Low Mg$^{2+}$ promotes phosphorylation of the response regulators PhoP and PmrA and transcription of their activated genes in Salmonella enterica. Using chromatin immunoprecipitation, we have now determined that the PhoP and PmrA proteins are recruited to the regulatory region of their target genes when bacteria experience low Mg$^{2+}$ but not when they are grown in high Mg$^{2+}$. Even when the PhoP protein was artificially produced at 4-fold higher levels than the wild-type strain, promoter occupancy required the low Mg$^{2+}$ signal. Substitution of the predicted phosphorylation site Asp-52 with a valine residue abolished phosphorylation of the PhoP protein, resulting in loss of PhoP binding to target promoters and transcription of PhoP-activated genes. Our results indicate that the promoter binding ability of the PhoP and PmrA proteins occurring in low Mg$^{2+}$ is correlated with phosphorylation of these proteins in vivo.

The PhoP/PhoQ two-component system governs the adaptation to low Mg$^{2+}$ virulence in mammals as well as several other physiological functions in Salmonella enterica and other Gram-negative bacteria (1). The Mg$^{2+}$ sensor PhoQ (2) controls the phosphorylated state of the response regulator PhoP by a combination of autokinase, phosphotransferase, and phospho-PhoP phosphatase activities (3–5): low Mg$^{2+}$ favors phosphorylation of the PhoP protein and transcription of PhoP-activated genes (2, 6), whereas high Mg$^{2+}$ promotes dephosphorylation of phospho-PhoP, thereby preventing expression of PhoP-activated genes (2, 3). Low Mg$^{2+}$ also stimulates transcription of genes under the control of the PmrA/PmrB two-component system (6) in a mechanism that involves the PhoP-activated PmrD protein (7) promoting the phosphorylated state of the PmrA protein (8).

In vitro experiments have demonstrated that phosphorylation of the PhoP protein promotes a conformational change in its C-terminal DNA-binding domain (9), which increases the affinity of the PhoP protein for its promoters (9, 10). Likewise, phospho-PmrA binds more tightly to its target promoters than unphosphorylated PmrA (11). Surprisingly, overexpression of the PhoP protein can reportedly promote gene transcription in a phoQ mutant or when the putative phosphorylation site of the PhoP protein has been mutated (12), suggesting that phosphorylation of the PhoP protein may not be required for binding to its target promoters. To examine the role of low Mg$^{2+}$-induced phosphorylation in PhoP- and PmrA-regulated gene expression, we investigated binding of the PhoP and PmrA proteins to their target DNAs in vivo using chromatin immunoprecipitation (ChIP).

