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Activated by Different Signals, the PhoP/PhoQ Two-Component System Differentially Regulates Metal Uptake▽†

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The PhoP/PhoQ two-component system controls several physiological and virulence functions in *Salmonella enterica*. This system is activated by low Mg^{2+}, acidic pH, and antimicrobial peptides, but the biological consequences resulting from sensing multiple signals are presently unclear. Here, we report that the PhoP/PhoQ system regulates different *Salmonella* genes depending on whether the inducing signal is acidic pH or low Mg^{2+}. When *Salmonella* experiences acidic pH, the PhoP/PhoQ system promotes Fe^{2+} uptake in a process that requires the response regulator RstA, activating transcription of the Fe^{2+} transporter gene *feoB*. In contrast, the PhoP-induced RstA protein did not promote *feoB* expression at neutral pH with low Mg^{2+}. The PhoP/PhoQ system promotes the expression of the Mg^{2+} transporter *mgtA* gene only when activated in bacteria starved for Mg^{2+}. This is because *mgtA* transcription promoted at high Mg^{2+} concentrations by the acidic-pH-activated PhoP protein failed to reach the *mgtA* coding region due to the *mgtA* leader region functioning as a Mg^{2+} sensor. Our results show that a single two-component regulatory system can regulate distinct sets of genes in response to different input signals.

Signal transduction mediated by two-component regulatory systems enables bacterial cells to rapidly adapt to and survive various stressful conditions. The PhoP/PhoQ two-component system is comprised of the response regulator PhoP and the sensor PhoQ. The PhoQ protein has been demonstrated to sense Mg^{2+} concentrations as a specific signal (14). When the environmental Mg^{2+} concentration is lowered to micromolar levels, PhoQ promotes the phosphorylated state of the PhoP protein (14, 32). In *Salmonella enterica*, the low-Mg^{2+}-activated PhoP protein directly regulates the transcription of many genes that are necessary for virulence in mammalian hosts, as well as other physiological processes (15). In particular, consistent with the environment where the system is activated, PhoP/PhoQ allows *Salmonella* to grow at limited concentrations of Mg^{2+} (14), which results from PhoP-activated expression of the *mgtA* and *mgtB* genes, encoding Mg^{2+} transporters (34). The PhoQ-mediated PhoP phosphorylation also occurs at acidic pH (31), and transcription levels of the PhoP-activated genes *pagA*, *phoN*, and *pmrD* increase in *Salmonella* experiencing acidic conditions (3, 28, 31). Taking into account that certain antimicrobial peptides promote the expression of a subset of the PhoP-regulated genes through the PhoP and PhoQ proteins (2), the activity of the PhoP/PhoQ system appears to respond to at least three different signals.

The PhoP/PhoQ system also regulates gene expression by controlling the levels and/or activity of other regulators (22). The RstA/RstB two-component system, which consists of a response regulator RstA and its partner sensor RstB, is the one whose expression is regulated by the PhoP/PhoQ system. In *Escherichia coli*, the PhoP protein binds to the *rstA* promoter, and transcription of the *rstA* gene is repressed by the PhoP/PhoQ system in cells grown at a high concentration of Mg^{2+} (26). The RstA protein promotes transcription of the *asr* gene, coding for a product necessary for adaptation to acidic stress (27). Consequently, at acidic pH, transcription of the *asr* gene is not fully activated in a strain lacking the *phoP* gene (27), due to the reduced levels of the RstA protein. In addition, the *rstA* gene has been identified as a multicopy suppressor of the essential genes *yjeE*, *yeaZ*, and *ygjD* (5, 16).

The *Salmonella* PhoP protein also directly binds to and activates the *rstA* promoter at low Mg^{2+} (Fig. 1) (I. Zwir et al., unpublished data). We have recently demonstrated that when overexpressed from a plasmid, the RstA protein specifically binds to the *feoA* promoter and promotes transcription of the *feoAB* operon encoding the ferrous iron (Fe^{2+}) transporter FeoB (Fig. 1) (20).

The PhoP/PhoQ system promotes the expression of the RstA protein when activated at either low Mg^{2+} or acidic pH (Fig. 1). We now report that the RstA protein promotes transcription of the *feoB* gene exclusively when activated at acidic pH, thereby enhancing the growth of *Salmonella* in environments with limited iron. We show that, in contrast to the PhoP-controlled Fe^{2+} uptake, the expression of the Mg^{2+} transporter MgtA occurs only when the PhoP/PhoQ system is activated at low Mg^{2+}. Thus, depending on the input signals, a single signal transduction system can differentially regulate its target genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella enterica* serovar

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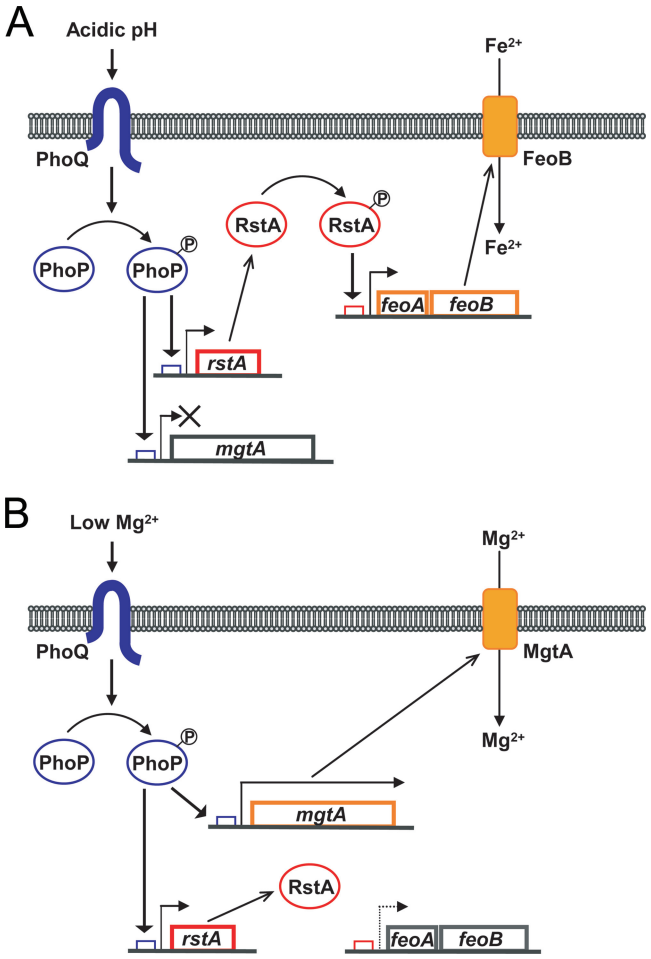

