Molecular Mechanism for Establishment of Signal-dependent Regulation in the PhoP/PhoQ System*

Wei Kong†, Natasha Weatherspoon‡, and Yixin Shi§†

From the †Center for Infectious Diseases and Vaccinology at the Biodesign Institute and §The School of Life Sciences, Arizona State University, Tempe, Arizona 85287-4501

In this report, we demonstrate that H-NS is essential for establishing the Mg$^{2+}$-responsive transcriptional regulation of the PhoP regulon in Salmonella. Deletion of this regulatory gene abolished the transcriptional repression of PhoP-activated genes when bacteria were grown in high environmental Mg$^{2+}$, thus stimulating expression of phoP and other PhoP regulon genes. In the absence of H-NS, transcriptional activation was PhoP-dependent for those genes only activated by PhoP, but was PhoP-independent for those genes activated by both PhoP and SlyA. The H-NS protein footprints the phoP promoter in a sequence located upstream of the PhoP box; mutation of this cis-acting factor abolished transcriptional repression of the phoP gene equivalent to the phenotype exhibited in the hns mutant. Further results showed that H-NS gel shifts other PhoP regulon promoters, indicating that a PhoP-activated gene would be transcriptionally repressed via direct H-NS binding and inhibition of its activator PhoP. Furthermore, H-NS footprints a newly identified SlyA box and the reverse PhoP box in the pagC promoter, suggesting that both SlyA and PhoP compete with this regulatory protein. Therefore, H-NS should pair with SlyA and PhoP to establish a forward regulatory loop to regulate expression of pagC, and perhaps other PhoP- and SlyA-dependent genes.

The PhoP/PhoQ two-component system controls several cellular functions essential for bacterial virulence in Salmonella typhimurium by responding to environmental Mg$^{2+}$, pH, and host-secreted antimicrobial peptides (1–4). The sensor protein PhoQ interacts with these signals and induces phosphorylation of the response regulator PhoP (5, 6), which then binds to its target promoters with higher affinity in vivo (7) and stimulates transcription of PhoP-activated genes (8). A conserved hexanucleotide repeat separated by 5 nucleotides, termed PhoP box, present in the promoter region of many PhoP-regulated genes (7, 9) is recognized by the PhoP protein. In addition, other regulators are also involved in activation of these PhoP-dependent loci. We and other researchers have demonstrated that MarR-type regulator SlyA is required for the transcriptional activation of two chromosomal loci, ugtL and pagC, through a feed-forward loop (10, 11). The ugtL and pagC genes are Salmonella-specific loci that are activated by PhoP (12, 13) and expressed in response to low Mg$^{2+}$ conditions that activate the PhoP/PhoQ system (1). The PhoP protein binds to the ugtL promoter by interacting with a reverse PhoP box, and substitutions of this sequence abolished its transcription (10). Meanwhile, SlyA, whose transcription appears to be activated by PhoP (14), footprints a DNA sequence located downstream of the transcription start (+1) of the ugtL promoter (10), implying that a direct protein-DNA interaction is essential for its function (10).

Contrary to the results from the in vivo expression (7), a protein-DNA interaction assay showed that the unphosphorylated PhoP protein exhibits a similar affinity to PhoP-dependent promoters in vitro when compared with the phosphorylated form (15). This raised the possibility that an unknown cellular factor is involved in establishing an in vivo transcriptional repression of PhoP-activated genes in response to high Mg$^{2+}$. Indeed, another study suggested that the unphosphorylated form of the PhoP protein could mediate transcriptional activation when it was overexpressed (16), probably because PhoP could compete in the promoter region with the unknown repressor when the concentration reached a high level.

DNA-binding protein H-NS is a global transcriptional regulator that specifically silences horizontally acquired virulence genes in Salmonella (17). This process is fulfilled through interactions between H-NS and AT-rich DNA regions with low specificity (for review see Ref. 18). Interestingly, a recent report claimed that a mutation at the hns locus derived from Salmonella wild-type 14028s was lethal, but was able to be suppressed when additional deletions occurred at the phoP or rpoS locus (17). It remains unknown whether bacterial viability was affected by simultaneously deleting the 90-bp upstream and a partial coding region in the hns gene or by a cryptic mutation existing in that particular resulting strain. Nevertheless, their results implied that H-NS might interact with various PhoP-dependent promoters throughout the Salmonella genome (17).

Here we report that H-NS controls transcriptional repression of horizontally acquired PhoP-activated genes when Salmonella cells experience high Mg$^{2+}$ conditions. We show that a strain derived from wild-type ATCC 14028s harboring the deleted hns coding region was viable, and transcription of these PhoP-regulated genes was kept activated under both low and high Mg$^{2+}$ conditions. This is because the unphosphorylated PhoP is able to bind to their promoters when H-NS is absent. H-NS undergoes a two-step interaction, i.e. binding to the reg-
H-NS Controls Regulatory Function of the PhoP/PhoQ System

**TABLE 1**

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| **S. enterica serovar typhimurium** | | |
| 14028s | Wild-type | ATCC |
| YSI1108 | Δhns | This work |
| YSI1150 | ΔphoP | This work |
| YSI1168 | ΔlysA | This work |
| YSI1195 | Δhns ΔphoP | This work |
| YSI1032 | pgcl-lacZ::Km<sup>r</sup> | This work |
| YSI11743 | pgcl-lacZ::Km<sup>r</sup> ΔphoP | This work |
| YSI11745 | pgcl-lacZ::Km<sup>r</sup> ΔlysA ΔphoP | This work |
| YSI11644 | pagC-lacZ::Km<sup>r</sup> Δhns ΔlysA | This work |
| YSI11664 | pagC-lacZ::Km<sup>r</sup> ΔlysA | This work |
| YSI11782 | pagC-lacZ::Km<sup>r</sup> ΔphoP | This work |
| YSI11780 | pagC-lacZ::Km<sup>r</sup> ΔlysA | This work |
| YSI11935 | pagC-lacZ::Km<sup>r</sup> Δhns ΔlysA | This work |
| YSI11932 | pagC-lacZ::Km<sup>r</sup> Δhns ΔphoP | This work |
| YSI11938 | pagC-lacZ::Km<sup>r</sup> Δhns ΔlysA ΔphoP | This work |
| YSI11535 | Δup-phoP::Cm<sup>r</sup> | This work |
| YSI11536 | Δup-phoP::Cm<sup>r</sup> up-6 | This work |
| YSI11368 | hns::HA | This work |
| YSI11370 | hns-HA ΔphoP::Cm<sup>r</sup> | This work |
| YSI11369 | hns-HA ΔlysA::Cm<sup>r</sup> | This work |
| YSI11398 | hns-HA ΔlysA ΔphoP::Cm<sup>r</sup> | This work |
| **E. coli** | | |
| DH5α | F<sup>−</sup> supE44 ΔlacU169 (680 lacZ ΔM15) hisdR1 recA1 endA1 gyrB96 thi-1 relA1 | 30 |