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains, Plasmids, and Growth Conditions—*Bacterial strains and plasmids used in this study are listed in Table I. *S. enterica serovar Typhimurium* strains used in this study are derived from strain 14028s. Phage P22-mediated transductions were performed as described (13). A strain with chromosomally encoded epitope-tagged proteins was constructed as follows. First, the Cm$^r$ cassette from plasmid pKD3 was amplified using primers 2566 and 2567 and integrated at the 3’ end of the phoP gene in the chromosome by selecting for Cm$^r$ transfectants (14). Primer 2566 (5’-AGGGCAGCGCGAGCGGGGCGG- AAATGGCGGCAAATTTCTTGCATGATATCCCTCTCGTAG-3’) harbors the sequence immediately downstream of the start codon of phoQ attached to priming site 2 of pKD3 harboring a ribosome binding site and start codon (14). Primer 2567 (5’-TACACCGTCAGCGGACACAGGATATCTTATGGACCTGCTATCGTATGATTGCTAGCTCTGATATGAGGCCTGGTTCATGCTGGTTGACACTGAGGAAAG-3’) was designed to encode the HA sequence immediately upstream of the stop codon of the phoQ gene following the priming site 1 sequence of pKD3 (14). The Cm$^r$ cassette was removed using plasmid pCP20 as described (14) and the resulting strain was designated EG13918. And second, we used primers 2491 (5’-TGGCCGCTTGCTGCTAGCTGTTCTCCACTGCGGAGAAG-CGACTAACAGGACGAGCTACAGAATGCTGCTAGCTGGACCTGCTGCTG-3’) and 2492 (5’-TTAACCCCGTGCAGAAGGCTACATGAGCTGCTGGACCTGCTGCTG-3’) to amplify the Cm$^r$ cassette from plasmid pKD3 and integrated the PCR product at the 3’ end of the pmrA gene in a strategy analogous to that described above. The pmrA-FLAG::Cm$^r$ allele was then transferred to strain EG13918 by P22 transduction and the Cm$^r$ cassette was removed using plasmid pCP20. The resulting strain was designated EG13922. The regions corresponding to the junctions between phoP-HA and phoQ, and of pmrA-FLAG and pmrB were amplified from the chromosome by PCR and were confirmed to have the predicted sequences by nucleotide sequencing. To construct a strain harboring a chromosomal deletion of the *phoP* and *phoQ* genes (EG15599), the Cm$^r$ cassette from plasmid pKD3 was amplified using primers 3726 and 4986 (14). Primer 4986 (5’-AAGCCTGACTTCTTCTTATGACAAACGGACGAGCTACAGAATGCTGCTAGCTGGACCTGCTGCTG-3’) carries the sequence immediately upstream of the start codon of the *phoQ* gene following priming site 1 sequence of pKD3 (14). Primer 3726 (5’-GGATGCTTAAACAGGATGCTGGACCTGCTGCTGCTG-3’) was designed to amplify the Cm$^r$ gene in the chromosome by selecting for Cm$^r$ transfectants (14). Primer 2566 (5’-AGGGCAGCGCGAGCGGGGCGGAAATGGCGGCAAATTTCTTGCATGATATCCCTCTCGTAG-3’) harbors the sequence immediately downstream of the start codon of *phoQ* attached to priming site 2 of pKD3 (14). The resulting PCR product was integrated into the chromosome as described above. Deletion of the *phoP* and *phoQ* genes was verified by PCR and transferred to the wild-type strain by P22 transduction. Plasmid pUHE-phoP-HA/phoQ (pEG13918) was constructed by cloning between the EcoRI and PstI sites of pUHE21–2ac $^+$ (15) a PCR fragment containing the *phoP-HA/phoQ* coding region generated with

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the and chromosomal DNA from strain EG13918 as template. Sequence of 38 mM glycerol, and the indicated concentration of MgCl₂. Ampicillin minimal medium, pH 7.7 (17), supplemented with 0.1% casamino acids, Bacteria were grown at 37 °C in Luria-Bertani broth (LB) (16) or N-
using the QuikChange II Site-directed Mutagenesis Kit (Stratagene). (D52V substitution) or PhoQ (T281R substitution) were constructed
nucleotide sequencing. Plasmids for overexpression of variants of PhoP
saline and opened by sonication. A whole cell lysate (30 l of lysis solution (50 mM Tris, pH 8.0, 100 mM EDTA, 50 mM NaF, and
amide gel. After drying the gel, autoradiography was performed for shown if larger than the resolution of the figure).
alkaline growth, formaldehyde (1%) was added to the cultures and placed at
duplicate. Error bars correspond to the standard deviation (and are only
duplicate and the activity was determined as described (18). All assays
4% SDS), which was 20-fold diluted with ice-cold IP buffer (50 mM Tris,
Western Blot Analysis—Bacteria were grown in N-minimal medium,
and isopropyl 1-thio-

cell lysate (30 l of protein) was run on a bis-Tris 4–12% gradient gel (Invitrogen) with MES-SDS running buffer, transferred to nitrocellulose membrane, and analyzed by Western blot using monoclonal anti-HA antibody (Sigma). Western blot was developed using anti-mouse IgG horseradish peroxidase-linked antibody and the ECL detection system (Amersham Biosciences).

