Modulation of the Regulatory Activity of Bacterial Two-component Systems by SlyA

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Activation of the transcriptional regulator SlyA by the PhoP/PhoQ two-component system controls intracellular expression of numerous factors influencing Salmonella virulence. By dissecting the SlyA regulon using stable isotope labeling with amino acids in cell culture analysis, we found that SlyA enhances overall transcription of PhoP-activated loci. This amplification of cellular responses to Mg²⁺ occurs when SlyA binds to the phoPQ promoter thereby activating phoP autoregulation via a positive feedback mechanism. SlyA footprints a DNA region located one helical turn upstream of the PhoP box, which overlaps the H-NS-binding motif required for signal-dependent phoP repression in high Mg²⁺ conditions. Therefore, binding of SlyA likely antagonizes H-NS and facilitates the interaction of PhoP to its own promoter, subsequently activating the phoPQ operon. Establishment of this regulatory circuit allows SlyA to exert its effect on the PhoP/PhoQ system specifically in Salmonella, which may confer an additional transcriptional regulation. Thus, our results provide a molecular mechanism that determines SlyA-dependent activation of PhoP-regulated genes in modulating Salmonella virulence. Evidence from this study also suggests a function of SlyA as a mediator in signal transduction from the PhoP/PhoQ system to other bacterial two-component systems in Salmonella.

The ability to sense hostile environments and trigger compensatory gene expression is critical for Salmonella typhimurium to survive within host cells (review see Refs. 1, 2). The PhoP/PhoQ two-component system governs regulatory signaling networks by responding to environmental changes, including acidic pH, low Mg²⁺, and host-derived antimicrobial peptides, which confer bacterial resistance to depletion of Mg²⁺ as well as bactericidal substances (3–5). When bacteria are grown under inducing conditions, kinase activity of the sensor in a two-component system is modulated to transfer a phosphoryl group from ATP to its cognate response regulator, which enhances the modified regulator to interact with its target promoters (for review see Ref. 6). Accordingly, PhoQ mediates phosphorylation of PhoP to facilitate binding of this regulator to the “PhoP box” sequence in promoter regions, thus giving rise to gene regulation (7, 8).

The PhoP/PhoQ system functions as a master regulatory system that controls expression of various transcriptional regulators such as the two-component systems PmrA/PmrB (9) and RstA/RstB (10), as well as the MarR family member SlyA (11, 12). SlyA, which is present in members of the family Enterobacteriaceae, has been recognized as a transcriptional regulator specifically modulating the intracellular expression of chromosomal loci required for Salmonella growth in macrophages (13) and resistance of internalized bacteria to oxidative stress (14). The PhoP/PhoQ system may contribute to Salmonella virulence, in part, by regulating slyA expression because observations from different laboratories suggest that SlyA is involved in regulation of a subgroup of PhoP-dependent genes (12, 15, 16). However, SlyA does not simply function as an intermediate regulator for the PhoP-directed regulation, but rather it includes more complicated regulatory circuits impinging on PhoP activity itself. Several previous results showed that a feedforward regulatory loop directs the expression of ugtL and pagC, whose promoter regions possess binding sites for both PhoP and SlyA (12, 16). Recently, we demonstrated that binding of PhoP and SlyA is to antagonize the inhibitory activity of the transcriptional repressor H-NS in these promoters, which occupies both the PhoP and SlyA boxes in signal-depleting conditions (17). As a result, transcription of ugtL and pagC is greatly activated only when both regulators are simultaneously present (12, 16). On the other hand, it remains to be determined whether SlyA could exert a direct regulatory effect on other PhoP- and SlyA-dependent loci identified from a transcriptomic analysis (16).