FIG. 1. Model illustrating Fe^{2+} and Mg^{2+} uptake regulated by the PhoP/PhoQ system and RstA protein in *Salmonella*. (A) When activated at millimolar Mg^{2+} concentrations by the acidic-pH signal, the phosphorylated PhoP protein promotes transcription of the *rstA* and *mgtA* genes. The induced RstA protein is activated, possibly by phosphorylation. The increase in *feoB* expression resulting from RstA binding to the *feoA* promoter enhances the FeoB-mediated Fe^{2+} uptake. In contrast, *mgtA* transcription fails to proceed due to Mg^{2+} binding to the *mgtA* leader. (B) The low-Mg^{2+} signal activates the PhoP/PhoQ system at neutral pH. Activation of *mgtA* transcription by phosphorylated PhoP increases production of the full length of the *mgtA* mRNA, leading to expression of the MgtA protein, which promotes Mg^{2+} uptake. In contrast, the RstA protein induced at neutral pH lacks the activity to promote transcription of the *feoB* gene.

Typhimurium strains are derived from strain 14028s. Phage P22-mediated transductions were performed as described previously (11). Bacteria were grown at 37°C in N-minimal medium (33), pH 7.7 or pH 5.7, supplemented with 0.1% Casamino Acids, 38 mM glycerol, and different concentrations of $MgCl_2$. Ampicillin, chloramphenicol, and kanamycin were used at 50 μg/ml, 25 μg/ml, and 50 μg/ml, respectively.

Construction of bacterial strains. The one-step gene inactivation method (10) was used for chromosomal gene deletion and epitope tagging. The sequences of primers used are indicated in Table S1 in the supplemental material. For construction of the *rstB* deletion strain, DS603, the Cm^r cassette from plasmid pKD3 (10) was amplified using primers DE-*rstB*-F and DE-*rstB*-R and integrated into the *rstB* region of strain 14028s. The EN323 strain, where 100 base pairs (bp) of the *mgtA* leader was replaced with 84 bp of the "scar" sequence, was constructed as described previously (9). The Km^r cassette from plasmid pKD4 (10) was amplified using primers DE-*mgtA*(5′UTR)-F and DE-*mgtA*(5′UTR)-R and was integrated into the chromosome of strain 14028s. The Km^r cassette was removed using plasmid pCP20 (10). To construct the EN252 strain, where the *ackA* and *pta* genes are deleted, the Km^r cassette was amplified using primers DE-*ackApta*-F and DE-*ackApta*-R and pKD4 as DNA template, and the purified PCR products were integrated into the chromosome of strain 14028s. The JH380 strain, in which both *sitABCD* and *mntH* are deleted, was constructed as follows. First, the Km^r cassette was amplified using primers DE-*sitABCD*-F and DE-*sitABCD*-R and pKD4 as DNA template; the Cm^r cassette was obtained by PCR amplification of pKD3 with primers DE-*mntH*-F and DE-*mntH*-R. The purified PCR products were introduced into strain 14028s, resulting in the Δ*sitABCD*::Km^r or Δ*mntH*::Cm^r strain, respectively. Second, the Δ*mntH*::Cm^r region was transferred into the Δ*sitABCD*::Km^r strain by phage P22-mediated transduction. Finally, both the Km^r and Cm^r cassettes were removed from the Δ*sitABCD*::Km^r Δ*mntH*::Cm^r strain using pCP20. Deletion of the corresponding genes was verified by colony PCR. The *Salmonella* Typhimurium strain DS604 encoding the RstA protein with a FLAG tag at the C terminus in the normal *rstA* chromosomal location was constructed as follows: the Km^r cassette was amplified by PCR using primers *rstA*-FLAG-F and *rstA*-FLAG-R and pKD4 as template and integrated at the 3′ end of the *rstA* gene. The Km^r cassette was removed from the resulting strain by using pCP20, and the presence of a FLAG tag at the C terminus of RstA was confirmed by nucleotide sequencing.

Plasmid construction. Plasmid pEN105 expressing the RstA-FLAG protein from the *lac* promoter was constructed as follows. The *rstA*-FLAG gene was amplified by PCR using primers CD-*rstA*-FLAG-F and CD-*rstA*-FLAG-R and chromosomal DNA from the DS604 strain. The PCR products were purified and introduced between the BamHI and PstI restriction sites of pUHE21-2*lacI*^{q} (36). Plasmid pEN106 is a derivative of pEN105 and expresses a variant of RstA-FLAG with a D52A substitution. This plasmid was constructed using a QuikChange II site-directed mutagenesis kit (Stratagene) with primers SM-*rstA*(D52A)-F and SM-*rstA*(D52A)-R and pEN105 as DNA template. For construction of the pDS303 plasmid in which PhoP*, a variant of the PhoP protein that can promote gene transcription independently of the PhoQ protein, is expressed from the *lac* promoter, the gene encoding PhoP* was amplified by PCR using primers CD-*phoP*-F and CD-*phoP*-R and chromosomal DNA from the EG10232 strain (7). The PCR products were purified and introduced between the BamHI and PstI restriction sites of pUHE21-2*lacI*^{q} (36). The se-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
S. enterica serovar Typhimurium strains		
14028s	Wild type	13
DS267	Δ*phoPQ*::*Cm*^{r}, same as EG15599	32
JH101	Δ*rstA*::*Km*^{r}	20
DS603	Δ*rstB*::*Cm*^{r}	This study
DS604	*rstA*-FLAG	This study
EN267	Δ*phoPQ*::*Cm*^{r} *rstA*-FLAG	This study
EN293	Δ*phoPQ*::*Cm*^{r} Δ*rstA*::*Km*^{r}	This study
EN252	Δ*ackA-pta*::*Km*^{r}	This study
EN323	Δ5′UTR-P*mgtA*	This study
EN331	Δ5′UTR-P*mgtA* Δ*phoPQ*::*Cm*^{r}	This study
JH380	Δ*sitABCD* Δ*mntH*	This study
EN256	Δ*sitABCD* Δ*mntH* Δ*rstA*::*Km*^{r}	This study
EN258	Δ*sitABCD* Δ*mntH* Δ*feoB*::*Cm*^{r}	This study
EN290	Δ*sitABCD* Δ*mntH* Δ*phoPQ*::*Cm*^{r}	This study
JH352	Δ*fur*	20
Plasmids		
pUHE21-2*lacI*^{q}	rep_{pMBI} Ap^r *lacI*^{q}	36
pKD3	$repR_{6K\gamma}$ Ap^r FRT Cm^r FRT	10
pKD4	$repR_{6K\gamma}$ Ap^r FRT Km^r FRT	10
pKD46	rep_{pSC101}(Ts) Ap^r P_{araBAD} γ β *exo*	10
pCP20	rep_{pSC101}(Ts) Ap^r Cm^r *c*I857 λP_R*flp*	10
pJH4	rep_{pMBI} Ap^r *lacI*^{q} *rstA*	20
pEN105	rep_{pMBI} Ap^r *lacI*^{q} *rstA*-FLAG	This study
pEN106	rep_{pMBI} Ap^r *lacI*^{q} *rstA*(*D52A*)-FLAG	This study
pDS303	rep_{pMBI} Ap^r *lacI*^{q} *phoP**	This study