In Vivo Phosphorylation of the PhoP Protein—Cells were grown in low phosphate N-minimal medium (0.3 mM phosphate) supplemented with 10 mM or 10 l MgCl₂. Cells in mid-exponential growth phase were taken and incubated with 100 lCi of 32-phosphorus (Amersham Biosciences) for 30 min. After centrifugation, cells were suspended in 50 l of lysis solution (50 mM Tris, pH 8.0, 100 mM EDTA, 50 mM NaF, and 4% SDS), which was 20-fold diluted with ice-cold IP buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 50 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). The PhoP protein was immunoprecipitated using monoclonal anti-HA antibody (Sigma) and a protein A affinity gel (Sigma) sequentially. After vigorously washing the beads (three times with IP buffer), the precipitates were eluted with 2× SDS sample buffer at 37 °C for 5 min and resolved on 12% SDS-polyacrylamide gel. After drying the gel, autoradiography was performed for detection of phosphorylated proteins.

β-Galactosidase Assay—β-Galactosidase assays were carried out in duplicate and the activity was determined as described (18). All assays correspond to mean values of three independent experiments done in duplicate. Error bars correspond to the standard deviation (and are only shown if larger than the resolution of the figure).

Chromatin Immunoprecipitation Assay—ChIP assays were performed as described (19) with the following modifications. After bacterial growth, formaldehyde (1%) was added to the cultures and placed at room temperature for 15 min, before quenching the reaction with gly

| Strain or plasmid | Description | Ref. or source |
|-------------------|-------------|---------------|
| S. enterica serovar Typhimurium | Wild-type | 28 |
| MS7951s | phoP7953::Tn10 | 28 |
| EG9241 | pbgP1::MudJ | 2 |
| EG9252 | phoP9252::MudJ phoP7953::Tn10 | 15 |
| EG9480 | pmgC9480::MudJ | 2 |
| EG9521 | mgtA9521::MudJ | 2 |
| EG9523 | mgtA9523::MudJ phoP7953::Tn10 | 2 |
| EG9526 | mgtC9526::MudJ phoP7953::Tn10 | 2 |
| EG13659 | pmrD-FLAG lacZY KmR | 29 |
| EG13828 | pmrA-FLAG CmR | This work |
| EG13917 | phoP-FLAG CmR | This work |
| EG13918 | phoP-HA | This work |
| EG13921 | phoP-HA pmrA-FLAG CmR | This work |
| EG13922 | phoP-HA pmrA-FLAG | This work |
| EG13923 | phoP-HA pmrA-FLAG mgtA9226::MudJ | This work |
| EG14334 | phoP-HA pmrA-FLAG pmrD-FLAG lacZY KmR | This work |
| EG14336 | phoP-HA pmrA-FLAG phoP552::MudJ | This work |
| EG14337 | phoP-HA pmrA-FLAG pmrC9505::MudJ | This work |
| EG15559 | ApoPQ::CmR | This work |
| EG16032 | ApoPQ::CmR mgtA9226::MudJ | This work |
| E. coli | | |
| DH5α | F’ supE44 lacU169 (Δ80 lacZDΔM15) hisΔR17 recA1 endA1 gyrA96 thi-1 relA1 | 30 |
| Primers | Target promoters | Sequences |
|-------------------|----------------|------------|
| 2777 | phoP forward | 5’-TGAAACAGTGTTATGATGCTTGCC-3’ |
| 2778 | phoP reverse | 5’-TGTCGGTAAATGCTATCCTCTC-3’ |
| 2779 | mgTA forward | 5’-GGCGAGTGCTTTGATGATGGC-3’ |
| 2780 | mgTA reverse | 5’-CTCCCTGTGAATATATATAATTTGGC-3’ |
| 2783 | pmrD forward | 5’-AGACGGTGAACCTCGCTGAAATG-3’ |
| 2784 | pmrD reverse | 5’-ACAGACCCAGAAACTGTCGC-3’ |
| 2785 | pmrC forward | 5’TGAATGGTGATCGGCGCTTGATTG-3’ |
| 2786 | pmrC reverse | 5’-TCAGCGGAGCGAGTATAATAAAGG-3’ |
| 300528 | pbgP forward | 5’-ATGGAAATTCGCCAGGAGACGAG-3’ |
| 3006 | pbgP reverse | 5’-GCCGCCGTTTTTTATAACATTGGGAGC-3’ |
| 3552 | mgTC forward | 5’-TACGTGCCAGGCATCATACAGGAGC-3’ |
| 3553 | mgTC reverse | 5’-TACGTGCCAGGCATCATACAGGAGC-3’ |
FIG. 1. A, schematic representation of chromosomal phoPQ and pmrCAB operons in strain EG13922, which encodes C-terminal HA-tagged PhoP and FLAG-tagged PmrA proteins. Arrows indicate transcription start sites of the phoPQ and pmrCAB operons. The black square indicates the “scar” sequence present at these loci as a result of the one-step inactivation method (14). Binding sites of the PhoP and PmrA proteins are indicated by striped boxes. B, β-galactosidase activities (Miller units) expressed by strains harboring lac-transcriptional fusions to the PhoP-activated mgtA and pmrD promoters and the PmrA-activated pmrC and pbgP promoters measured both in the wild-type (EG13923, EG14334, EG14337, and EG14336) and tagged (EG9521, EG13659, EG9460, and EG9241) strains grown in N-minimal medium, pH 7.7, with 10 mM (H) or 10 μM (L) Mg²⁺.

