNA levels were determined by qRT-PCR. Wild-type (14028s) and *phoPQ*-deletion (*ΔphoPQ*, DS267) strains were grown in N-minimal medium containing 2 mM (H, high) or 50 μM (L, low) Mg^{2+} and adjusted to pH 7.7 (H, high) or 5.7 (L, low). The relative mRNA levels in the y-axis correspond to the mRNA levels of each gene divided by those of *gyrB*. The mean values and standard deviations of three independent experiments are shown. Also, note that the data for *rstA* transcription has been reported previously (Choi et al., 2009).

been identified to date. Interestingly, DNA microarray analysis revealed that growth of the wild-type strain in LB medium resulted in elevation of the transcription levels of several genes that have been reported to be activated by a response regulator of the PhoP/PhoQ two-component system (PhoP) in response to IPTG-induced RstB (Table 1).

Once activated, the PhoP response regulator binds to promoter of the *phoPQ* operon and promotes its own levels (Lejona et al., 2003; Soncini et al., 1995). This positive feedback loop appears to be necessary for PhoP to control vast numbers of target genes (Bijlsma and Groisman, 2003). When the wild-type strain was grown in LB medium, the *phoP* mRNA levels were found to increase by 3.5-fold (Fig. 2A), while the PhoP protein levels were changed accordingly upon RstB-induction (Fig. 2B). These findings suggested that the RstB could influence PhoP-regulated transcription in a process dependent on the PhoP/PhoQ system. Indeed, the absence of the *phoPQ* genes was found to abolish the regulatory effect of RstB on the PhoP-targets. qRT-PCR determined that RstB-induction in the wild-type strain resulted in production of the *pmrD* and *mgtC* mRNAs at 5- and 28-fold higher levels, respectively, but that this increase was not observed in a strain lacking the *phoPQ* genes (Fig. 2C).

We also evaluated other sensor proteins to determine if they influenced expression of the PhoP-regulated genes because bacterial sensors share homology in their amino acid sequences (Stock et al., 2000). However, induction of the EnvZ or PmrB sensor protein in the wild-type strain did not change the transcription levels of the *pmrD* and *mgtC* genes (data not shown), suggesting that RstB-mediated regulation of the PhoP-target genes was specific.

Neither kinase nor phosphatase activities of the RstB sensor are involved in RstB-mediated gene regulation

Although it is rare, a certain two-component system controls activity of the other via crosstalk. In this system, the phosphorylation state of a response regulator is modulated by a non-cognate sensor protein (Laub and Goulian, 2007). Thus, the RstB protein may enhance the PhoP activity, which would in turn increase the expression of the PhoP-regulated genes. We reasoned that, in such a case, the lack of kinase or phosphatase activity of RstB could impair its regulation of PhoP-targets because a sensor protein determines the levels of the phosphorylated response regulator based on these two en-