[a] UTR, untranslated region; FRT, FLP recombination target.

quences of the *rstA*-FLAG gene and PhoP*-encoding regions on the recombinant plasmids were confirmed by nucleotide sequencing. The sequences of the primers used are indicated in Table S1 in the supplemental material.

RNA isolation and quantitative real-time reverse transcription-PCR (qRT-PCR) analysis. RNA was isolated from mid-exponential-phase culture (optical density at 600 nm [OD_{600}] of 0.5 to 0.6) grown in 20 ml of N-minimal medium. One-half milliliter of the culture was removed and mixed with 1 ml of RNAprotect bacterial reagent (Qiagen), and RNA was isolated using an RNeasy mini kit (Qiagen). The RNA sample was treated further with RNase-free DNase (Ambion). By using Omnitranscript reverse transcription reagents (Qiagen) and random primers (Invitrogen), cDNA was synthesized from 0.5 μg of RNA. Transcripts were quantified by real-time PCR using SYBR green PCR master mix (Applied Biosystems) on an ABI7300 sequence detection system (Applied Biosystems). The primers used for detection of transcripts of each gene are listed in Table S2 in the supplemental material. The transcription levels of each gene were calculated from a standard curve obtained by PCR with the same primers and serially diluted genomic DNA. The mRNA levels of target genes were normalized to 16S rRNA levels.

Western blot analysis. *Salmonella* strains harboring the *rstA*-FLAG gene were grown in 20 ml of N-minimal medium. When the cells' OD_{600} reached ~0.5, bacterial cells were washed once with phosphate-buffered saline, suspended in 0.5 ml of phosphate-buffered saline, and opened by sonication. Total protein concentrations were determined by the bicinchoninic acid method. Whole-cell lysates containing 25 μg of total proteins were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed by Western blotting using monoclonal anti-FLAG (Sigma) or anti-DnaK antibody (Stressgen). Blots were developed by using horseradish peroxidase-linked anti-mouse immunoglobulin G antibody (GE Healthcare) and an ECL detection system (GE Healthcare). We verified that signals corresponding to the RstA-FLAG proteins were within the linear range of detection by conducting the experiments with cell lysates containing 12.5, 25, and 50 μg of total protein.

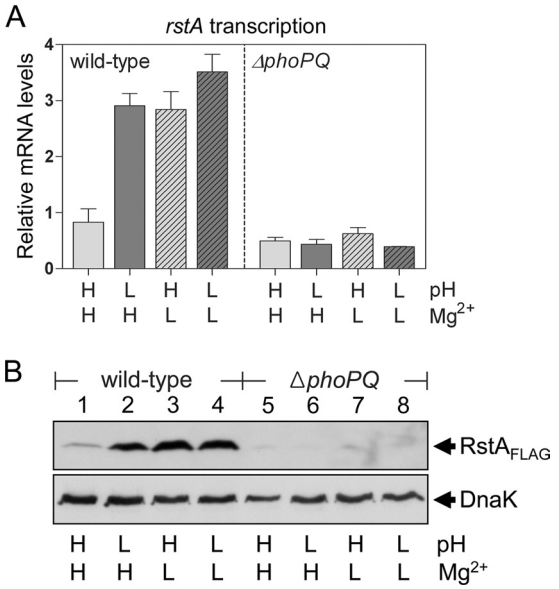

FIG. 2. The PhoP/PhoQ system activated by acidic pH or low Mg^{2+} promotes expression of the *rstA* gene. *Salmonella* strains were grown to an OD_{600} of 0.5 to 0.6 in N-minimal medium containing 2 mM (H, high) or 50 μM (L, low) Mg^{2+} and adjusted to pH 7.7 (H, high) or 5.7 (L, low). (A) The *rstA* mRNA levels in wild-type (14028s) and *phoPQ* deletion (DS267) strains were determined using qRT-PCR. Shown are the mean values and standard deviations from three independent experiments. (B) Western blot analysis of crude extracts prepared from wild-type (DS604) and *phoPQ* mutant (EN267) strains and probed with anti-FLAG and anti-DnaK antibodies.

RESULTS

The PhoP/PhoQ system promotes RstA expression at similar levels when activated at either low Mg^{2+} or acidic pH. We explored whether *rstA* expression could be controlled by acidic pH because low pH also plays a role as a signal activating the PhoP/PhoQ system (31). We isolated RNA from *Salmonella* strains grown in minimal medium containing activating (i.e., micromolar Mg^{2+} or acidic pH) or repressing (i.e., neutral pH with millimolar Mg^{2+}) signals for the PhoP/PhoQ system. The results of qRT-PCR revealed that the *rstA* mRNA levels were threefold higher in the wild-type strain grown at pH 7.7 with 50 μM Mg^{2+} or at pH 5.7 with 2 mM Mg^{2+} than at pH 7.7 with 2 mM Mg^{2+} (Fig. 2A). This activation was dependent on the PhoP/PhoQ system, because there was no *rstA* transcription in a strain with the *phoPQ* operon deleted (Fig. 2A). In agreement with the transcription data, Western blot analysis using the cell extracts prepared from strains expressing the RstA-FLAG protein from the normal chromosomal location showed the RstA protein in the wild-type strain only following growth in low Mg^{2+} or acidic pH (Fig. 2B, lanes 2 and 3). The RstA protein was hardly detected in the *phoPQ* deletion mutant, regardless of the Mg^{2+} concentration and pH value (Fig. 2B, lanes 5 to ~7), or in the wild-type strain that was grown at pH 7.7 with 2 mM Mg^{2+} (Fig. 2B, lane 1). Therefore, the data showed that both the low-Mg^{2+} and acidic-pH signal can promote RstA expression to similar levels in a process that is dependent on the PhoP/PhoQ two-component system.