| Plasmids | | |
|-----------------|-------------|-----|
| pACYC184 | rep<sub>122</sub>, Cm<sup>r</sup> F<sup>r</sup> | 31 |
| pKD3 | rep<sub>122</sub>, γAp<sup>r</sup> FRT Cm<sup>r</sup> FRT | 21 |
| pKD46 | rep<sub>122</sub>, Ap<sup>r</sup> F<sup>r</sup> p<sub>regA</sub>, γβ exo | 32 |
| pCP20 | rep<sub>122</sub>, Ap<sup>r</sup> Cm<sup>r</sup> thi<sup>r</sup> | 22 |
| pCE37 | rep<sub>122</sub>, Km<sup>r</sup> FRT lacZY<sup>+</sup> | 22 |
| pUHE21-2lac<sup>α</sup> | rep<sub>122</sub>, lac<sup>α</sup> | 22 |
| pYS1000 | rep<sub>122</sub>, Cm<sup>r</sup> <sup>−</sup> lacZ<sup>−</sup> | This work |
| pYS1100 | rep<sub>122</sub>, Cm<sup>r</sup> P<sup>−</sup> phoP, lacZ<sup>−</sup> | This work |
| pYS1115 | rep<sub>122</sub>, Cm<sup>r</sup> P<sup>−</sup> phoP, up-6 lacZ<sup>−</sup> | This work |
| pYS1244 | rep<sub>122</sub>, Ap<sup>r</sup> lac<sup>α</sup> | This work |
| pYS1118 | rep<sub>122</sub>, Ap<sup>r</sup> lac<sup>α</sup> hns | This work |
| pYS1119 | rep<sub>122</sub>, Ap<sup>r</sup> lac<sup>α</sup> hns-HA | This work |

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains used in this study are described in Table 1. All *Salmonella enterica* serovar *typhimurium* strains are derived from the wild-type strain 14028s. Phage P22-mediated transductions in *Salmonella* are performed as described previously (19). Bacteria are grown at 37°C in Luria-Bertani broth or in N minimal medium, pH 7.4, supplemented with 0.1% casamino acids and 38 mM glycerol (20). MgCl<sub>2</sub> is added to the required concentrations. When necessary, antibiotics were added at final concentrations of 50 μg/ml of ampicillin, 20 μg/ml for chloramphenicol, and 50 μg/ml for kanamycin. *Escherichia coli* DH5α is used as a host for the preparation of plasmid DNA.

**DNA and Oligonucleotides**—Chromosomal DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). DNA primers used as probes, and for the construction of plasmids and strains, are described in Table 2.

**Construction of Chromosomal Mutations, lac Fusions, and Epitope-tagged Proteins**—PCR products were used to generate a deletion in the coding region of a gene or introduce the HA<sup>2</sup> epitope sequence in bacterial chromosome as described previously (21). Primers are listed as pairs for individual genes in Table 2 and plasmid pKD3 was used as the template (21). All resulting strains were confirmed using colony PCR and DNA sequencing. A lac gene was integrated behind a coding region in chromosome using plasmid pCE37 (22) into the FLP recombination target sequence generated after the Cm<sup>r</sup> cassette, which was derived from a one-step gene disruption using primers in Table 2, was removed using plasmid pCP20 (21). Δup-phoP::Cm<sup>r</sup> up-6 (YS11536) was constructed as follows: the Cm<sup>r</sup> cassette was introduced 36-bp upstream of the PhoP box in the *phoP* promoter using PCR fragments synthesized with primers 395 and 396 from pKD3 to generate Δup-phoP::Cm<sup>r</sup> (YS11535). Then PCR amplification was carried out using the chromosomal DNA of this strain and primers 395 and 575. This DNA product was electroporated into the wild-type strain harboring pKD46 and chloramphenicol-resistant colonies were selected. The hexamer substitution (up-6) located 12 to 6 bp upstream of the PhoP box was confirmed using colony PCR and DNA sequencing. The Cm<sup>r</sup> cassette was removed using plasmid pCP20.

**Construction of Plasmids**—Plasmid pYS1000 was constructed as following: plasmid pACYC184 was digested with HindIII and Sall, and the resulting 3.6-kb fragment was purified with the Gel Extraction Kit (Qiagen). This fragment was ligated with the HindIII and Sall digests of a DNA fragment amplified from the *E. coli* lacZ gene using primers 1 and 2 (Table 2). Plasmid pYS1100 was constructed by using a PCR fragment containing 108 bp of the *phoP* promoter region, covering one promoter (P<sub>phoP</sub>) generated with primers 409 and 410, and strain 14028s chromosomal DNA, used as the template, which were digested with Sall and Xhol, and then ligated between the Sall and Xhol sites of pYS1000. Plasmids pYS1115 and pYS1244 were constructed with primers 538 and 539, and primers 775 and 776 using the Gene Tailor Site-directed Mutagenesis System (Invitrogen) with Platinum *Taq* Polymerase High Fidelity (Invitrogen). Plasmids pYS1118 and pYS1119 were constructed by using PCR fragments containing the 430-bp wild-type hns gene generated with primers 594 and 595, or primers 595 and 596, respectively, and strain 14028s chromosomal DNA, used as the template, which were digested with BamHI and HindIII, and then ligated between the BamHI and HindIII sites of pUHE21. All plasmids were confirmed by DNA sequencing.

**Reverse Transcription-PCR (RT-PCR)**—Bacterial cells were grown for 4 h in N medium supplemented with 0.01 (low) or 10 mM (high) MgCl<sub>2</sub>. Expression of the hns gene was induced from strains harboring pYS1118 by adding 0.2 mM IPTG under the same growth conditions. Total RNA was isolated from bacterial cells.
All oligonucleotides were purchased from IDT (Integrated DNA Technologies).

