The PhoP/PhoQ two-component system stabilizes the alternative sigma factor RpoS in Salmonella enterica

Xuanlin Tu1, Tammy Latifi1, Alexandre Bougdour2, Susan Gottesman1*, and Eduardo A. Groisman1**

1Department of Molecular Microbiology and 2Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110; and 3Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by Susan Gottesman, July 19, 2006

The sigma factor RpoS regulates the expression of many stress response genes and is required for virulence in several bacterial species. We now report that RpoS accumulates when Salmonella enterica serovar Typhimurium is growing logarithmically in media with low Mg2+ concentrations. This process requires the two-component regulatory system PhoP/PhoQ, which is specifically activated in low Mg2+. We show that PhoP controls RpoS protein turnover by serving as a transcriptional activator of the iraP (yaiB) gene, which encodes a product that enhances RpoS stability by interacting with RssB, the protein that normally delivers RpoS to the ClpXP protease for degradation. Mutation of the phoP gene rendered Salmonella as sensitive to hydrogen peroxide as an rpoS mutant after growth in low Mg2+. In Escherichia coli, low Mg2+ leads to only modest RpoS stabilization, and iraP is not regulated by PhoP/PhoQ. These findings add the sigma factor RpoS to the regulatory proteins and two-component systems that are elevated in a PhoP/PhoQ-dependent fashion when Salmonella face low Mg2+ environments. Our data also exemplify the critical differences in regulatory circuits that exist between the closely related enteric bacteria Salmonella and E. coli.

The alternative sigma factor RpoS of bacterial RNA polymerase is necessary for bacterial survival in stationary phase, and under various stresses including high osmolarity, oxidative stress, acid pH, temperature shock, and starvation for different nutrients (reviewed in refs. 1 and 2). Also known as σ38 or σ7, RpoS regulates expression of ∼10% of Escherichia coli genes (3), including katE and xthA, which encode hydroperoxidase II catalase and exonuclease III, respectively (4, 5). In Salmonella enterica serovar Typhimurium, RpoS plays a major role in virulence by controlling expression of the spv plasmid genes (6, 7), which govern Salmonella’s growth rate in deep organs and are required for systemic infection in mice (8–10). The production and accumulation of the RpoS protein are tightly controlled at the levels of transcription, translation, and protein stability (1, 2). Regulated proteolysis is a particularly critical mechanism that ultimately determines the levels of the RpoS protein (11). Thus, E. coli cells that are exponentially growing in defined medium with glucose as sole carbon source contain low amounts of RpoS because of its continuous rapid degradation (12). When the bacterium experiences a variety of different stresses, including nutrient starvation, hyperosmolarity, and heat shock, RpoS accumulates rapidly, which promotes expression of numerous RpoS-dependent genes, many of which confer protection against the specific stress conditions (1, 13).

RpoS stability is dependent upon several proteins, including the ATP-dependent ClpXP protease, which governs this process (14). RpoS degradation also requires the RssB protein (15, 16), an atypical response regulator that lacks a DNA-binding domain and exerts its activity by binding to RpoS and delivering it to the ClpXP protease (15–18). Also called SprE in E. coli (16, 19) and MviA in Salmonella (16, 19), RssB appears to be the rate-limiting factor in RpoS degradation (20). It has been recently shown that the small protein IraP (previously known as YaiB) stabilizes RpoS by binding to the RssB protein; IraP is particularly important for stabilization of RpoS during phosphate starvation in E. coli (21).

The Salmonella PhoP/PhoQ system governs the adaptation to low Mg2+ environments (22–24), intramacrophage survival, and virulence in mice (25–27). The sensor protein PhoQ responds to low Mg2+ by promoting phosphorylation of the response regulator PhoP (28–31), which binds to its target promoters to stimulate transcription of PhoP-activated genes (24, 32). The Salmonella PhoP/PhoQ system controls expression of ∼3% of the genome (33).