The RstA protein, induced at acidic pH by the PhoP/PhoQ system, promotes transcription of the *feoB* gene. We have recently reported that overexpression of the RstA protein from a plasmid activates transcription of the *feoAB* operon encoding the ferrous iron transporter FeoB by direct RstA binding to the *feoA* promoter (20). Thus, we explored whether the RstA protein induced at low Mg^{2+} and acidic pH by the PhoP/PhoQ system could promote transcription of the *feoB* gene.

We determined that, when grown in the presence of 2 mM Mg^{2+}, the wild-type strain expressed fourfold higher levels of *feoB* mRNA at pH 5.7 than at pH 7.7 (Fig. 3A). The *feoB* activation required the PhoP/PhoQ-dependent RstA protein, because there was no *feoB* transcription in response to acidic pH in strains with *rstA* or *phoPQ* deleted (Fig. 3A). Transcription of the *feoB* gene was not activated in the wild-type strain grown at pH 7.7 with 50 μM Mg^{2+} (Fig. 3A). This is in spite of the fact that *Salmonella* bacteria experiencing low Mg^{2+} produced the RstA protein at levels similar to the levels in bacteria grown at acidic pH (Fig. 2B, lanes 2 and 3). When both signals were present (i.e., low Mg^{2+} and acidic pH), *feoB* transcription took place in an RstA- and PhoPQ-dependent manner (Fig. 3A). This result was not due to differences in the RstA protein levels between *Salmonella* experiencing pH 5.7 with 50 μM Mg^{2+} versus pH 7.7 with 50 μM Mg^{2+} (Fig. 2B, compare lanes 3 and 4). In sum, our experiments demonstrated that acidic pH can promote *feoB* transcription in an RstA-dependent fashion, whereas the low-Mg^{2+} signal is unable to elicit *feoB* transcription.

The RstB sensor is dispensable for activation of *feoB* transcription at acidic pH. Because the phosphorylation of a response regulator is primarily mediated by its cognate sensor kinase, we next asked whether the RstB sensor is responsible for the acidic-pH-promoted RstA activity. However, the RstB protein does not appear to affect *feoB* transcription because an *rstB* deletion mutant grown in medium with high (i.e., 2 mM)

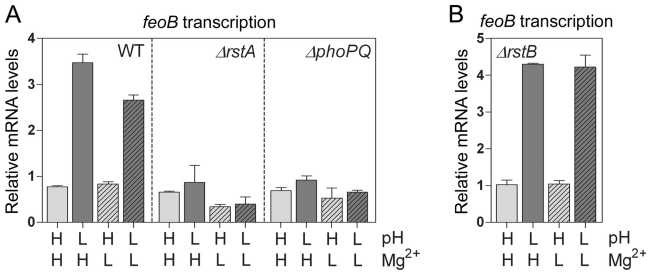

FIG. 3. Activation of *feoB* transcription at acidic pH is dependent on the PhoP/PhoQ and RstA proteins but independent of the RstB protein. Wild-type (WT; 14028s), *rstA* deletion (JH101), *phoPQ* deletion (DS267), and *rstB* deletion (DS603) strains were grown in N-minimal medium at pH 7.7 (H, high) or 5.7 (L, low) with 2 mM (H, high) or 50 μM (L, low) Mg^{2+}. The *feoB* mRNA levels were determined using qRT-PCR. Shown are the mean values and standard deviations from three independent experiments.

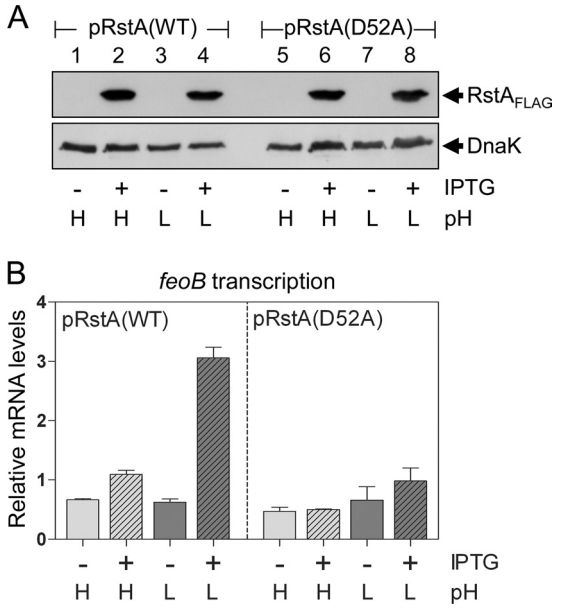

FIG. 4. RstA-promoted *feoB* transcription requires the acidic-pH signal and its putative phosphorylation site even when the *rstA* gene is transcribed from a heterologous promoter. Plasmids pEN105 [pRstA(WT)] and pEN106 [pRstA(D52A)] express the FLAG-tagged wild-type RstA and D52A RstA protein, respectively, from the *lac* promoter. Strains were grown in N-minimal medium containing 2 mM Mg^{2+} and buffered at pH 7.7 (H, high) or 5.7 (L, low) in the presence (+) or absence (−) of 0.1 mM IPTG. (A) Western blot analysis of crude extract prepared from the Δ*rstA* (JH101) strain harboring pEN105 or pEN106 and probed with anti-FLAG and anti-DnaK antibodies. (B) *feoB* mRNA levels produced by the Δ*rstA* (JH101) strain harboring pEN105 or pEN106. Shown are the mean values and standard deviations from three independent experiments.

or low (i.e., 50 μM) Mg^{2+} expressed fourfold higher levels of the *feoB* mRNA at pH 5.7 than at pH 7.7 (Fig. 3B), just like the wild-type strain. Thus, our data suggest that phosphorylation of the RstA protein could be mediated by a phosphodonor other than RstB during the growth of *Salmonella* at acidic pH.