### TABLE 2
Primer No. | Sequence
--- | ---
**Construction of deletion mutants**

| ΔphoP     | tgT tct tat tgt taa cac aag gag gaa gac cat atg aat atgc tct ctt ag |
| ΔslyA     | ata ata act tag caa gcta at taa gag cat atg aat atgc tct ctt ag |
| Δhns      | cgg cgg gag ttt aag cat cca gga agt aaa gtt tag gct gga gct gtc |
| Δup-phoP  | tgg cgc tta aat aat gcc gtc tct ccc cat atg aat atgc tct ctt ag |
| Δup-phoP up-6 | tgg gag taa aat aac cat ccc cat atg aat atgc tct ctt ag |

### Construction of chromosomal HA epitope in *hns* locus

| Primer | Sequence |
|--------|----------|
| phoP   | aag caa atg tac taa aac gag gcc ctg tga gat gtt |
| hns    | aag caa atg tac taa aac gag gcc ctg tga gat gtt |
| pagC   | aac cat atg aat atgc tct ctt ag |

### Construction of chromosomal lacZ fusion

| Primer | Sequence |
|--------|----------|
| pgl    | cgg cgg tga ttt aag cat cca gga aag gtt tag gct gga gct gtc |
| pagC   | ggc ttc aac gtc ggg gtt gta cag cgt tgc aag gtt ggg aag gag gcc ctg tga gat gtt |

### Construction of plasmids

| Primer | Sequence |
|--------|----------|
| pYS1100 | ggt gta atc acg gcc tgt ctt aag gtt tag gct gga gct gtc |
| pYS1115 | cgc ctc cat atg aat atgc tct ctt ag |
| pYS1118 | cgg gat cca gtt gta gat tac taa aag gag gc |
| pYS1119 | cgg gat cca gtt gta gat tac taa aag gag gc |
| pYS1244 | aat aat gcc gcc tcc ctt ccc cat atg aat atgc tct ctt ag |

Reverse transcription-PCR

| Primer | Sequence |
|--------|----------|
| phoP | atg atg gca gta cgg gta cgg gga gaa |
| pgl | gta gta aat acg gcc tct ctt gtt gga gct gtc |
| pagK | aag ggt gtt gta cgg gta cgg ccc cat atg aat atgc tct ctt ag |

ChIP assay

| Primer | Sequence |
|--------|----------|
| phoP | aag ggt gtt gta cgg gta cgg ccc cat atg aat atgc tct ctt ag |
| pgl | gta gta aat acg gcc tct ctt gtt gga gct gtc |
| pagK | aag ggt gtt gta cgg gta cgg ccc cat atg aat atgc tct ctt ag |

EMSA assay and footprinting assay

| Primer | Sequence |
|--------|----------|
| phoP | aag ggt gtt gta cgg gta cgg ccc cat atg aat atgc tct ctt ag |
| pgl | gta gta aat acg gcc tct ctt gtt gga gct gtc |
| pagK | aag ggt gtt gta cgg gta cgg ccc cat atg aat atgc tct ctt ag |

H-NS Controls Regulatory Function of the PhoP/PhoQ System

*Table 2—Continued*

| Primer | Sequence |
|--------|----------|
| phoP | atg atg gca gta cgg gta cgg gga gaa |
| pgl | gta gta aat acg gcc tct ctt gtt gga gct gtc |
| pagK | aag ggt gtt gta cgg gta cgg ccc cat atg aat atgc tct ctt ag |

*Note: The table continues with more rows and columns, providing sequences for other primers and constructs. The text mentions the use of Taq polymerase (BioLabs) and performed in a thermocycler (Bio-Rad). The isolation and purification of H-NS-HA was done using the SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions.*
Western blotting substrate (Pierce). Anti-HA antibody. Protein signals were detected using ECL.

Protein was separated in 15% SDS-PAGE, transferred to nitrocellular membrane, and incubated with monoclonal anti-HA antibody. Protein signals were detected using ECL Western blotting substrate (Pierce).

**Electrophoretic Mobility Shift Assay (EMSA)**—1 pmol of 32P-labeled DNA fragments amplified with primers in Table 2 were incubated at room temperature for 30 min with various amounts of H-NS-HA in 15 μl of an EMSA buffer consisting of 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl, and 10 mM MgCl₂. 100 pmol of unlabeled DNA was added to 32P-labeled DNA when required. 1.8 μg of anti-HA antibody (Sigma) was added to the H-NS-HA and DNA mixture when required. After addition of the DNA dye solution (40% glycerol, 0.05% bromphenol blue, 0.05% xylene cyanol), the mixture was directly subjected to 5% PAGE. Signals were detected by autoradiography.

**DNase I Protection Assay**—DNase I protection assays were carried out using DNA fragments amplified by PCR using *Salmonella* chromosomal DNA as template. Prior to the PCR, primers 470 and 514 (Table 2) were labeled with T4 polynucleotide kinase and 32P/ATP (GE Healthcare). The phoP promoter region was amplified with primers 469 and 32P-470 and the pagC promoter region was amplified with primers 513 and 32P-514. Approximately 25 pmol of labeled DNA and 0, 50, 100, or 200 pmol of the H-NS-HA protein were used in a 100-μl reaction. DNase I digestion was carried out as described previously (23). DNase I was purchased from Invitrogen, and 0.05 units were used per reaction. Samples (3 μl) were analyzed by 6% denaturing polyacrylamide electrophoresis by comparison with a DNA sequence ladder generated with the appropriate primer by using a Maxam and Gilbert A+G reaction. The positions of radioactive DNA fragments in the gels were detected by autoradiography.

**Chromatin Immunoprecipitation (ChIP) Assay**—Strains harboring chromosomally encoded H-NS protein with a C-terminal HA epitope were grown in 25 ml of N medium as described above for 4 h, washed with PBS once to remove Tris (amino group), and resuspended in 25 ml of PBS. H-NS was crosslinked to promoter DNA by adding formaldehyde to 1% final concentration. ChIP assays were performed as described previously (24). Enriched promoter DNA fragments were detected by PCR using the primers listed in Table 2.

**Immunoblot Analysis of HA-tagged H-NS Proteins**—Bacterial suspensions for Western blot analysis were ultrasonicated. Total protein of whole cell lysates were determined using the BCA Protein Assay Kit (Pierce), and normalized to the same amount before being treated with SDS loading buffer. Expression of the hns gene was induced from strains harboring pYS1119 by adding IPTG to the required concentrations. H-NS protein was separated in 15% SDS-PAGE, transferred to nitrocellular membrane, and incubated with monoclonal anti-HA antibody. Protein signals were detected using ECL Western blotting substrate (Pierce).

**β-Galactosidase Assay**—β-Galactosidase assays were carried out in triplicate, and the activity was determined as described previously (25). Data corresponds to at least three independent assays conducted in duplicate.