Mutations in phoP and rpoS have some similar phenotypes, suggesting a possible connection between PhoP/PhoQ and RpoS. Inactivation of either the phoP or rpoS genes results in Salmonella strains that are highly attenuated for virulence in mice (6, 25–27, 34). The PhoP and RpoS regulons are both activated inside macrophages (35–37) and by sublethal concentrations of the cationic antimicrobial peptide polymyxin (38). Both the phoP and rpoS genes negatively regulate bacterial growth within fibroblasts (39), phoP and rpoS mutants also both display increased susceptibility to acid pH (40). Moreover, PhoP has been reported to control expression of the RpoS-regulated spv virulence genes under certain conditions (41, 42).

In this article, we establish that the PhoP/PhoQ system stabilizes RpoS when Salmonella experience low Mg2+, primarily by transcriptional control of IraP. Even though low Mg2+ also promotes RpoS accumulation in E. coli, this process differs from that taking place in Salmonella in that it requires PhoP but not IraP.

Results

The RpoS Protein Levels Increase in Low Mg2+ in a PhoP-Dependent Fashion. To explore whether the PhoP/PhoQ system controls RpoS protein levels, we grew Salmonella wild-type cells in N-minimal media (pH 7.7) with 10 mM Mg2+ overnight and then diluted the cells and grew them in N-minimal media with different Mg2+ concentrations until the cultures reached an OD600 of 0.35–0.4. Extracts from these logarithmically growing bacteria were prepared, and the levels of RpoS were determined by Western blot analysis using anti-RpoS antibodies. The RpoS protein was strongly induced in extracts prepared from cells grown in ≤20 μM Mg2+ but not from those organisms grown in ≥40 μM Mg2+ (Fig. L4).

We hypothesized that the PhoP/PhoQ system might be responsible for the increase in RpoS protein levels taking place in ≤20 μM Mg2+ because low Mg2+ is the signal that activates the PhoP/PhoQ system (22, 32). Indeed, after bacterial growth for 4 h in 10 μM Mg2+, RpoS was detected in the wild-type strain but not in the isogenic phoP mutant (Fig. 1B). The phoP mutant grew as well as the wild-type strain at the time cells were

Conflict of interest statement: No conflicts declared.

FREELY AVAILABLE ONLINE THROUGH THE PNAS OPEN ACCESS OPTION.

*To whom correspondence may be addressed. E-mail: groisman@biorch.wustl.edu or susan@helix.nih.gov.

© 2006 by The National Academy of Sciences of the USA
harvested (Fig. 7A, which is published as supporting information on the PNAS web site). A strain defective in PhoP’s cognate sensor kinase PhoQ did not accumulate the RpoS protein during logarithmic growth in low Mg$^{2+}$ (data not shown), consistent with PhoQ’s established role in activation of the PhoP protein (31, 43). On the other hand, near wild-type RpoS levels were detected in a strain deleted for the chromosomal copy of the RpoS gene, and the RpoS- and PhoP-independent corA gene, from wild-type (14028s), phoP (MS7953s), rpoS (EG14794), and rpoQ phoP (EG14978) strains grown in N-minimal media (pH 7.7) with 10 μM Mg$^{2+}$ as described in Materials and Methods. The same amount of RNA was applied for real-time PCR. Fold induction is calculated as described in Supporting Materials and Methods. The data correspond to mean values of two independent experiments performed in duplicate. Error bars correspond to the SD.

Expression of RpoS-Regulated Genes Is Induced in Low Mg$^{2+}$ in a PhoP-Dependent Manner. Because RpoS protein levels increase when cells experience low Mg$^{2+}$, the expression of RpoS-dependent genes should be induced under these conditions. In fact, the mRNA levels corresponding to the RpoS-dependent katE gene were 20-fold higher in the wild-type strain than in the phoP mutant (Fig. 1D). Mutation of the rpoS gene abolished expression of the katE gene but not of the PhoP-activated RpoS-independent mgtC gene (Fig. 1E). As expected, the transcription levels of the PhoP- and RpoS-independent corA gene were unaffected by mutation of the phoP or rpoS genes (Fig. 1D and E). These experiments demonstrated that both the phoP and rpoS genes are required for katE gene transcription in low Mg$^{2+}$ conditions and support the hypothesis that the PhoP/PhoQ system effects on the RpoS pathway are physiologically relevant.