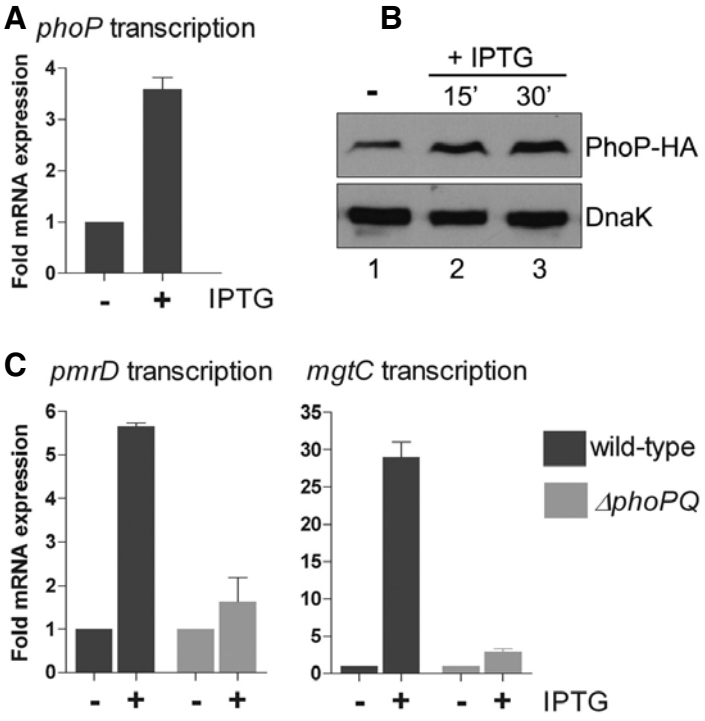

Fig. 2. Heterologous expression of the RstB sensor enhances PhoP-regulated transcription in a PhoP/PhoQ-dependent fashion. (A, C) Wild-type (14028s) and Δ*phoPQ* (DS267) strains carrying pRstB plasmid were grown in LB medium. RNA was extracted from the cultures immediately before (-) and 30 min after (+) IPTG-addition, and the *phoP*, *pmrD*, and *mgtC* mRNA levels were determined by qRT-PCR. The fold mRNA expression values in the y-axis correspond to the relative mRNA levels of each gene in the presence of IPTG to those in the absence IPTG. Relative mRNA levels were determined as described in the legend of Fig. 1. The mean values and standard deviations of three independent experiments are shown. (B) Strain DS202 expresses the PhoP protein with a HA-tag at the C-terminus from the normal chromosomal location. DS202 harboring pRstB was grown in LB medium. Western blot analysis was performed using cell lysates that were obtained from the culture immediately before (lane 1) and 15- (lane 2) and 30 min (lane 3) after IPTG-addition. The DnaK protein served as a control.

zyme activities (Stock et al., 2000). To explore this idea, we constructed two derivatives of the plasmid pRstB, one expressing mutant RstB with a T225R substitution (RstB^{T225R}) and another with a H221A substitution (RstB^{H221A}). The RstB^{T225R} protein was predicted to be phosphatase-defective because T225 is a conserved residue that is crucial for the phosphatase activity of sensors (Dutta et al., 2000; Kato and Groisman, 2004; Shin and Groisman, 2005), whereas the RstB^{H221A} protein would lack the kinase function because H221 is a conserved site for autophosphorylation. However, we found that neither of these mutations was able to eliminate RstB-regulation from the PhoP-target genes: in the wild-type strain, expression of the RstB^{T225R} or RstB^{H221A} protein increased the *pmrD* and *mgtC* transcripts to levels similar to those achieved by expression of the wild-type RstB protein (compare Figs. 2C and 3A).

The N-terminal RstB domain lacking catalytic activities is responsible for RstB-promoted expression of the PhoP-regulated genes

Like other typical sensor proteins, RstB consists of the signal sensing N-terminal domain (i.e., the periplasmic and transmembrane regions) and the catalytic C-terminal cytoplasmic domain (Fig. 3B). We wanted to assess the effect of these two domains on PhoP-regulated transcription separately. To accomplish this, we constructed two derivative plasmids, pRstB$^{1-167}$ and pRstB$^{168-433}$, which expressed the N-terminal 167 amino acids and C-terminal 266 amino acids of RstB, respectively, from the *lac* promoter. qRT-PCR determined that the IPTG-induced cytoplasmic domain of RstB (i.e., RstB$^{168-433}$) failed to increase transcription of *pmrD* and *mgtC* in the wild-type strain (Fig. 3C), emphasizing that the enzyme activities of RstB are not involved in regulation of the PhoP-targets (Fig. 3A). Conversely, heterologous expression of the RstB$^{1-167}$ protein elevated levels of the *pmrD* and *mgtC* mRNA by up to 5- and 25-fold, respectively, similar to the full-length of RstB (Fig. 3C). Therefore, these results reveal that it is not the catalytic C-terminus, but the non-catalytic N-terminus of RstB that exerts the regulatory function of the PhoP-regulated genes.

The RstB sensor targets the PhoQ sensor to control the PhoP-regulated genes

We assumed that the RstB protein could act on the PhoQ sensor rather than the PhoP response regulator (Fig. 4A) because the catalytic domain of RstB showed no effect on expression of the PhoP-regulated genes (Fig. 3C). To investigate this assumption, we employed a strain (*phoP* phoQ*::Tn10) that carries a *phoP* allele expressing a variant PhoP protein, PhoP*, and lacks the functional *phoQ* gene. The PhoP* protein has been reported to constitutively regulate transcription of the PhoP-target genes, even in the absence of PhoQ (Chamnongpol and Groisman, 2000). qRT-PCR revealed that, in the *phoPQ* deletion strain grown without IPTG, the *pmrD* mRNA levels were only 17% of the wild-type levels, while they were generally unchanged by RstB induction (Fig. 4B). Similarly, the IPTG-induced RstB protein failed to increase *pmrD* transcription in the *phoP* phoQ*::Tn10 strain (Fig. 4B). However, it should be noted that this occurred despite the fact that PhoP* maintained the wild-type levels of *pmrD* transcription, even in the absence of PhoQ (Fig. 4B). qRT-PCR conducted on the same RNA samples revealed that transcription of the *mgtC* gene also displayed the *pmrD*-like patterns in response to RstB-induction (data not shown). Thus, these results suggest that the RstB sensor acts on the PhoQ sensor to control the PhoP-regulated genes.