Acidic pH promotes RstA activity, possibly via phosphorylation. The results presented above suggest that the activity of the RstA protein is promoted at acidic pH. To further explore the activation of the RstA protein, we constructed plasmid pEN105, where expression of the RstA-FLAG protein is under the control of the *lac* promoter. Western blot analysis revealed that the *rstA* deletion strain harboring pEN105 expressed the RstA-FLAG protein only in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), regardless of the pH of the medium (Fig. 4A, lanes 2 and 4). (These RstA-FLAG protein levels were similar to those expressed from a strain with a chromosomal *rstA*-FLAG gene experiencing acidic pH [data not shown]). The *feoB* mRNA levels were threefold higher in organisms grown at pH 5.7 than in those grown at pH 7.7 (Fig. 4B). Because the *rstA* gene is expressed from the *lac* promoter, these data indicate that RstA activity (as opposed to RstA level) is increased in acidic pH.

The activation of response regulators usually results in phosphorylation at a conserved aspartic acid residue (38). Thus, we reasoned that RstA phosphorylation might be responsible for the acidic-pH-promoted RstA activity. To test this idea, we constructed plasmid pEN106, expressing a mutant form of RstA-FLAG with a single amino acid substitution in the predicted phosphorylation site (i.e., a D52A substitution). We determined that when the *rstA* deletion strain carrying pEN106 was grown in the presence of IPTG, the *feoB* mRNA levels were slightly higher at pH 5.7 than at pH 7.7 but failed to reach the threefold difference exhibited by the isogenic strain with the original RstA-FLAG protein (Fig. 4B). The levels of the mutant RstA-FLAG protein were similar to those of the wild-type RstA-FLAG protein (Fig. 4A, compare lanes 4 and 8), indicating that the differences in *feoB* mRNA levels were not due to altered RstA-FLAG amounts. Cumulatively, these results suggest that the activity of the RstA protein is promoted under acidic pH conditions, possibly via phosphorylation of the conserved aspartic acid which, in turn, activates transcription of the *feoB* gene.

The PhoQ protein does not affect the activity of the RstA protein at acidic pH. Though it is rare, some response regulators can be phosphorylated by noncognate sensors (24). As RstB was not required for the RstA-promoted transcription of *feoB*, we wondered whether the PhoQ protein, whose activity is promoted at acidic pH (31), might be responsible for activation of the RstA protein at acidic pH. To test this idea, we compared *feoB* transcription between isogenic *phoPQ*$^+$ and *phoPQ*$^-$ strains that had the chromosomal copy of the *rstA* gene deleted and expressed the *rstA* gene from the *lac* promoter. This allowed us to explore the contribution of the PhoP/PhoQ system to RstA activity independently from its role in RstA expression. We determined that, when RstA protein was induced by IPTG, both the Δ*rstA* and Δ*rstA* Δ*phoPQ* strains displayed ~2.8-fold-higher levels of *feoB* transcription at pH 5.7 than at pH 7.7 (Fig. 5A), indicating that the PhoP and PhoQ proteins are not required for the acidic-pH-promoted RstA activity.

PhoP* is a variant of the PhoP protein that can promote gene transcription independently of the PhoQ protein (7). Thus, to further assess the participation of the PhoQ protein in RstA activity, we constructed a strain with the *phoPQ* operon deleted and carrying the pDS303 plasmid expressing the PhoP* protein from the *lac* promoter. We determined that the

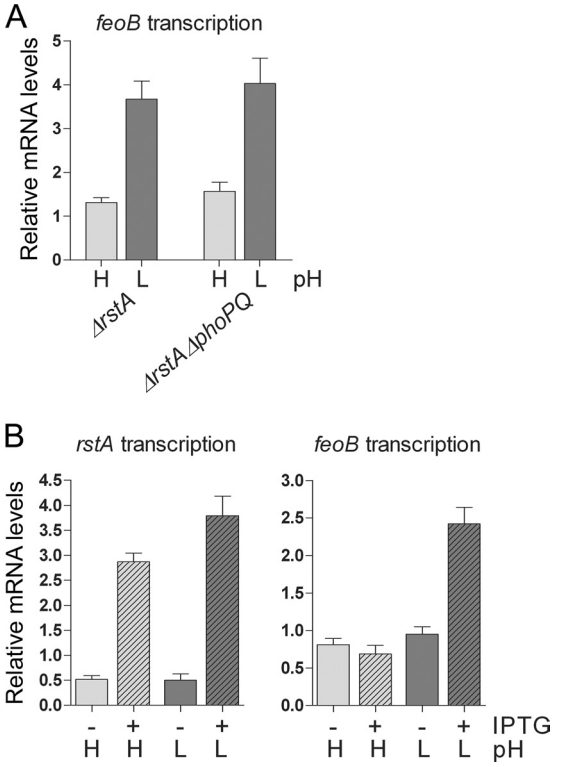

FIG. 5. The PhoP/PhoQ system is not required for RstA-promoted *feoB* transcription at acidic pH. (A) *feoB* mRNA levels produced by the Δ*rstA* (JH101) and Δ*rstA* Δ*phoPQ* (EN293) strains harboring pJH4, which expresses RstA from the *lac* promoter. The strains were grown in N-minimal medium containing 2 mM Mg^{2+} and buffered at pH 7.7 (H, high) or 5.7 (L, low) in the presence of 0.1 mM IPTG. (B) *rstA* and *feoB* mRNA levels produced by the Δ*phoPQ* strain (DS267) harboring pDS303, which expresses the PhoP* protein from the *lac* promoter. The strain was grown in N-minimal medium containing 2 mM Mg^{2+} and buffered at pH 7.7 (H, high) or 5.7 (L, low) in the presence (+) or absence (−) of 0.5 mM IPTG. Shown are the mean values and standard deviations from three independent experiments.