The PhoP/PhoQ System Controls the RpoS Protein Levels Posttranslationally. A phoP mutation had no effect on the expression of a transcriptional fusion of the rpoS promoter to lac at high or low Mg$^{2+}$ (Fig. 7B). PhoP also did not affect the stability of the rpoS mRNA; the half-life of the rpoS message was the same in both strains (i.e., 5 min) after 4 h of growth in N-minimal media with 10 μM Mg$^{2+}$ (data not shown).

To examine whether PhoP exerted its effect posttranslationally, we investigated the levels of an RpoS-FLAG protein in strains deleted for the chromosomal copy of the rpoS gene and expressing rpoS from a derivative of the plac promoter in a plasmid vector that also provided the ribosome-binding site. The amounts of RpoS-FLAG protein were 8-fold higher in the phoP$^+$ strain than in the isogenic phoP mutant (Fig. 1C). Because the only rpoS-derived sequences in the plasmid were those corresponding to the rpoS ORF, these results are most consistent with PhoP controlling the RpoS protein levels posttranslationally, likely via a PhoP-regulated gene product(s).

The iraP Gene Leads to Stabilization of RpoS in Low Mg$^{2+}$ in Salmonella. Our data suggest that PhoP controls RpoS at a posttranslational level. Regulation of RpoS stability is highly regulated and provides an important point of control for RpoS (44). The half-life of RpoS was determined in cells grown logarithmically in N-minimal media with 10 μM (A) or 10 mM (B) Mg$^{2+}$. Protein synthesis was inhibited with chloramphenicol (200 μg/ml). Samples were removed at indicated time points and analyzed by Western blotting with anti-RpoS antibodies. RpoS half-lives (t$_{1/2}$) were calculated by regression analysis of the exponential decay of RpoS. Because the Western blot analysis of total crude extracts prepared from wild-type (14028s), phoP (MS7953s), and iraP (EG17133) strains grown logarithmically in Mops media after phosphate starvation using anti-RpoS and anti-CorA antibodies as described in Materials and Methods. (C) Western blot analysis of total crude extracts prepared from wild-type (14028s), phoP (MS7953s), and iraP (EG17133) strains grown logarithmically in N-minimal 10 μM Mg$^{2+}$ media by using anti-RpoS and anti-CorA antibodies as described in Materials and Methods. (D) Western blot analysis of total crude extracts prepared from wild-type (14028s), phoP (MS7953s), and iraP (EG17133) strains grown logarithmically in N-minimal 10 μM Mg$^{2+}$ media with or without isopropyl β-D-thiogalactoside (IPTG) (0.5 mM) by using anti-RpoS and anti-CorA antibodies as described in Materials and Methods.
RpoS half-life was similar in iraP media with 10 M Mg\(^{2+}\) than in the wild-type strain (Fig. 2C).

In addition, the iraP mutant was as susceptible as an rpoS mutant when cells were grown in N-minimal media with the indicated concentrations of Mg\(^{2+}\) (Fig. 3C). PhoP induced RpoS transcription using isogenic wild-type and iraP mutant when cells were grown in 10 M Mg\(^{2+}\) and a PhoP-repressed site that was present in larger amounts in the wild-type strain after growth in high Mg\(^{2+}\) and in the phoP mutant under both conditions (Fig. 3C and data not shown).

Thus, we tested the possibility of PhoP regulating irap transcription using isogenic wild-type and phoP strains deleted for the iraP gene and harboring a chromosomal lacZY transcriptional fusion to the iraP promoter. The β-galactosidase activity produced by the phoP\(^{-}\) strain was ~2- to 3-fold higher than that produced by the phoP mutant when cells were grown in 10 M Mg\(^{2+}\) (Fig. 3D). PhoP induced irap gene transcription only in organisms grown in ~20 M Mg\(^{2+}\) (Fig. 3D), which is in striking parallel with the Mg\(^{2+}\) concentrations that stimulate accumulation of the RpoS protein in a PhoP-dependent manner (Fig. 1A).