**RESULTS**

The Regulatory Gene hns Controls Signal-responsive Repression in Horizontally Acquired PhoP-activated Loci—The unphosphorylated PhoP protein binds to target promoter DNA with a similar affinity to the phosphorylated PhoP protein *in vitro* (15); when overexpressed, it induces PhoP-activated genes *in vivo* (16). This raised the possibility that repression of PhoP-activated transcription may require other regulatory proteins in high Mg2+ conditions, in which dephosphorylation of the PhoP protein is facilitated (5, 6). To see if this repression process could be mediated by a universal transcriptional regulator, we constructed strains harboring a deletion mutation in chromosomal loci that were identified as global transcriptional regulators, including hns (26). We conducted RT-PCR to determine mRNA levels of the phoP transcripts in wild-type and isogenic strains carrying a mutation in those genes. When wild-type bacteria were grown in high (10 mM) and low (0.01 mM) Mg2+, expression of a PhoP-activated gene is repressed and activated, respectively (1). Therefore, the wild-type mRNA level was lower when bacteria were grown in high Mg2+ than in low Mg2+ (Fig. 1A). Different from the wild-type and other mutant strains we tested, the phoP mRNA level in the hns mutant grown in high Mg2+ was as high as that grown in low Mg2+ (Fig. 1A and data not shown). This suggests that H-NS should be the cellular factor that mediates transcriptional repression of the phoP gene in high Mg2+ conditions, thus, deletion of this locus results in derepressing phoP expression in the signal depleting condition (*i.e.*, high Mg2+) for the PhoP/PhoQ system. We compared transcription of those PhoP-activated genes, identified specifically from *Salmonella*, in wild-type and the isogenic hns mutant. The rationale for choosing these loci was because a previous report suggested that H-NS specifically silences horizontally acquired genes (17). We classified these genes as type I, which is activated by PhoP, including phoP, pglL, pagK, and phoN (12, 27, 28) and type II, which is activated by PhoP and SlyA, including ugtL and pagC (10, 11). Consistent with an established model (1), expression of these loci was repressed under the high Mg2+ condition because the mRNA level from wild-type was reduced when bacteria were grown in high Mg2+ (Fig. 1A). Mutation of the hns gene, however, abolished the transcriptional repression of both type I and II genes in this signal-depleting condition because their mRNA levels in the hns mutant grown in high Mg2+ were as high as that grown in low Mg2+ (Fig. 1A). The phenotype of the transcriptional activation in the hns mutant strain is solely the result of a lack of hns gene function because transcription of these genes including phoP could be restored to wild-type levels, *i.e.*, the mRNA level was higher in low Mg2+ than in high Mg2+, by a plasmid (pYS1118) containing a wild-type copy of the hns gene (Fig. 1A). Deletion of the phoP gene in hns mutant strains inhibited transcription of the type I genes under all tested conditions (Fig. 1A), suggesting that removing transcriptional repressor H-NS is not sufficient to activate their transcription. Different from the type I genes, transcriptional derepression by deleting the hns locus allows activation of the type II genes even in the absence of PhoP (Fig. 1A). PhoP appears to be unrelated to hns expression because the protein level of H-NS in a phoP mutant...
H-NS Controls Regulatory Function of the PhoP/PhoQ System

FIGURE 1. H-NS is required for repression of PhoP-dependent transcription in high Mg$^{2+}$ conditions. A, RT-PCR analysis of mRNA level of PhoP-activated genes: type I, phoP, pglL, pagK, and phoN; type II, pagC and ugtL; synthesized from bacteria was determined in wild-type (wt, 14028s), hns mutant (YS11708), hns mutant (YS11708) harboring pUHE21–2lac$^+$ with a wild-type copy of the hns gene (pHNS, pYS1118), phoP mutant (YS11590), and hns phoP mutant (YS11945). Wild-type chromosomal DNA (chr) was used as control. The constitutively transcribed rpoD gene indicated that similar amounts of total RNA were used. The protein levels of H-NS were determined in the same growth conditions from strains harboring hns-HA fusion in the chromosome or in plasmid pYS1119, whose sequence is identical to pYS1118 except a HA tag sequence at the C terminus of the hns gene (Table 1). B, P$_{phoP-lacZ}$ transcriptional fusion expressed by bacteria harboring pYS1100 was determined in wild-type (14028s), phoP mutant (YS11590), hns mutant (YS11708), hns mutant (YS11708) harboring pYS1118, and hns phoP mutant (YS11945) strains. C, pglL-lacZ transcriptional fusion expressed by bacteria was determined in wild-type (YS10382), phoP mutant (YS11743), hns mutant (YS11744), hns mutant (YS11744) harboring pYS1118, and hns phoP mutant (YS11745) strains. D, pagC-lacZ transcriptional fusion expressed by bacteria was determined in wild-type (YS11644), slyA mutant (YS11664), hns mutant (YS11782), hns mutant (YS11780), hns mutant (YS11780) harboring pYS1118, hns slyA mutant (YS11935), hns phoP mutant (YS11932), hns phoP slyA mutant (YS11938), and hns phoP slyA mutant (YS11938) harboring pYS1118 strains. Bacteria were grown in N minimal medium, pH 7.4, with 0.01 mM (low or L) and 10 mM (high or H) Mg$^{2+}$. Complementation required the addition of 0.2 mM IPTG. All graphed values (Miller unit) are mean ± S.D., and data correspond to three independent assays conducted in duplicate.

was similar to that in wild-type (Fig. 1A). Thus, our results demonstrate that PhoP functions as a transcriptional anti-repressor and activator for the type I genes, and as a transcriptional anti-repressor for the type II genes.

Further evidence came from β-galactosidase assays using strains harboring lacZ fusions under control of the phoP promoter, type I promoter, and type II promoter. To compare the in vivo phoP transcription without interfering with the regulatory function of the PhoP/PhoQ system, we constructed a plasmid (pYS1100) carrying lacZ fused to a 108-bp DNA fragment including a phoP promoter ($P_{pho-1}$ fragment from −123 to −34, Fig. 2C). This DNA could initiate a PhoP-dependent transcription from −34 (or P1 in Fig. 2C, also see Ref. 33). Meanwhile, we constructed strains harboring a chromosomal pglL-lacZ fusion and a chromosomal pagC-lacZ fusion in addition to a set of isogenic strains with mutations in the regulatory genes. Expression of all three lacZ fusions were Mg$^{2+}$-responsive and PhoP-dependent because β-galactosidase activity from wild-
type strains was higher in low Mg$^{2+}$ than in high Mg$^{2+}$, and because this activity from wild-type strains was higher than in \textit{phoP} mutants in low Mg$^{2+}$ (Fig. 1, B--D). In addition, expression of \textit{pagC-lacZ} was SlyA-dependent because $\beta$-galactosidase activity from wild-type was higher than in a slyA mutant (Fig. 1D). Consistent with RT-PCR results (Fig. 1A), activation of the \textit{phoP}, \textit{pcgL}, and \textit{pagC} genes in the high Mg$^{2+}$ condition occurred when the \textit{hns} gene was deleted because $\beta$-galactosidase activity from \textit{hns} mutants grown in high Mg$^{2+}$ was greatly induced (Fig. 1, B--D). The \textit{lacZ} induction in these \textit{hns} mutants is solely the result of a lack of \textit{hns} gene function because transcription of \textit{lacZ} could be restored to wild-type levels by pYS1118, \textit{i.e.} $\beta$-galactosidase activity was greatly reduced in high Mg$^{2+}$ (Fig. 1, B--D). Mutation of the \textit{phoP} locus resulted in inhibition of \textit{lacZ} transcription from promoters of \textit{phoP} and \textit{pcgL}. No matter whether H-NS was present or absent (Fig. 1, B and C). Thus, H-NS binding to a \textit{PhoP}-activated promoter is to prevent unphosphorylated PhoP, which is produced in high Mg$^{2+}$ conditions, from initiating transcription of this gene.