phosphate-buffered saline twice. The cells were lysed in 0.5 ml of lysis solution (10 mM Tris, pH 8.0, 50 mM NaCl, 10 mM EDTA, 20% sucrose, 10 mg/ml lysozyme) and 0.5 ml of 2%H₁₁₀₀₃ RIPA solution (100 mM Tris, pH 8.0, 300 mM NaCl, 2% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS). The cell extract was sonicated to fragment DNA to an average size of 500 bp. Fifty microliters of the extract was removed for total DNA preparation. For immunoprecipitation of PhoP cross-linked DNA, a portion of the extract (400 μl) was incubated with 4 μl of monoclonal anti-HA H7 antibody (Sigma) at 4 °C for 4 h. PmrA cross-linked DNA was immunoprecipitated with 4 μl of monoclonal anti-FLAG M2 antibody (Sigma) using the same cell extract. After incubation with Protein G affinity gel (Sigma) for 1 h at 4 °C, the beads were washed twice with 1× RIPA solution, then twice with LiCl/detergent solution (10 mM Tris, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and finally with TE buffer. The immunoprecipitated material was eluted with 100 μl of elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS). Cross-linking of immunoprecipitated and total DNA was reversed by incubation at 65 °C overnight. After Proteinase K (Invitrogen) treatment, the immunoprecipitated and total DNA were purified using phenol extraction, precipitated with ethanol, and resuspended in TE buffer.

Quantitative PCR Analysis—Analysis of the immunoprecipitated DNA was performed using quantitative PCR as described (19). All promoter-specific primers used in this study are listed in Table II. Reactions were performed in 25 μl containing 1/100 of immunoprecipitated DNA (or 1/10,000 of total DNA), 1 μM of each primer, 0.1 mM dNTPs, and 0.1 mCi/ml of [γ⁻³²P]dATP with 27 cycles of amplification. To determine the linearity of the reactions, the PCRs were performed with each set of primers and serially diluted total DNA as templates. The PCR products were resolved on a 5% polyacrylamide gel and the signals of the products were quantified using a FLA-5000 Imaging System (Fuji Film). For quantification of the immunoprecipitated DNA, real-time PCR was used. The reaction containing the 1/50 of immunoprecipitated DNA