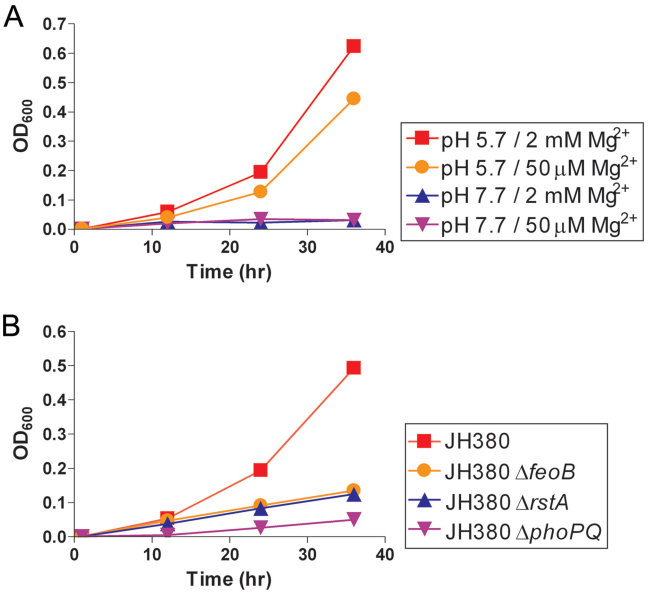

FIG. 6. FeoB induction at acidic pH by the PhoP/PhoQ and RstA proteins enhances *Salmonella* growth upon iron starvation. (A) Growth of *Salmonella* strain (JH380) lacking both the *sitABCD* and *mntH* genes in N-minimal medium at pH 7.7 or 5.7 with 2 mM or 50 μM Mg^{2+}. (B) Growth of the JH380 strain and its isogenic *feoB* (EN258), *rstA* (EN256), and *phoPQ* (EN290) deletion mutants in N-minimal medium at pH 5.7 with 2 mM Mg^{2+}. All media contained 2 mM sodium ascorbate and 10 μM diethylenetriaminepentaacetic acid. The results shown are representative of three experiments.

IPTG-induced PhoP* protein increased the expression of the *rstA* gene at both high and low pH (Fig. 5B). However, activation of *feoB* transcription was observed only at low pH (Fig. 5B). Note that the *rstA* expression levels promoted by the PhoP* protein were similar to those achieved by the low-Mg^{2+}- or acidic-pH-activated PhoP protein in the wild-type strain (compare Fig. 2A and 5B). This indicates that the RstA protein was active for *feoB* transcription in the absence of PhoQ, and it reinforces the notion that the PhoP/PhoQ system only controls the RstA protein levels.

Acidic pH promotes *feoB* transcription in a strain lacking acetyl phosphate production. We hypothesized that acetyl phosphate might be necessary for RstA activity at acidic pH because this small molecule serves as a phosphodonor for many response regulators (41). To test this, we determined the *feoB* transcription levels in a strain with both the *ackA* and *pta* genes, encoding the enzymes that are required for acetyl phosphate production, deleted (41). The Δ*ackA-pta* strain still produced threefold-higher levels of *feoB* mRNA at pH 5.7 than at pH 7.7, though the lack of *ackA* and *pta* genes slightly reduced the wild-type levels of *feoB* transcription at acidic pH (see Fig. S1 in the supplemental material). Thus, this result suggests that the RstA protein can be activated at acidic pH in the absence of acetyl phosphate.

Acidic activation of *feoB* transcription enhances *Salmonella* growth under iron-depleted conditions. We previously reported that RstA-promoted expression of the *feoAB* operon increases Fe^{2+} uptake in *Salmonella* (20). Thus, we hypothesized that FeoB expression at acidic pH might contribute to *Salmonella*'s ability to grow under Fe^{2+}-depleted conditions. However, wild-type *Salmonella* grew equally well in minimal medium that contained sodium ascorbate as an iron-reducing agent and the iron chelator diethylenetriaminepentaacetic acid for restriction of Fe^{2+} and was buffered at pH 7.7 and 5.7 (data not shown). Because *Salmonella* can also import Fe^{2+} using other transporters, such as SitABCD and MntH (4, 23), we reasoned that Fe^{2+} uptake via these routes might mask the effect of FeoB in our experimental conditions. Thus, we re-evaluated bacterial growth using strain JH380, which has both the *sitABCD* and *mntH* genes deleted. Upon Fe^{2+} restriction, strain JH380 grew in the medium at pH 5.7 but not at pH 7.7, regardless of the Mg^{2+} concentration (Fig. 6A). This growth difference is due to FeoB-mediated Fe^{2+} transport, because deletion of the *feoB* gene greatly impaired the growth of the JH380 strain in the Fe^{2+}-depleted medium adjusted to pH 5.7 (Fig. 6B). Moreover, consistent with the regulatory roles of the PhoP/PhoQ and RstA proteins in promoting *feoB* transcription (Fig. 3A), deletion of the *rstA* or *phoPQ* genes prevented the growth of JH380 experiencing acidic pH and Fe^{2+} limitation (Fig. 6B). Cumulatively, these results suggest that activation of the PhoP/PhoQ system and the RstA protein at acidic pH

increases FeoB-mediated Fe^{2+} uptake under iron-restricted conditions.

Iron represses *feoB* transcription at acidic pH. When bacteria are grown in the presence of iron, the Fur protein associates with Fe^{2+} to repress transcription of genes involved in iron acquisition (1). Because the *feoAB* operon is one of the Fur-regulated targets (20, 21), we explored how transcriptional activation of the *feoB* gene at acidic pH is affected by iron. On one hand, iron repressed *feoB* transcription in both the wild-type and the *rstA* deletion strain, but the effect was much stronger in the wild-type strain, demonstrating the RstA requirement in *feoB* transcription (see Fig. S2 in the supplemental material). On the other hand, deletion of the *fur* gene allowed *feoB* expression at acidic pH in both the presence and the absence of iron (see Fig. S2 in the supplemental material).

The mRNA leader sequence allows expression of the *mgtA* gene only when the PhoP/PhoQ system is activated at low Mg^{2+}. Given that *feoB* expression was promoted exclusively when the PhoP/PhoQ system was activated at acidic pH (Fig. 3A), we wondered whether there might be a PhoP-dependent gene(s) whose expression occurs only at low Mg^{2+}. To explore this possibility, we examined transcription of the Mg^{2+} transporter *mgtA* gene, which is directly activated by the PhoP protein (25). It has been previously reported that the β-galactosidase activity produced by a strain harboring a *lacZ* fusion within the *mgtA* coding region was 12 times higher in organisms grown in N-minimal medium at pH 7.7 with 10 μM Mg^{2+} than in those grown at pH 5.8 with 10 mM Mg^{2+} (35). Why is *mgtA* not being fully expressed despite the acidic-pH activation of the PhoP protein?