On the other hand, PhoP is not essential for activation of the type II gene \textit{pagC} when H-NS is absent because \textit{lacZ} expression from the \textit{pagC} promoter was similar in a \textit{hns} mutant and a \textit{hns} \textit{phoP} double mutant (Fig. 1D). This indicates that PhoP functions differently in the type I and II genes. Because expression of the \textit{pagC} gene is also SlyA-dependent (11), we compared the transcriptional level of the \textit{pagC-lacZ} fusion from wild-type and isogenic slyA mutant strains carrying a deletion at the \textit{hns} locus. \textit{lacZ} expression was inhibited in a slyA mutant, but remained activated in a \textit{hns slyA} mutant under all tested conditions (Fig. 1D). This result, and the above result from a \textit{hns phoP} mutant, suggested that PhoP and SlyA are not required for \textit{pagC} transcription when H-NS is absent. Indeed, we found that the transcriptional level of the \textit{pagC-lacZ} fusion in a \textit{hns phoP} \textit{slyA} mutant was as high as that in the \textit{hns} mutant (Fig. 1D). These experiments demonstrate that PhoP and SlyA are designed to antagonize H-NS, which represses transcription of the type II PhoP-dependent loci. Thus, the \textit{pagC} promoter, perhaps \textit{igtL} as well, could be regarded as a “constitutively activated” promoter in the absence of H-NS.

The \textit{H-NS} Protein Binds to a DNA Region Located Upstream of the \textit{PhoP} Box in the Promoter of the \textit{phoP} Gene—We conducted an EMSA using C-terminal HA-tagged H-NS protein (H-NS-HA) and a 235-bp DNA fragment including the \textit{purB-phoP} intergenic region (see “Experimental Procedures”). We found that this H-NS protein could gel shift the DNA fragment (Fig. 2A), indicating the presence of a binding site for H-NS in this DNA region. Specific H-NS binding was further identified by a supershift reaction, in which a monoclonal anti-HA antibody (Sigma) could bind to the H-NS protein and further decrease the mobility of the protein-DNA complex in a polyacrylamide gel (Fig. 2A). Thus, we conducted DNase I footprinting assays and found that the H-NS protein protects three DNA regions in the \textit{purB-phoP} intergenic region (Fig. 2B): the $-112$ to $-100$ sequence, 11 bp downstream of the \textit{purB} coding region; the $-86$ to $-74$ sequence, 6 bp upstream of the PhoP box; and the $-30$ to $-21$ sequence, 4 bp downstream of the PhoP-dependent transcription start P1 (the first nucleotide of the \textit{phoP} start codon as +1, Fig. 2C). The $-30$ to $-20$ sequence was not present in the DNA fragment cloned in pYS1100 (see “Experimental Procedures”). This ruled out the possibility that this region is required for H-NS repression because the upstream region of P1 is sufficient to confer a Mg$^{2+}$-responsive \textit{lacZ} expression (Fig. 1B). To examine the $-86$ to $-74$ sequence adjacent to the PhoP box, we constructed a strain (the \textit{up-6} mutant) harboring a chromosomal substitution from actatt to tgtaag (Fig. 2C) at the H-NS binding site (UP) present in the \textit{phoP} promoter, and a pYS1100-derived plasmid (pyp-6) carrying this hexamer substitution. Determination of \textit{phoP} transcripts from the \textit{up-6} mutant indicated that mRNA levels of the \textit{phoP} gene were similar in this mutant grown in low and high Mg$^{2+}$ conditions (Fig. 2D). Furthermore, the wild-type strain harboring plasmid pyp-6 exhibited similar $\beta$-galactosidase activities in low and high Mg$^{2+}$ conditions (Fig. 2E), which was the same phenotype displayed by the \textit{hns} mutant carrying the wild-type plasmid (Fig. 1B). On the other hand, \textit{lacZ} expression from wild-type harboring another pYS1100-derived plasmid (pYS1244) with a heptamer substitution from ttttctt to atatgaa within the $-112$ to $-100$ sequence was similar to that from wild-type harboring a wild-type plasmid (Fig. 2E). These results indicate that binding of the H-NS protein to the UP region adjacent to the PhoP box in the \textit{phoP} promoter is required for its transcriptional repression in high Mg$^{2+}$.

The \textit{H-NS} Protein Represses Type I \textit{PhoP}-activated Genes by Binding to Their Promoters—To examine whether transcriptional repression of type I genes results from H-NS binding to their promoters, we conducted EMSA using H-NS-HA protein and PCR-generated DNA fragments carrying regulatory sequences present upstream of \textit{pcgL}, \textit{pagK}, and \textit{phoN} coding regions (see “Experimental Procedures”). We found that the H-NS protein could gel shift all tested DNA fragments, which were supershifted by anti-HA antibodies (Fig. 3A). This result indicates that a binding site for H-NS is present in these type I promoters. Thus, we compared the H-NS binding ability in these promoters in a strain harboring a chromosomal \textit{hns}-HA fusion with that expressed by the isogenic strain with a mutation in the regulatory gene \textit{phoP}, and carried out a ChIP experiment (29) using bacteria grown in low and high Mg$^{2+}$. A stronger enrichment of DNA fragments present in the \textit{phoP}, \textit{pcgL}, \textit{pagK}, and \textit{phoN} promoters was observed for the HA-tagged wild-type strain grown in high Mg$^{2+}$ (Fig. 3B, lane \textit{d}) than in low Mg$^{2+}$ (Fig. 3B, lane \textit{c}). Furthermore, a dramatic enrichment was observed for the \textit{phoP} mutant strain grown in low Mg$^{2+}$, which was stronger than the wild-type strain grown in high Mg$^{2+}$ (Fig. 3B, lanes \textit{e} and \textit{d}). The ChIP assay was specific because there was no significant enrichment of H-NS-bound DNA to the examined promoters when a control (\textit{i.e.} wild-type strain lacking a HA-tag) strain was used (Fig. 3B, lanes \textit{a} and \textit{b}). We ruled out the possibility that strong binding of the H-NS protein to these type I promoters in wild-type cells grown in high Mg$^{2+}$ (Fig. 3B, lane \textit{d}) or in the \textit{phoP} mutant strain (Fig. 3B, lane \textit{e}) could be caused by high expression of the H-NS protein under these conditions (as opposed to a low level of the PhoP protein), because immunoblot analysis in cell lysates from ChIP experiments revealed that the H-NS protein level was constant in wild-type grown in both Mg$^{2+}$ conditions and in a \textit{phoP} or slyA mutant grown in low Mg$^{2+}$ (Fig. 3C). This
H-NS Controls Regulatory Function of the PhoP/PhoQ System

indicates that expression of H-NS is independent of PhoP and SlyA, as well as Mg2+ concentration. Taken together, our observations demonstrate that the H-NS protein competes with PhoP to repress transcription directed by type I PhoP-dependent promoters.