FIG. 2. The PhoP and PmrA proteins are recruited to their target promoters in response to the low Mg²⁺ signal. A, in vivo PhoP and PmrA binding to target promoters in wild-type (14026s) and tagged (EG13922) strains examined using ChIP assay. Immunoprecipitated (IP) and total (input) DNA were subject to PCR using primers specific to target promoters. B, the results of the PCR shown in A were quantified using a phosphorimager. The “relative IP” of each promoter represents the value of the immunoprecipitated signal divided by the particular input signal, which corresponds to the total DNA before precipitation.
(or 1/1,000 of total DNA) was performed using SYBR Green PCR Master Mix (Applied Biosystems) and amplification of the PCR product was analyzed using the ABI 7000 Sequence Detection System (Applied Biosystems). For amplification of the mgtA, phoP, and rpoD promoter regions, primers 3041 (5'-TCAAGCAGGGCAATGG-3') and 3042 (5'-ATGGGATGTATCAGGTTAGC-3'), and primers 3039 (5'-ATCGGTGCTGACTTG-3') and 3040 (5'-AGAGGGTGAGGCAGGCATT-3'), and primers 4149 (5'-ACCCTGCAAAATGATGCT-3') and 4150 (5'-TCCGGCAGCTTATCGT-3') were used, respectively. The amount of PCR product was calculated from standard curves obtained from PCR with primer-specific primers and serially diluted total DNA.

RESULTS

A Strain Expressing Chromosomally Encoded Epitope-tagged PhoP and PmrA Proteins That Displays a Wild-type Response to Mg$^{2+}$—To facilitate the recovery of DNA fragments bound by the PhoP and PmrA proteins, we constructed a strain (EG13922) that expressed a PhoP protein with an HA epitope tag at its C terminus and a PmrA protein with a FLAG epitope tag at its C terminus from their respective wild-type promoters and chromosomal locations as described under “Experimental Procedures” (Fig. 1A).

We established that strain EG13922 displayed a normal response to Mg$^{2+}$ as transcription of the PhoP-activated mgtA and pmrD genes, and of the PmrA-activated pmrC and pbgP genes was induced in low (10 μM) Mg$^{2+}$ and repressed in high (10 mM) Mg$^{2+}$-minimal medium, pH 7.7 (17) (Fig. 1B). Furthermore, the epitope-tagged PhoP and PmrA proteins promoted gene transcription at similar levels as the wild-type PhoP and PmrA proteins (Fig. 1B). These results indicate that the HA and FLAG tags do not interfere with the ability of the PhoP and PmrA proteins to regulate gene transcription.

Binding of the PhoP and PmrA Proteins to Their Target Promoters Is Dependent on Low Mg$^{2+}$ Signal—To examine binding of the PhoP and PmrA proteins to their target promoters in vivo, we carried out a ChIP experiment (19) using bacteria experiencing either high (10 mM) or low (10 μM) Mg$^{2+}$. When PCR was performed on the DNA that was precipitated with the anti-HA antibody (to recover DNA fragments bound by the PhoP protein) using primers specific for the mgtA and pmrD promoters, a dramatic enrichment of PhoP-associated DNA was observed for the epitope-tagged strain grown in low Mg$^{2+}$ (Fig. 2, A and B). On the other hand, PhoP barely bound to the mgtA and pmrD promoters in cells experiencing high Mg$^{2+}$ (Fig. 2, A and B). Analysis of the PmrA-dependent pmrC and pbgP promoters showed the same binding pattern: PmrA-associated DNA was observed only in the epitope-tagged strain grown in low Mg$^{2+}$ (Fig. 2, A and B). The ChIP assay was specific because: first, there was no significant enrichment of PhoP- and PmrA-bound DNA to the examined promoters when a wild-type (i.e. lacking epitope tags) strain was used (Fig. 2, A and B). And second, no products were recovered when the PCR was performed using primers specific for the phoP coding region or the rpoD promoter, which lack sequences resembling the PhoP box (data not shown). These results demonstrate that the PhoP and PmrA proteins bind to their target promoters in response to low Mg$^{2+}$.