DISCUSSION

In general, the phospho-transfer reaction between a sensor protein and a response regulator of a two-component system is specific (Laub and Goulian, 2007; Stock et al., 2000). However, some two-component systems promote or suppress each other. In this phenomenon, which is known as crosstalk, a sensor

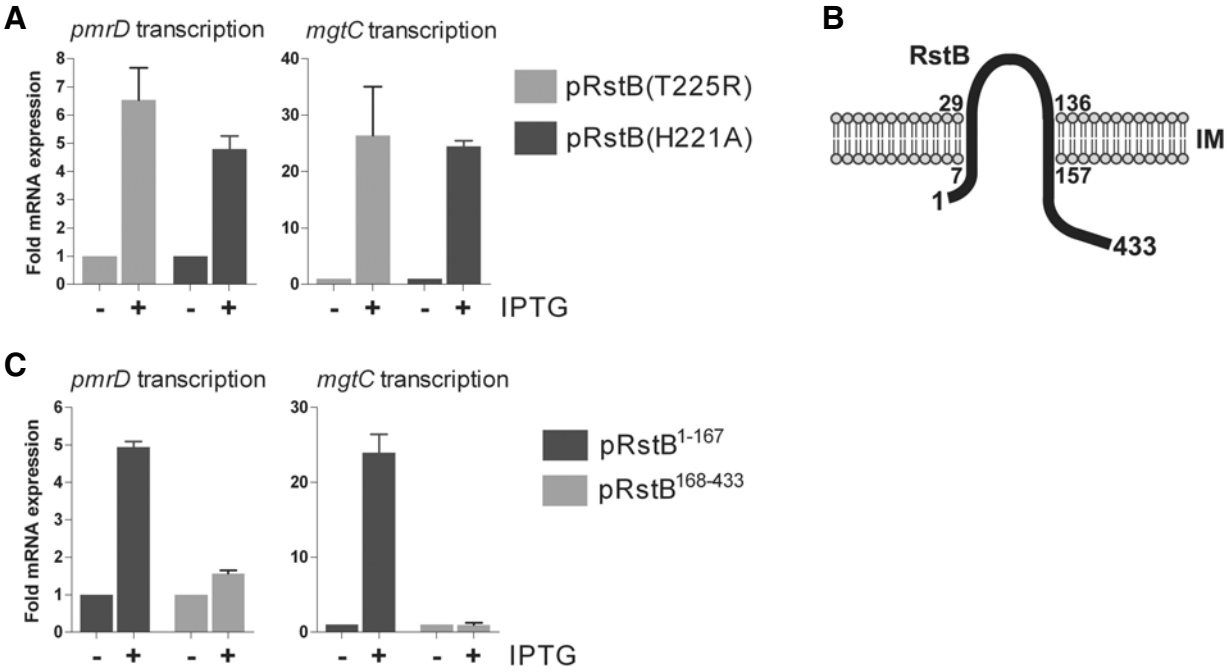

Fig. 3. The N-terminal domain of RstB lacking enzymatic activities is responsible for enhanced PhoP-regulated transcription. (A, C) Wild-type strain (14028s) carrying the plasmid pRstB(T225R) or pRstB(H221A) and pRstB$^{1-167}$ or pRstB$^{168-433}$ was grown in LB medium. The *pmrD* and *mgtC* transcription levels were determined using qRT-PCR conducted on RNA that was isolated from the cultures immediately before (-) and 30 min after (+) adding IPTG. The fold mRNA expression values in the y-axis correspond to the relative mRNA levels of each gene in the presence of IPTG to those in the absence IPTG. The mean values and standard deviations of three independent experiments are shown. (B) Predicted topology of the RstB sensor in the inner membrane (IM). Numbers correspond to amino acid positions.