Salmonella phoP and iraP Mutants Are Sensitive to Hydrogen Peroxide when Grown in Low Mg\(^{2+}\). Inactivation of the rpoS gene renders Salmonella hypersusceptible to hydrogen peroxide (46), possibly because of RpoS control of the antioxidant enzyme exocunelse III (4). Because functional phoP and iraP genes are necessary for normal RpoS levels during growth in low Mg\(^{2+}\), we hypothesized that mutants defective in these genes would exhibit increased susceptibility to hydrogen peroxide after growth under such conditions. Consistent with this notion, the Salmonella phoP and iraP mutants were as susceptible as an rpoS mutant when experiencing low Mg\(^{2+}\) compared with the wild-type strain (Fig. 2D), whereas it exhibited a normal growth rate (data not shown). The iraP mutant could be rescued by a plasmid expressing the iraP gene from a heterologous promoter but not by the plasmid vector itself (Fig. 2E). The iraP-expressing plasmid could partially rescue the phoP mutant as well (Fig. 2E). These results indicate that the PhoP-dependent increase in RpoS displayed by Salmonella in low Mg\(^{2+}\) is primarily dependent upon stabilization of RpoS by IraP. However, other PhoP-regulated gene products likely participate in this process because there were higher levels of RpoS in the iraP mutant than in the phoP\(^{-}\) mutant (Fig. 2D).

**Fig. 3.** The Salmonella iraP gene is under direct transcriptional control of the PhoP protein. (A) DNA sequence of the promoter region of the Salmonella iraP gene. The boxed bold sequences indicate the predicted PhoP boxes. Regions footprinted by the PhoP protein are underlined. The transcription start sites are marked by bent arrows. The sequences of the predicted first four amino acids of the iraP ORF are indicated below the nucleotide sequence. (B) DNase I footprinting analysis of the iraP promoter performed with probes for the coding and noncoding strands was carried out as described in Supporting Materials and Methods with increasing amounts of the PhoP protein (0, 5, 15, 30, and 90 pmoI). Solid vertical lines correspond to regions of the iraP promoter protected by the PhoP protein. (C) Primer extension assay of RNAs extracted from the wild-type (14028s) and phoP (MS7953s) strains grown in N-minimal media with 10 M (L) or 0 M (H) Mg\(^{2+}\). AG corresponds to the Maxam-Gilbert DNA ladder of the target sequence. The sequences spanning the two transcription start sites are shown, and the start sites are indicated with arrows. (D) β-galactosidase activity (Miller units) from an iraP-lac transcrip- tion fusion to the lacZY gene is under direct transcriptional control of the PhoP protein than the ORF-proximal region (Fig. 3B). Primer extension experiments and S1 mapping revealed the presence of two transcription start sites: a PhoP-activated site that was observed only in the wild-type strain experiencing low Mg\(^{2+}\) and a PhoP-repressed site that was present in larger amounts in the wild-type strain after growth in high Mg\(^{2+}\) and in the phoP mutant under both conditions (Fig. 3C and data not shown). Thus, we tested the possibility of PhoP regulating iraP transcription using isogenic wild-type and phoP strains deleted for the iraP gene and harboring a chromosomal lacZY transcriptional fusion to the iraP promoter. The β-galactosidase activity produced by the phoP\(^{-}\) strain was ~2- to 3-fold higher than that produced by the phoP mutant when cells were grown in 10 M Mg\(^{2+}\) (Fig. 3D). PhoP induced iraP gene transcription only in organisms grown in ~20 M Mg\(^{2+}\) (Fig. 3D), which is in striking parallel with the Mg\(^{2+}\) concentrations that stimulate accumulation of the RpoS protein in a PhoP-dependent manner (Fig. 1A).
organisms were grown in low Mg\(^{2+}\) (Fig. 4A) but exhibited wild-type resistance to hydrogen peroxide when organisms were harvested at stationary phase after growth in high Mg\(^{2+}\), which are PhoP-repressing conditions (Fig. 4B).

**Low Mg\(^{2+}\) Promotes Accumulation of RpoS in *E. coli* in a PhoP-Dependent but IraP-Independent Fashion.** The stabilization of RpoS after starvation for Mg\(^{2+}\) is less dramatic in *E. coli* than in *Salmonella*. The half-life of RpoS went from 2 min in high Mg\(^{2+}\)-growing cells to 6 min under Mg\(^{2+}\) starvation conditions, and this increase was not affected by a mutation in *iraP* (Fig. 5A), unlike in *Salmonella* (Fig. 2A and B).