We focused on the recent finding that in response to intracellular Mg^{2+} levels, the expression of the *mgtA* gene is controlled by a riboswitch on the leader sequences (9). When Mg^{2+} concentration attains certain levels in the cytoplasm, Mg^{2+} binds to the *mgtA* leader to form a structure that does not favor transcription elongation into the coding region (9). Thus, we hypothesized that *mgtA* transcription that has been initiated at acidic pH fails to proceed unless cytoplasmic Mg^{2+} levels are sufficiently low. To test this idea, we examined *mgtA* transcription by conducting qRT-PCR with two primer sets, one specific to the coding region and the other targeting the first 100 nucleotides of the *mgtA* leader (Fig. 7A). We found that in the wild-type strain, the *mgtA* leader mRNA levels were 70- and 140-fold higher at pH 5.7 with 10 mM Mg^{2+} and at pH 7.7 without Mg^{2+}, respectively, than at pH 7.7 with 10 mM Mg^{2+} (Fig. 7B). In contrast, the *mgtA* coding region mRNA level was 1,000-fold higher at pH 7.7 without Mg^{2+} but only 6-fold higher at pH 5.7 with 10 mM Mg^{2+} than at pH 7.7 with 10 mM Mg^{2+} (Fig. 7C). As expected, both low-Mg^{2+}- and acidic-pH-mediated *mgtA* induction was greatly impaired in the Δ*phoPQ* mutant (Fig. 7B and C). These results suggest that *mgtA* transcription initiated in cells experiencing acidic pH fails to reach the coding region.

To evaluate the role of the *mgtA* leader region, we constructed a strain where the sequence corresponding to positions 148 to 247 of the *mgtA* leader was replaced with the 84-bp "scar" sequence (10) (Fig. 7A). This mutation has been previously shown to abolish the Mg^{2+}-sensing ability of the *mgtA* leader (9). We determined that the *mgtA* leader mutant strain expressed the *mgtA* coding region even at pH 5.7 with 10 mM

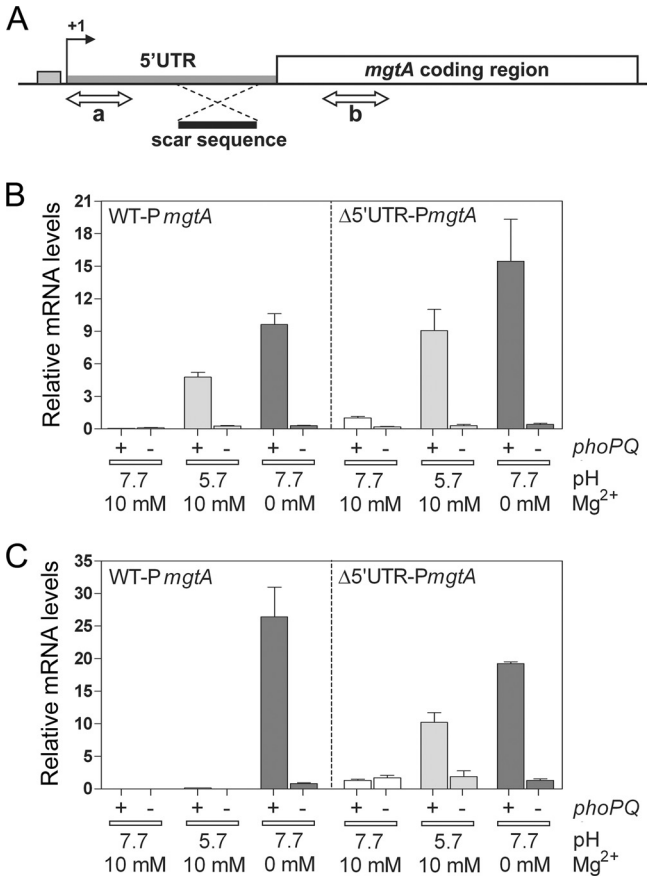

FIG. 7. The leader sequence allows the *mgtA* gene to be expressed only when *Salmonella* is starved for Mg^{2+}. (A) Schematic representation of the leader and coding regions of the *mgtA* gene. The double-headed arrows marked as "a" and "b" indicate the regions amplified by qRT-PCR using two pairs of primers. A part of the leader replaced with the "scar" sequence (10) in strain EN323 is also shown. (B) The wild-type *Salmonella* strain (14028s) and its *phoPQ* deletion mutant (DS267), *mgtA* leader mutant (EN323), and EN323 with the *phoPQ* deletion (EN331) were grown to an OD_{600} of 0.4 in N-minimal medium at pH 7.7 with 2 mM Mg^{2+} and transferred to medium at pH 7.7 or 5.7 in the presence or absence of Mg^{2+}. After 30 min, RNA was isolated from the strains, and the levels of *mgtA* mRNA corresponding to region "a" were determined by qRT-PCR. (C) By conducting qRT-PCR on the same RNA samples used for the experiment described for panel B, the *mgtA* transcripts corresponding to region "b" were quantified. Shown are the mean values and standard deviations from three independent experiments. UTR, untranslated region; WT, wild type; +, present; −, absent.

Mg^{2+} at levels that were 53% of those expressed in organisms grown at pH 7.7 without Mg^{2+} (Fig. 7C). This was contrary to the behavior of the wild-type strain grown at pH 5.7 with 10 mM Mg^{2+}, where the *mgtA* coding region mRNA level was only 0.5% of the level produced following growth at pH 7.7 without Mg^{2+} (Fig. 7C).

The *mgtCB* operon encodes another Mg^{2+} transporter, MgtB, whose expression is also determined at the transcription level by the PhoP protein and at the posttranscription level by the leader preceding the first gene in the operon (9, 37). To examine whether the differential pH and Mg^{2+} regulation displayed by the *mgtA* gene also applies to *mgtB*, we determined the mRNA levels for the *mgtB* coding region in the wild-type

and Δ*phoPQ* strains experiencing different growth conditions. The PhoP-dependent expression of the *mgtB* gene took place at low Mg^{2+} but not at acidic pH with high Mg^{2+} (see Fig. S3 in the supplemental material), indicating that *mgtB* transcription can reach the coding region only when intracellular Mg^{2+} levels are sufficiently low. Our results suggest that the leader regions allow the MgtA and MgtB proteins to be produced only when *Salmonella* faces limiting Mg^{2+} levels, dramatically decreasing the expression of these Mg^{2+} transporters when the PhoP/PhoQ system is activated by the acidic-pH signal at high Mg^{2+} concentrations.