PhoP Recruitment to Target Promoters Requires the Low Mg$^{2+}$ Signal Even When the PhoP Protein Is Produced at Non-physiological Higher Levels—Because transcription of the phoPQ operon is positively autoregulated (15), binding of the PhoP protein to the mgtA and pmrD promoters in cells experiencing low Mg$^{2+}$ (Fig. 2, A and B) could be mediated by the PhoP protein present at higher levels (as opposed to higher levels of phosphorylated PhoP protein). To address this issue, we constructed a strain that lacked the ability to positively autoregulate phoPQ transcription because it was deleted for the chromosomal copy of the phoP gene and harbored plasmid pEG13918, which expresses the phoP-HA and phoQ genes from the lac promoter. Western blot analysis revealed that, as expected, production of the PhoP protein was dependent on the inducer of the lac promoter and independent of the Mg$^{2+}$ concentration (Fig. 3A). When grown in low Mg$^{2+}$ and in the presence of IPTG (final concentration 0.5 mM), the PhoP protein levels were 4-fold higher in the strain with the plasmid-encoded PhoP-HA than in the strain producing the chromosomally encoded PhoP-HA protein (data not shown).

We established that binding of the PhoP protein to the PhoP-activated phoP, mgtA, and mgtC promoters required both IPTG and low Mg$^{2+}$, as cells experiencing IPTG and high Mg$^{2+}$ displayed weak PhoP binding to these promoters (Fig. 3, B and...
No significant binding of the PhoP protein was observed in cells grown without IPTG regardless of the Mg\(^{2+}\)/H\(_{11001}\) levels (Fig. 3, B and C). The transcriptional activities of the three examined promoters were consistent with the results of the ChIP experiment: expression was observed only in cells grown with IPTG and low Mg\(^{2+}\)/H\(_{11001}\) (Fig. 3D), even though this strain produced similar levels of PhoP protein in high and low Mg\(^{2+}\)/H\(_{11001}\) (Fig. 3A).

The D52V Mutation in the Putative Phosphorylation Site of the PhoP Protein Prevents PhoP Binding to Its Target Promoters in Vivo—To examine the role of phosphorylation on promoter occupancy, we conducted a ChIP experiment using cells that expressed a mutant form of PhoP with the D52V amino acid substitution in its predicted phosphorylation site. The D52V mutation abolishes transcription of PhoP-regulated genes. Binding to the target promoters was not detected with the PhoPD52V protein in cells experiencing the low Mg\(^{2+}\)/H\(_{11001}\) signal (Fig. 4, A and B), which is in contrast to what we observed using the PhoP protein with the intact phosphorylation site (Fig. 3, B and C).

The DNA Binding Ability of the PhoP Protein Is Correlated with Its Phosphorylation State in Vivo—To examine the role of phosphorylation on promoter occupancy, we conducted a ChIP experiment using cells that expressed a mutant form of PhoP with the D52V amino acid substitution in its predicted phosphorylation site. The D52V mutation abolishes transcription of PhoP-regulated genes. Binding to the target promoters was not detected with the PhoPD52V protein in cells experiencing the low Mg\(^{2+}\) signal (Fig. 4, A and B), which is in contrast to what we observed using the PhoP protein with the intact phosphorylation site (Fig. 3, B and C).

The DNA Binding Ability of the PhoP Protein Is Correlated with Its Phosphorylation State in Vivo—To examine the role of phosphorylation on promoter occupancy, we conducted a ChIP experiment using cells that expressed a mutant form of PhoP with the D52V amino acid substitution in its predicted phosphorylation site. The D52V mutation abolishes transcription of PhoP-regulated genes. Binding to the target promoters was not detected with the PhoPD52V protein in cells experiencing the low Mg\(^{2+}\) signal (Fig. 4, A and B), which is in contrast to what we observed using the PhoP protein with the intact phosphorylation site (Fig. 3, B and C).