Consistent with a lack of IraP-dependent stabilization after Mg\(^{2+}\) starvation, similar levels of *iraP* transcription were obtained in high versus low Mg\(^{2+}\), and in isogenic wild-type and *phoP* strains (data not shown). In addition, the PhoP-binding sites that are present in the *Salmonella* *iraP* promoter are not found in the *E. coli* sequence (Fig. 5C). Despite being regulated differently in *Salmonella* and *E. coli*, the *iraP* genes of these two species encode functionally equivalent proteins as the *Salmonella* *iraP* gene could rescue an *E. coli* *iraP* mutant and vice versa (data not shown).

PhoP still plays a role in RpoS accumulation in *E. coli*; in Mg\(^{2+}\) starvation conditions, the levels of RpoS were much lower in a *phoP* mutant (Fig. 5B). In an *rssB* mutant, in which RpoS is stable, the *phoP* mutation lowered RpoS levels <2-fold after Mg\(^{2+}\) starvation (Fig. 5B). This finding suggests that there is a PhoP-dependent, IraP-independent RpoS stabilization.

**Discussion**

The RpoS protein levels are tightly controlled in response to a variety of signals by several transcriptional and posttranscriptional regulators (1, 2). We have now determined that, when *Salmonella* experiences low Mg\(^{2+}\), RpoS becomes stable, which promotes expression of RpoS-dependent genes. This stabilization requires the PhoP and PhoQ proteins, consistent with the known activation of the PhoP/PhoQ two-component system by low Mg\(^{2+}\) (22, 32). Surprisingly, the stabilization of RpoS is less dramatic in *E. coli* experiencing low Mg\(^{2+}\) conditions.

The PhoP protein is a DNA-binding transcriptional regulator (33, 45) that increases RpoS levels indirectly because inactivation of the *phoP* gene had no effect on *rpoS* transcription. In *Salmonella*, PhoP increases RpoS levels by promoting transcription of the *iraP* (i.e., *yaiB*) gene (Fig. 6), which encodes a small protein recently shown to stabilize RpoS during phosphate starvation in *E. coli* (21). IraP acts by binding to RssB, probably sequestering it from binding to RpoS and delivering RpoS to the ClpXP protease (Fig. 6). However, synthesis of IraP and the resulting stabilization of RpoS may not be the full explanation for lack of RpoS accumulation in a *Salmonella* *phoP* mutant because there were lower levels of the RpoS protein in a *phoP* mutant than in an *iraP* mutant (Fig. 2B). Moreover, when *rpoS* was transcribed from a heterologous promoter in a *phoP* mutant, the RpoS levels were higher in a construct that also provided a foreign ribosome-binding site than in one harboring the 5′ UTR region of the *rpoS* gene (Fig. 1C). Thus, the PhoP-dependent up-regulation of RpoS levels taking place when *Salmonella* experiences low Mg\(^{2+}\) is mediated by both IraP and a yet to be identified PhoP-dependent product.

The IraP proteins of *Salmonella* and *E. coli* are 83% identical and can substitute for each other in vivo. It is likely that the IraP proteins use a similar mechanism to stabilize RpoS (Figs. 2 and 5) (21). In addition, the *iraP* gene is necessary for stabilization after phosphate starvation in both *E. coli* and *Salmonella* (21) (Fig. 2C). However, it is striking that these organisms appear to regulate *iraP* expression in different ways: the *Salmonella* PhoP
protein regulates iraP transcription directly by binding to distinct sites in the iraP promoter (Fig. 3B). In contrast, PhoP does not appear to regulate the initiation of iraP transcription in E. coli. Sequences resembling the PhoP-binding site are not present in the iraP promoter region (Fig. 5C), and the Salmonella PhoP protein does not footprint the E. coli promoter (unpublished results). Consistent with these observations, RpoS stability is only slightly greater during Mg\(^{2+}\) starvation in E. coli, and mutations in iraP do not affect RpoS accumulation or turnover under low Mg\(^{2+}\) growth conditions (Fig. 5A).