DISCUSSION

Certain two-component regulatory systems are activated by multiple signals. For example, the PhoP/PhoQ system is activated when *Salmonella* is grown in low Mg^{2+} (14), in an acidic pH (31), or with certain antimicrobial peptides (2). In the case of the PmrA/PmrB system, both ferric iron (42) and acidic pH (28) promote PmrB activity. These facts raise the question of whether a single regulatory system controls the expression of distinct sets of genes when activated by different signals. We have now demonstrated that the acidic-pH activation of the PhoP/PhoQ system promotes transcription of the Fe^{2+} transporter-encoding gene *feoB*, whereas its activation by low Mg^{2+} promotes the expression of the Mg^{2+} transporter-encoding *mgtA* gene (Fig. 1).

Regulation of *feoB* expression by the PhoP/PhoQ system and the RstA protein. We demonstrated that the PhoP/PhoQ system promotes the expression of the FeoB Fe^{2+} transporter when activated by acidic pH but fails to do so when activated at neutral pH by the low-Mg^{2+} signal (Fig. 3A). This control requires a second response regulator, RstA, which functions as the direct activator of *feoB* transcription (20). PhoP binds to the *rstA* promoter region (I. Zwir et al., unpublished) and activates *rstA* transcription (29, 30). Even though the low-Mg^{2+} and acidic-pH signals can promote the expression of the RstA protein equally well (Fig. 2), transcription of the RstA-dependent *feoB* gene took place only when bacteria experienced an acidic pH (Fig. 3A).

We propose that acidic pH controls RstA activity via phosphorylation, because the RstA protein expressed from a heterologous promoter activated *feoB* transcription at pH 5.7 but not at pH 7.7 and because the activation required RstA's predicted phosphorylation site (i.e., D52) (Fig. 4). Although phosphorylation of a response regulator is generally mediated by its cognate sensor kinase, intriguingly, the acidic-pH activation of *feoB* transcription still took place in a mutant with a deletion of the *rstB* gene, which codes for the cognate sensor for RstA (Fig. 3B). That the RstA protein promoted *feoB* transcription at acidic pH in the absence of PhoQ (Fig. 5) argues against the possibility of cross-phosphorylation of RstA by the noncognate sensor PhoQ. In addition, the finding that acidic pH still activates *feoB* transcription in a strain that does not synthesize acetyl phosphate rules out the possibility of RstA phosphorylation by this small-molecule phosphodonor (see Fig. S1 in the supplemental material). Therefore, it is likely that a sensor kinase other than RstB and PhoQ might phosphorylate the RstA protein, because some response regulators are phosphorylated by noncognate sensors (24).

PhoP/PhoQ-mediated iron homeostasis. Under aerobic conditions at neutral pH, iron is present in an oxidized ferric form. Consistent with the notion that iron is reduced to a ferrous form under anaerobic conditions, transcription of the *feoAB* operon increases when *E. coli* is grown without oxygen (21). What, then, is the biological significance of the pH-regulated *feoB* expression? It has been found that *Salmonella* possesses extracellular enzyme activities to reduce iron (40) and that an acidic pH keeps ferrous iron stable in the presence of oxygen (6). Thus, FeoB induction resulting from activation of the PhoP/PhoQ system and RstA protein could enhance Fe^{2+} uptake in *Salmonella* growing in acidic environments with limited iron.

In bacterial cells grown with oxygen, cytoplasmic Fe^{2+} participates in the Fenton reaction and catalyzes the formation of hydroxyl radicals, which causes DNA damage (39). The Fur protein is a primary regulator that senses intracellular Fe^{2+} levels (1). When associated with Fe^{2+}, the Fur protein represses the expression of genes for iron acquisition, which minimizes the accumulation of free Fe^{2+} in the cytoplasm (1, 12). Not surprisingly, the Fur protein repressed *feoB* transcription even at acidic pH when iron was plentiful (see Fig. S2 in the supplemental material).

The PhoP/PhoQ system is also necessary for the survival of *Salmonella* under Fe^{2+}-dependent oxidative stress (8). Although the CorA protein is a Mg^{2+} transporter, it has been reported to import Fe^{2+} as well (17). When aerobically grown at low Mg^{2+}, a *Salmonella phoP* mutant displayed increased Fe^{2+} accumulation in a process dependent on the CorA protein (8). The finding that neither the expression level of the CorA protein nor its membrane location is affected by the PhoP protein implies the presence of a PhoP-regulated gene product(s) regulating CorA activity (8). Indeed, the *phoP* mutant was hypersensitive to Fe^{2+}-dependent oxidative-stress-mediated killing, which was rescued by inactivation of the *corA* gene (8). Taking these findings together, the PhoP/PhoQ system is likely to play dual roles in iron homeostasis: PhoP enhances Fe^{2+} uptake when iron is scarce at acidic pH, whereas downregulation of CorA activity at low Mg^{2+} protects *Salmonella* from Fe^{2+}-mediated killing.

PhoP/PhoQ-controlled Mg^{2+} uptake. *Salmonella* imports Mg^{2+} via three transporters: CorA, MgtA, and MgtB (18, 19, 33). The *corA* gene is constitutively expressed (8), whereas transcription of the *mgtA* and *mgtB* genes is directly activated by the PhoP protein (25, 43). As opposed to the PhoP-controlled Fe^{2+} uptake at acidic pH, we determined that MgtA expression takes place only at low Mg^{2+} (Fig. 7). This is because the *mgtA* leader functions as an RNA sensor (9) such that when the cytoplasmic Mg^{2+} concentration reaches a certain high level, Mg^{2+} binds to the *mgtA* leader to form a structure that prevents RNA polymerase from proceeding to the *mgtA* coding region (9). The full-length transcript, including the *mgtA* coding region, is produced when intracellular Mg^{2+} is low enough to promote the formation of a different structure in the leader region (9).

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