Cells harboring either pEG13920 or pEG13921 were grown with 32-phosphorus and the PhoP protein was immunoprecipitated with anti-HA antibody. Phosphorylation of the wild-type PhoP protein was observed in cells grown in media containing IPTG (Fig. 5A). Phosphorylation was detected even in high...
Mg\(^{2+}\) concentrations, presumably because PhoQ\(^{T281R}\) protein fails to dephosphorylate phospho-PhoP (Fig. 5A). In contrast, the Δ52V mutation abrogated phosphorylation of the PhoP protein (Fig. 5A). The absence of phosphorylation is not due to decreased protein levels because the Western blot assay showed that both the wild-type and mutant PhoP proteins were expressed at similar levels when IPTG was added to the media (Fig. 5B). ChIP analysis revealed that PhoP binding to its target promoters is correlated with the phosphorylation state of the PhoP protein in vivo: PhoP bound to the mgtA and phoP promoters only when PhoP was phosphorylated (Fig. 5C). Transcriptional activity of mgtA was consistent with the result of the ChIP assay: binding of phosphorylated PhoP protein to mgtA promoter resulted in transcription of mgtA (Fig. 5D). These results indicate that phosphorylation of PhoP is necessary for PhoP binding to its target promoters and transcriptional activation of target genes.

**DISCUSSION**

In bacterial two-component systems, sensor proteins modify the phosphorylation state of their cognate response regulators usually in response to external stimuli (20). Response regulators are typically DNA binding transcription factors whose phosphorylation can have two possible roles: (i) it can promote DNA binding, possibly by stimulating response regulator oligomerization (21–23); and/or (ii) modulate activities of the regulators taking place after DNA binding (e.g. the interaction between the response regulator and RNA polymerase) (24).

Our in vivo experiments with the response regulator PhoP have demonstrated that, when the *Salmonella* PhoP/PhoQ system is physiologically activated by low Mg\(^{2+}\), binding of the PhoP protein to its target promoter sequences is correlated with phosphorylation of the PhoP protein because: (i) PhoP bound to its target promoters when *Salmonella* experienced the low Mg\(^{2+}\) inducing signal (Fig. 2), which has been shown to promote the phosphorylated form of the PhoP protein (3–5); (ii) PhoP did not bind to its target promoters when *Salmonella* experienced the high Mg\(^{2+}\) repressing signal even when the PhoP protein was produced at levels that were four times higher than those normally produced from the autoregulated *phoPQ* operon (Fig. 3, B and C); and (iii) Δ52V substitution in the PhoP protein prevented both PhoP phosphorylation and the ability of PhoP to bind DNA (Figs. 4 and 5).

Phosphorylation may be important to promote dimerization of the PhoP protein, which may be the form that binds DNA because PhoP appears to recognize a direct hexanucleotide repeat (9). Therefore, conditions that promote PhoP dimerization may bypass the requirement for PhoP phosphorylation in DNA binding and gene transcription. For example, in vitro experiments have shown that unphosphorylated PhoP protein can specifically bind to DNA (10, 25) and promote transcription from the mgtA promoter (9). Likewise, overexpression of the PhoP protein at >10-fold higher levels than those normally produced by the wild-type strain from its promoters allowed transcription of PhoP-regulated genes even in the absence of the sensor protein PhoQ, which is required for PhoP phosphorylation (12).

The stimulation of DNA binding resulting from phosphorylation may be a general feature of response regulators because binding of the PhoP protein to its target promoters was also signal dependent: it was detected in organisms experiencing low Mg\(^{2+}\) but not in those exposed to high Mg\(^{2+}\) (Fig. 2). Low Mg\(^{2+}\) promotes the synthesis of the PhoP-activated PmrD protein, which binds to the phosphorylated form of the PmrA protein protecting it from dephosphorylation by PmrB, the cognate sensor of PmrA (8). Taken together with the finding that phosphorylated PmrA binds its target promoters with higher affinity than unphosphorylated PmrA (11), the stimulation in gene transcription that takes place in low Mg\(^{2+}\) (7) is likely because of increased binding by phosphorylated PmrA. Finally, it has been proposed that binding of *E. coli* OmpR to its specific target sequences stimulates OmpR phosphorylation (26). Whereas this implies that unphosphorylated OmpR binds efficiently to DNA, others did not find DNA to have a stimulatory effect on OmpR phosphorylation by its cognate sensor EnvZ (27), and our results with the PhoP and PmrA proteins indicate that it is unlikely to be a general phenomenon.

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