In E. coli under low Mg\(^{2+}\) conditions, there is a PhoP-dependent accumulation of RpoS (Fig. 5B), even though RpoS is not fully stable. Because this PhoP-dependence is mostly bypassed by an rssB mutation, it would seem that, under Mg\(^{2+}\) starvation conditions, RpoS is degraded extremely rapidly and that a PhoP-dependent product other than iraP acts to help accumulate RpoS. Such a PhoP-regulated product may also exist in Salmonella, because the effect of a phoP mutation on RpoS accumulation in low Mg\(^{2+}\) is significantly more dramatic than the effect of an iraP mutation (Fig. 2D).

The findings reported in this article provide further support to the notion that the PhoP/PhoQ two-component system is a central regulator that controls the expression and/or activity of other regulatory proteins and systems when cells experience low Mg\(^{2+}\), including the two-component system RstA/RstB of E. coli, Salmonella SpiR/SrrB two-component system, and the Salmonella transcription factor SlyA. In addition, the PhoP/PhoQ system has the ability to activate the PmrA/PmrB system posttranslationally by promoting expression of the small basic protein PmrD (50), which has been shown to bind to the phosphorylated form of the response regulator PmrA protein and protect it from dephosphorylation by the sensor protein PmrB (51). This ability allows Salmonella to express PmrA-activated genes in response to the low Mg\(^{2+}\) signal sensed by the PhoQ protein. The enhanced RpoS levels resulting from activation of the PhoP/PhoQ system is reminiscent of that activating the PmrA/PmrB system because it is mediated to a large extent by a small protein (IraP) that is under transcriptional control of PhoP (Fig. 3) and shown to bind to the response regulator RssB (21). Thus, the PhoP-regulated IraP protein endows Salmonella with the ability to express RpoS-regulated genes in response to low Mg\(^{2+}\) (Fig. 1 D and E).

Finally, the low Mg\(^{2+}\)-promoted RpoS stabilization depended on the IraP protein in Salmonella; E. coli showed less stabilization and did not regulate IraP under low Mg\(^{2+}\). This finding demonstrates that closely related bacterial species can adopt different regulatory strategies to govern expression of conserved proteins, which likely contribute to their ability to occupy distinct niches.

Materials and Methods

Bacterial Strains, Plasmids, Primers, and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table 1, which is published as supporting information on the PNAS web site, and the primers used for this study are listed in Table 2, which is published as supporting information on the PNAS web site. The construction of plasmids and strains is described in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

All S. enterica serovar Typhimurium strains used for this study were derived from the wild-type strain 14028s. Phase P22-mediated transductions were performed as described (52). Bacteria were grown at 37°C with aeration in Luria-Bertani (LB) broth or in N-minimal media (53) (pH 7.7), supplemented with 0.1% casein, 38 mM glycerol, and 10 μM or 10 mM MgCl\(_2\). When necessary, antibiotics were added at the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; rifampicin, 500 μg/ml, and tetracycline, 10 μg/ml. E. coli DH5α was used as a host for the preparation of plasmid DNA. All E. coli strains are derived from MG1655 unless otherwise indicated.

Western Blot Assay for RpoS. Cells were harvested from an overnight culture grown in N-minimal media at pH 7.7 with 10 mM Mg\(^{2+}\), washed twice with N-minimal media at pH 7.7 without Mg\(^{2+}\), and diluted 1:50 into 2.5 ml of N-minimal media, pH 7.7, with 10 μM or 10 mM Mg\(^{2+}\). When indicated, isopropyl β-D-thiogalactoside (IPTG) (0.01 mM or 0.5 mM) was added. Bacteria were grown for 4 h at 37°C with aeration (OD\(_{600}\) of 0.35–0.40). The cells were then harvested, washed once with PBS, and resuspended in 0.4 ml of PBS. The cells were opened by sonication. Cell debris was removed by centrifugation at 20,000 × g for 10 min at 4°C. Protein concentrations were determined by a modified Lowry method, with BSA used as a standard. A whole cell lysate (10 μg of protein) was run in an SDS-10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and developed by using an anti-RpoS monoclonal antibody (Neoclon, Madison, WI), an anti-CorA serum (a generous gift from M. E. Maguire, Case Western Reserve University, Cleveland, OH), an anti-mouse IgG horseradish peroxidase-linked antibody, an anti-rabbit IgG horseradish peroxidase-linked antibody, and the ECL detection system (Amer sham Biosciences, Piscataway, NJ).