H-NS Represses Transcription of the Type II Promoters by Binding to the PhoP Box and SlyA Box—We conducted EMSA using H-NS-HA protein and the 250-bp DNA fragment containing regulatory motifs of the pagC promoter.3 We found that H-NS could gel shift the pagC promoter DNA (Fig. 4A), which indicates the presence of a possible binding site for H-NS in this type II promoter region. Gel shift resulted specifically from H-NS binding because a monoclonal anti-HA antibody (Sigma) was able to further decrease the mobility of the H-NS protein and pagC DNA complex in a polyacrylamide gel (Fig. 4A). We then conducted DNase I footprinting assays and found that the H-NS protein binds to the pagC promoter throughout the DNA region from −122 to −54 (Fig. 4, B and D). We identified the pagC sequence atatt- (N10)-attatt from −109 to −88 as the SlyA-binding site (the SlyA box) and the sequence taatt- (N5)-taacc from −76 to −61 as the PhoP-binding site (the PhoP box, Fig. 4D) because substitutions in these sequences resulted in a complete inhibition in pagC transcription.3 The region protected by the H-NS protein extends through the whole SlyA box (Fig. 4), indicating the presence of a possible binding site for H-NS in this promoter region. H-NS could gel shift the type II promoter DNA because a monoclonal anti-HA antibody (Sigma) was able to further decrease the mobility of the H-NS protein (Fig. 4A). Gel shift resulted specifically from H-NS binding because a monoclonal anti-HA antibody (Sigma) was able to further decrease the mobility of the H-NS protein and pagC DNA complex in a polyacrylamide gel (Fig. 4A). We then conducted DNase I footprinting assays and found that the H-NS protein binds to the pagC promoter throughout the DNA region from −122 to −54 (Fig. 4, B and D). We identified the pagC sequence atatt- (N10)-attatt from −109 to −88 as the SlyA-binding site (the SlyA box) and the sequence taatt- (N5)-taacc from −76 to −61 as the PhoP-binding site (the PhoP box, Fig. 4D) because substitutions in these sequences resulted in a complete inhibition in pagC transcription.3 The region protected by the H-NS protein extends through the whole SlyA box and PhoP box (Fig. 4D), suggesting that binding of the H-NS protein to this promoter should compete with PhoP and the

3 W. Kong, N. Weatherspoon, and Y. Shi, submitted for publication.
PhoP-activated SlyA (14). We carried out a ChIP experiment to compare the H-NS binding ability in this type II promoter using the strain harboring the chromosomal *hns*-HA fusion and an isogenic strain with mutations in both *phoP* and *slyA* genes grown in low and high Mg$^{2+}$. A dramatic enrichment of the DNA fragment corresponding to the *pagC* promoter was observed for the HA-tagged wild-type strain grown in high Mg$^{2+}$ (Fig. 4C, lane c), but very weak H-NS binding was observed in this strain grown in low Mg$^{2+}$ (Fig. 4C, lane b). Furthermore, DNA enrichment was even stronger for the *phoP* slyA mutant strain grown in low Mg$^{2+}$ (Fig. 3B, lane d). The ChIP assay was specific because there was no significant enrichment of H-NS-bound DNA to the *pagC* promoter when the control (i.e., wild-type strain lacking a HA-tag) was used (Fig. 4C, lane a). Thus, these results suggest that the H-NS protein establishes Mg$^{2+}$-dependent transcription of the type II *pagC* promoter by competing with PhoP and SlyA.

The Molecular Mechanism of Mg$^{2+}$-dependent Regulation Mediated by the PhoP/PhoQ System and H-NS—We titrated the H-NS protein that could mediate repression of the type I gene *pcgL* and the type II gene *pcgL* using *hns* mutants harboring a chromosomal *lacZ* fusion and a plasmid (pYS1118) with an inducible *hns* gene. When bacteria were grown in medium without IPTG, β-galactosidase activity from each strain grown in low (0.01 mM) and high (10 mM) Mg$^{2+}$ was determined (see the actual values in lower panel of Fig. 5A) and set as 100% (Fig. 5A). In the signal-depleting condition (i.e., high Mg$^{2+}$), *lacZ* expression controlled by the *pcgL* promoter was fully repressed, like a wild-type strain, when supplemented IPTG concentrations reached 0.05 mM or higher (Fig. 5A). On the contrary, in the signal-stimulating condition (i.e., low Mg$^{2+}$), transcription remained at 78% maximum in the same IPTG concentration (Fig. 5A). This result, and a similar result in Fig. 1C, indicates that only the phosphorylated PhoP could antagonize H-NS at the *pcgL* promoter (Fig. 6). We determined the H-NS protein levels induced by IPTG using the *hns* mutant harboring a plasmid (pYS1119), which has an identical sequence to pYS1118 with a C-terminal HA tag added to the *hns* gene. As revealed by an immunoblot analysis, induction of the H-NS protein is proportional to the concentration of supplemented IPTG (Fig. 5B).

The SlyA amount was maintained at similar levels in low and high Mg$^{2+}$ (11), thus, Mg$^{2+}$-responsive transcription of the *pagC* gene should depend primarily on the phosphorylation of PhoP. Indeed, this *pagC* expression was inhibited as rapidly as the *pcgL* gene in high Mg$^{2+}$ (Fig. 5A). Unexpectedly, low IPTG concentrations resulted in transcriptional repression in low Mg$^{2+}$ equal to high Mg$^{2+}$, which was followed by a slight reduction of *pcgl* transcription even when the IPTG concentration became higher (Fig. 5A). We therefore divided H-NS-mediated transcriptional repression of the *pagC* gene into two stages, the first stage different from that of the *pcgl* gene when H-NS concentrations are low, and the second stage the same as the *pcgl* gene when H-NS concentrations are high. Because the H-NS protein induced in the first stage is lower than the actual amount in bacterial cytoplasm (data not shown), this Mg$^{2+}$-independent process implies the possibility that SlyA may facilitate binding of the unphosphorylated PhoP to the *pagC* promoter exhibited at the first stage. Our results demonstrate that the phosphorylated PhoP is much more resistant to H-NS repression exhibited in the second stage of *pagC* expression equivalent to expression of type I genes in low Mg$^{2+}$ (Fig. 5A).