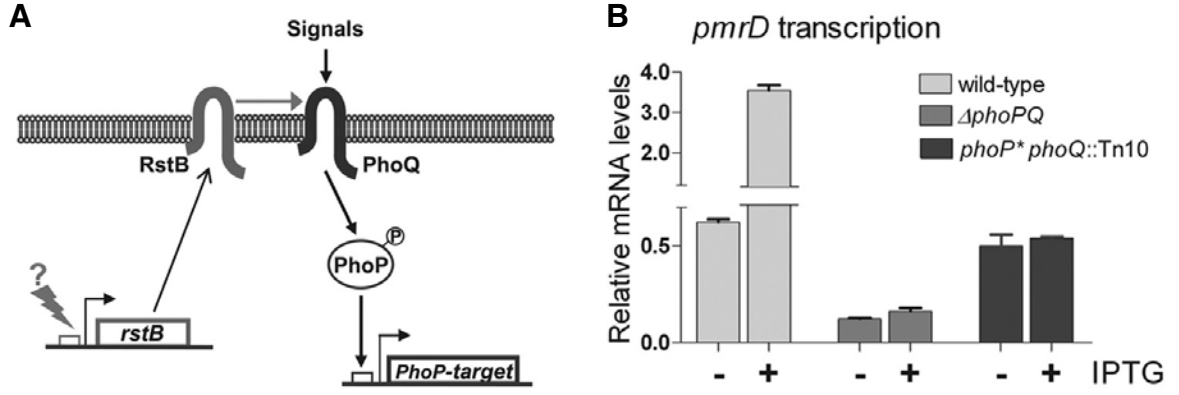

Fig. 4. The RstB protein acts on the PhoQ sensor to control PhoP-regulated transcription. (A) Model illustrating RstB-controlled expression of the PhoP-regulated genes. When *rstB* expression is triggered under a certain condition, the RstB protein acts on the PhoQ protein to further its activity. As a result, the levels of phosphorylated PhoP increase to enhance PhoP-dependent transcription. (B) Wild-type (14028s), *ΔphoPQ* (DS267), and *phoP* phoQ*:: Tn10 (EG10232) strains harboring the plasmid pRstB were grown in LB medium. The *pmrD* mRNA levels were quantified using qRT-PCR conducted on RNA that was isolated from the cultures immediately before (-) and 30 min after (+) IPTG-induction. The relative mRNA levels in the y-axis correspond to the *pmrD* mRNA levels divided by the *gyrB* mRNA levels. The mean values and standard deviations from three independent experiments are shown.

protein participates in phosphorylation or dephosphorylation of a non-cognate response regulator (Laub and Goulian, 2007). In the present study, we report a novel type of crosstalk between the RstA/RstB and PhoP/PhoQ two-component systems.

We propose that the RstB sensor acts on the PhoQ sensor to control PhoP-regulated transcription (Fig. 4A), which is opposed to the initial possibility that the regulatory function of RstB might work through the PhoP response regulator. This idea is supported by the following explanation. Expression of mutant RstB protein that lacks phosphatase activity or a site for phosphorylation normally enhanced transcription of the PhoP-targets (Fig. 3A). In addition, the truncated RstB protein that contains only periplasmic and transmembrane regions could still control the PhoP-regulated genes (Fig. 3C). Finally, in a strain displaying the normal PhoP activity without the PhoQ sensor, the RstB-promoted transcription of the PhoP-targets was abolished

(Fig. 4B).

However, these findings do not reveal the mechanism by which the RstB sensor controls PhoP-dependent expression via the PhoQ sensor protein. Recently, third components that modulate the activity of sensor proteins have been identified in two-component systems. In *Bacillus subtilis*, it has been proposed that a membrane protein, YycH, interacts with the YycG sensor to suppress the YycG/YycF system (Szurmant et al., 2005; 2007). Notably, in the *Escherichia coli* PhoP/PhoQ system, activity of the PhoQ can be further controlled by two small membrane proteins, B1500 and MgrB. Both the B1500 and MgrB proteins directly interact with PhoQ (Eguchi et al., 2007; Lippa and Goulian, 2009). However, expression of the former promotes the activity of PhoQ, while the latter inhibits this activity (Eguchi et al., 2007; Lippa and Goulian, 2009). Thus, it is plausible that the RstB protein might interact with the PhoQ protein to enhance its activity, which in turn could increase PhoP-phosphorylation (Fig. 4A). We speculate that expression of the *rstB* gene is silenced by an unknown factor because highly conserved promoter sequences (i.e., -10 and -35 boxes) and a ribosome binding site are found upstream of the *rstB* coding region (Fig. 1A). If *Salmonella* is subjected to conditions that could trigger RstB expression, the magnitude of the activity of the PhoP/PhoQ system could be increased (Fig. 4A). Maintenance of PhoP-mediated regulation assisted by the RstB protein could enable *Salmonella* to survive stressful conditions in which the levels of PhoQ-stimulating signals are low.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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