Assay for Salmonella RpoS Degradation in Vivo. Cells were grown in N-minimal media as described above or in Mops as described (21). Logarithmic phase cells were treated with chloramphenicol (200 μg/ml), and 950-μl samples were removed at the indicated time points and precipitated with 5% ice-cold tricarboxylic acid. Precipitated pellets were washed with 500 μl of 80% cold acetone and then resuspended in a volume of SDS sample buffer normalized to the OD\(_{600}\). Western blot analysis of samples was carried out as described above. For quantitative analysis of the blots, we used Imagequage (Storm; Amersham Pharmacia).

Assay for E. coli RpoS Degradation in Vivo. Cells were grown overnight in M9 minimal media, supplemented with 10 μM FeSO\(_4\), 2 mM MgSO\(_4\), and 100 μM CaCl\(_2\), and then diluted into 30 ml of the same fresh media with starting OD\(_{600}\) = 0.01 and growth continued to OD\(_{600}\) ∼ 0.3. Fifteen milliliters of culture
were filtered and washed twice with 50 ml of prewarmed media without Mg^{2+}/Ca^{2+}; cells were resuspended from the filter with 15 ml of media without Mg^{2+}/Ca^{2+}, grown an additional 45 min, and assayed for RpoS degradation. Fifteen milliliters of culture without filtering were used to assay RpoS degradation before starvation. The sample collection and assay were carried out as described (21). Note that Ca^{2+} as well as Mg^{2+} can repress the PhoP/PhoQ system. Although the N-minimal medium used for Salmonella experiments does not contain Ca^{2+}, Mg^{2+} was used for E. coli, and therefore it was necessary to limit both for these experiments.

**Hydrogen Peroxide Killing Assay.** Bacteria were grown to logarithmic phase in N-minimal media with 10 μM Mg^{2+} as described above or grown to stationary phase in N-minimal media (pH 7.7) with 10 μM or 10 mM Mg^{2+}. Fifty microliters of the diluted bacterial culture was mixed with 50 μl of 30 mM hydrogen peroxide in N-minimal media (pH 7.7) with 10 μM or 10 mM Mg^{2+} and was placed in a 96-well plate (Cell Culture Cluster; Costar, Corning, NY). After 0, 5, 10, 20, and 30 min incubation at 37°C with aeration, cultures were serially diluted in cold LB and plated onto LB agar plates and incubated overnight at 37°C to determine the number of cfu. The percentage survival was calculated as follows: (cfu of hydrogen peroxide-treated culture/cfu of untreated culture) × 100. The statistical significance of the hydrogen peroxide susceptibility data was analyzed by a two-tailed Student’s t test by using Excel software (Microsoft, Redmond, WA).

**β-Galactosidase Assay.** Strains were grown in N-minimal media (pH 7.7) supplemented with either 10 μM or 10 mM Mg^{2+}. Activity was determined as described (54) after 4 h of growth at 37°C. Assays were performed in duplicate. PCR, DNase I Footprinting, and Primer Extension assays are as described in Supporting Materials and Methods.

We thank M. E. Maguire (Case Western Reserve University, Cleveland, OH) for anti-CorA antibodies; A. Kato, C. Mouslim, C. Perez, Y. Shi, and H. Huang for discussions; S. Wickner for comments on the manuscript; and J. Lee and E. Lee for technical support. This work was supported in part by National Institutes of Health (NIH) Grant AI49561 (to E.A.G., who is an Investigator of the Howard Hughes Medical Institute), and in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.
Supplemental Fig 1S. X. Tu

A

![Graph showing OD₆₀₀ over time for wild-type and phoP samples](image)

B

![Bar graph showing β-galactosidase activity with Mg²⁺ concentrations](image)