**DISCUSSION**

We have identified the regulatory mechanism governing transcriptional repression of the PhoP/PhoQ two-component signaling network of *S. enterica*. We have established that transcriptional regulation of the PhoP regulon is controlled by the H-NS protein, which competes with PhoP protein, as well as SlyA in particular cases. This model is supported by the following data. (i) The transcriptional repression of *phoP* and all...
H-NS Controls Regulatory Function of the PhoP/PhoQ System

We ruled out the possibility that the H-NS protein level could be influenced in different mutants or when bacteria were grown under all tested conditions in this study (Fig. 3C), therefore, H-NS-mediated transcriptional repression solely depends on its selective binding to the target promoters. H-NS not only binds to the promoter of the master regulator PhoP (Fig. 2, A and B), but also binds to promoters of individual PhoP-activated genes (Figs. 3A and 4A), suggesting that H-NS and PhoP should form a counteracting regulatory pair in these horizontally acquired PhoP-activated genes. Most PhoP-activated promoters (i.e., type I promoters in this study) described to date harbor a conserved hexanucleotide repeat separated by 5 nucleotides, termed the PhoP box, located 12 bp upstream of the −10 region (9). We found one H-NS binding site (termed the UP element here) located in one helical turn upstream of the PhoP box in the phoP promoter; this spatial adjacency could explain competition between H-NS and phosphorylated PhoP when they approach these binding sites as demonstrated in this study (Fig. 3B) and illustrated in Fig. 6. This competition appears to be specific in vivo. Although H-NS could bind several DNA regions in phoP promoter in vitro (Fig. 2, B and C), only the hexamer mutation in the UP site could abolish repression of the phoP transcription in high Mg\(^{2+}\) (Fig. 2, D and E). This feature of the phoP promoter should be applicable for other type I promoters that also possess the AT-rich sequences located upstream from the identified PhoP box (7). Although no UP consensus sequence could be found, H-NS is able to bind to these promoter DNA regions (Fig. 3, A and B).

Regulatory activity of PhoP is modulated by changing its phosphorylation level, which likely changes its conformation, and therefore, changes its in vivo affinity to target promoters when the PhoP/PhoQ system responds to environmental Mg\(^{2+}\) (5, 6). On the other hand, both unphosphorylated and phosphorylated forms of PhoP could bind to promoters with similar affinities in vitro (15). Taken together, it is likely that additional cellular factor(s) could be required for preferential binding of the phosphorylated PhoP in vivo. One example is SlyA, which mediates a feedback activation of the PhoP/PhoQ system by interacting with the H-NS binding site, i.e., the UP element in the phoP promoter. Two functions of PhoP were suggested based on results in this study. (i) As a transcriptional anti-repressor. In wild-type, Mg\(^{2+}\)-responsive transcription could be because the phosphorylated PhoP, but not unphosphorylated PhoP, was able to counteract H-NS in these promoters (Fig. 3B). (ii) As a transcriptional activator. Under high Mg\(^{2+}\) conditions, removing H-NS in the phoP mutant could activate type II promoters (Fig. 1, A and D), but not activate type I promoters probably because an unphosphorylated PhoP is still required to turn on their expression (Fig. 1, A and C–E). Because the PhoP box is located within the −35 region of type I promoters (7), it is possible that PhoP is required to directly interact with RNA polymerase for their transcription initiation. Transcriptional activation of phoP and other PhoP-activated genes in the hns mutants can be turned on under PhoP-dephosphorylated conditions (high Mg\(^{2+}\)), possibly because unphosphorylated PhoP protein is able to bind to their promoter regions and function as a transcriptional activator, like the phosphorylated PhoP protein, in the absence of the competitor H-NS.

tested PhoP-activated genes was abolished in hns mutants when bacteria were grown under PhoP-dephosphorylated conditions (Fig. 1, A–E). (ii) The H-NS protein gel shifted all tested PhoP-dependent promoter DNA (Figs. 2A, 3A, and 4A) and footprinted the phoP promoter (Fig. 2B). (iii) The H-NS protein footprinted both the SlyA binding site and the PhoP binding site in the pagC promoter (Fig. 4B). (iv) Mutation of a H-NS binding site in the phoP promoter abolished phoP transcriptional repression to levels observed in a hns mutant (Fig. 2, D and E). (v) H-NS binding to all tested PhoP-dependent promoters was enhanced in a phoP mutant grown under PhoP-phosphorylated conditions and in wild-type bacteria grown under PhoP-dephosphorylated conditions (Fig. 3B). (vi) H-NS binding to the PhoP-, SlyA-dependent pagC promoter was enhanced in a phoP slyA mutant grown under PhoP-phosphorylated conditions and in wild-type bacteria grown under PhoP-dephosphorylated conditions (Fig. 4C). (vii) Inducing H-NS selectively repressed pgcL and pagC transcription in hns mutants grown under PhoP-dephosphorylated conditions (Fig. 5A).

Figure 6. Model illustrating the H-NS-dependent Mg\(^{2+}\) response of the PhoP/PhoQ system. In wild-type, the PhoP protein is phosphorylated during growth in low Mg\(^{2+}\). The type I PhoP-activated genes (left) are transcriptionally activated via competition of the phosphorylated PhoP, which binds with high affinity (probably facilitated by other factors) to the PhoP box (the red box) over H-NS near the UP motif (the green box), whereas type II PhoP-activated genes (green circles) are activated during growth in low Mg\(^{2+}\) when PhoP-activated SlyA, along with the phosphorylated PhoP, bind to the promoters replacing H-NS in the SlyA box and PhoP box, and stimulating their transcription in S. typhimurium. Competition over the unphosphorylated PhoP allows the H-NS protein to occupy these chromosomal regions and repress transcription of the type I and II genes when wild-type bacteria are grown in high Mg\(^{2+}\) conditions. In the absence of H-NS (Hns\(^{-}\) strain), the unphosphorylated PhoP, regardless of its low DNA affinity, is able to bind to type I promoters, which facilitates transcriptional activation of these genes even in high Mg\(^{2+}\) conditions. Meanwhile, type II promoters become independent of SlyA and PhoP in Hns\(^{-}\) bacteria.
Type II promoters (ugtL and pagC in this study) are atypical in that they contain a PhoP box located on the opposite strand (Fig. 4D), and that they require SlyA for their activation (10, 11). A reverse PhoP box and SlyA box are all located farther upstream from the putative −35 region of the ugtL and pagC promoters. Contrary to the type I promoters, PhoP is not essential for transcription of these type II genes in the absence of H-NS (Fig. 1, A and D), indicating that this regulator functions solely to antagonize H-NS, i.e. as an anti-repressor. This difference is possibly derived from the involvement of the additional regulator SlyA. Although SlyA was transcriptionally activated by PhoP (10, 14), protein levels of SlyA appeared to be kept at similar levels when bacteria were grown under both low and high Mg$^{2+}$ conditions (11). However, SlyA could only bind weakly to the pagC promoter in high Mg$^{2+}$ (data not shown), indicating a coordinate binding of the PhoP and SlyA proteins onto type II promoters with an unknown mechanism. H-NS binds to both the PhoP box and SlyA box present in the type II promoters although their nucleotide sequences are different (Fig. 4D). Our results, therefore, provide a molecular mechanism for the feed-forward loop designed for the transcriptional activation of type II promoters, i.e. to counteract the inhibitory effect of H-NS in the upstream elements, PhoP box and SlyA box. PhoP and SlyA proteins should simultaneously bind to DNA and remove this repressor (Fig. 6). It is unknown how these distal regulatory elements enhance transcriptional initiation of the RNA polymerase complex. One possibility is that RNA polymerase requires an “entrance passage” located upstream of the −35 sequence, where, in high Mg$^{2+}$, H-NS functions as a “gate-keeper” by occupying these regions and preventing polymerase complex from approaching.

In summary, we provide a molecular mechanism for the signal-dependent gene regulation controlled by the PhoP/PhoQ system, and perhaps by other two-component regulatory systems. Results in this study show that a type I PhoP-dependent promoter interacts with H-NS and PhoP in the following order: phosphorylated PhoP > H-NS > unphosphorylated PhoP. Therefore, H-NS antagonizes low DNA-affinity PhoP when it is unphosphorylated in high Mg$^{2+}$, but not high DNA-affinity PhoP when it is phosphorylated in low Mg$^{2+}$. Results in this study also show that PhoP and SlyA antagonize H-NS in type II PhoP-dependent promoters, in which the Mg$^{2+}$ concentration obviously determines PhoP binding to these promoters. However, it remains to be investigated which cellular signal molecule could influence SlyA binding to these promoters.

Acknowledgments—We thank Roy Curtiss III and Josephine Clark-Curtiss for discussions and Guozheng Qin for technical support. We also thank one anonymous reviewer for thoughtful comments.

REFERENCES

1. Garcia Vescovi, E., Soncini, F. C., and Groisman, E. A. (1996) Cell 84, 165–174
2. Prost, L. R., Daley, M. E., Le Sage, V., Bader, M. W., Le Moual, H., Klevit, R. E., and Miller, S. I. (2007) Mol. Cell 26, 165–174
3. Bader, M. W., Sanowar, S., Daley, M. E., Schneider, A. R., Cho, U., Xu, W., Klevit, R. E., Le Moual, H., and Miller, S. I. (2005) Cell 122, 461–472
4. Prost, L. R., and Miller, S. I. (2008) Cell Microbiol. 10, 576–582
5. Castelli, M. E., Cauerhoff, A., Amongero, M., Soncini, F. C., and Vescovi, E. G. (2003) J. Biol. Chem. 278, 23579–23585
6. Montagne, M., Martel, A., and Le Moual, H. (2001) J. Bacteriol. 183, 1787–1791
7. Lejona, S., Aguirre, A., Cabeza, M. L., Garcia Vescovi, E., and Soncini, F. C. (2003) J. Bacteriol. 185, 6287–6294
8. Soncini, F. C., Garcia Vescovi, E., Solomon, F., and Groisman, E. A. (1996) J. Bacteriol. 178, 5092–5099
9. Minagawa, S., Ogasawara, H., Kato, A., Yamamoto, K., Eguchi, Y., Oshima, T., Mori, H., Ishihama, A., and Utsumi, R. (2003) J. Bacteriol. 185, 3696–3702
10. Shi, Y., Latifi, T., Cromie, M. J., and Groisman, E. A. (2004) J. Biol. Chem. 279, 38618–38625
11. Navarre, W. W., Halsey, T. A., Walthers, D., Frye, J., McClelland, M., Potter, J. L., Kenney, L. J., Gunn, J. S., Fang, F. C., and Libby, S. J. (2005) Mol. Microbiol. 56, 492–508
12. Hilbert, F., Garcia-del Portillo, F., and Groisman, E. A. (1999) J. Bacteriol. 181, 2158–2165
13. Miller, S. I., Kukral, A. M., and Meekalanan, J. I. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5054–5058
14. Norte, V. A., Stapleton, M. R., and Green, J. (2003) J. Bacteriol. 185, 3508–3514
15. Perron-Savard, P., De Crescenzo, G., and Le Moual, H. (2005) Microbiology 151, 3979–3987
16. Lejona, S., Castelli, M. E., Cabeza, M. L., Kenney, L. J., Garcia Vescovi, E., and Soncini, F. C. (2004) J. Bacteriol. 186, 2476–2480
17. Navarre, W. W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S. J., and Fang, F. C. (2006) Science 313, 236–238
18. Dorman, C. J. (2007) Nat. Rev. Microbiol. 5, 157–161
19. Davis, R. W., Belstein, D., and Roth, J. R. (1980) Advanced Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Snavely, M. D., Miller, C. G., and Maguire, M. E. (1991) J. Biol. Chem. 266, 815–823
21. Datsenko, K. A., and Wanner, B. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6640–6645
22. Ellermeier, C. D., Janakiraman, A., and Slauch, J. M. (2002) Gene (Amst.) 290, 153–161
23. Kato, A., Tanabe, H., and Utsumi, R. (1999) J. Bacteriol. 181, 5516–5520
24. Shin, D., and Groisman, E. A. (2005) J. Biol. Chem. 280, 4089–4094
25. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26. Hengge-Aronis, R. (1999) Curr. Opin. Microbiol. 2, 148–152
27. Kier, L. D., Weppelman, R. M., and Ames, B. N. (1979) J. Bacteriol. 138, 155–161
28. Belden, W. J., and Miller, S. I. (1994) Infect. Immun. 62, 5095–5101
29. Kuras, L., and Struhl, K. (1999) Nature 399, 609–613
30. Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
31. Chang, A. C., and Cohen, S. N. (1978) J. Bacteriol. 134, 1141–1156
32. Cherepanov, P. P., and Wackerenagel, W. (1995) Gene (Amst.) 158, 9–14
33. Soncini, F. C., Vescovi, E. G., and Groisman, E. A. (1995) J. Bacteriol. 177, 4304–4317