Transcription of the phoPQ operon is positively autoregulated (18). This regulation is also responsible for transcriptional expression in other two-component systems, e.g. ompR/envZ (19). Regardless of the important role played by the PhoP/PhoQ system in bacterial virulence, it is not clear whether other regulatory mechanisms are involved in controlling expression of the phoPQ operon in Salmonella. Here we demonstrate that SlyA fine-tunes the cellular level of the PhoP/PhoQ system. Our results provide evidence that SlyA
TABLE 1
Bacterial strains used in this study

| S. enterica serovar | Strain or plasmid | Description | Ref. or source |
|---------------------|------------------|-------------|---------------|
| Typhimurium         | 14028s           | Wild type   | ATCC          |
|                     | YS11590          | ΔphoP        | 17            |
|                     | YS11068          | ΔslyA        | This work     |
|                     | YS14047          | ΔslyA ΔphoP  | This work     |
|                     | YS11591          | up-pho-P HA  | This work     |
|                     | YS11592          | up-pho-P HA  | This work     |
|                     | YS10075          | slyA-FLAG    | This work     |
|                     | YS11249          | pho-24       | A derivative from 35 |
|                     | YS11250          | pho-24 ΔslyA | This work     |
|                     | YS11620          | STM3595-lacZ::KmR | This work |
|                     | YS11754          | pgl-lacZ::KmR | This work     |
|                     | YS11621          | STM3595-lacZ::KmR | This work |
|                     | YS10382          | lac1–6 lacZ  | 17            |
|                     | YS11743          | pgcl-lacZ::KmR | This work     |
|                     | YS14071          | pacG-FLAG-lacZ | This work |
|                     | YS11644          | mgbT-FLAG    | 17            |
|                     | YS15021          | mgtB-FLAG    | This work     |
|                     | YS15022          | ompr-HA      | This work     |
|                     | YS10849          | ompr-HA ΔslyA | This work     |
|                     | YS10170          | cpxR-HA      | This work     |
|                     | YS10173          | cpxR-HA ΔslyA | This work     |
|                     | YS10666          | pmra-FLAG    | This work     |
|                     | YS10665          | pmra-FLAG ΔslyA | This work |
|                     | YS14033          | arcA-ΔHA     | This work     |
|                     | YS14034          | arcA-ΔslyA   | This work     |
|                     | YS11477          | corA-FLAG-CmR | This work     |
|                     | YS14099          | corA-FLAG-CmR ΔslyA | This work |

E. coli

| Strain or plasmid | Description | Ref. or source |
|-------------------|-------------|---------------|
| DH5α              | F supE44 ΔluxU169 (680 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | 38 |
| BL21 (DE3)        | F ompT hsdS (rK30) m15 gal dcm (DE3) | 39 |
| MC1000            | F Δ(argR-lacU169 araD1399 ptsL150 tpsI25 ibbS501 ribR deoC recA1) | This work |
| YS14200           | MC1401 ΔslyA::KmR | This work |

Plasmids

| Strain or plasmid | Description | Ref. or source |
|-------------------|-------------|---------------|
| pKD3              | rep lac FRT | 21 |
| pKD46             | rep lac FRT | 21 |
| pCP20             | rep lac FRT | 472 bp of the wild-type slyA gene, generated with primers 45 and 46, and 675 bp of the wild-type phoP gene, generated with primers 88 and 89, and strain 14028s chromosomal DNA as template, with BamHI and HindIII and cloning between the EcoRI and HindIII sites of pUHE21. Plasmid pYS1055 was constructed by digesting a PCR fragment containing 437 bp of the wild-type slyA gene, generated with primers 478 and 479 and strain 14028s chromosomal DNA as template, with EcoRI and BamHI and cloning between the EcoRI and BamHI sites of pET11a. Inserted DNA sequences in plasmids were confirmed by DNA sequencing. | This work |

The abbreviations used are: SILAC, stable isotope labeling with amino acids in cell culture; WT, wild type; HA, hemagglutinin; IPTG, isopropyl 1-thio-β-D-galactoside; RT, reverse transcription; ChIP, chromatin immunoprecipitation; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.

participates in a positive feedback loop, which facilitates transcription of the phoPQ loci and, in turn, stimulates transcription of the PhoP regulon. Our data also suggest that SlyA functions as a connecting mediator that transmits signals from the PhoP/PhoQ system to several other two-component systems in Salmonella.

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. Escherichia coli was derived from the wild-type strain MC4100. Bacteria were grown at 37 °C in Luria-Bertani broth or in N minimal medium (pH 7.4) supplemented with 38 mM glycerol and 0.1% casamino acids, except SILAC experiments. MgCl2 is added to required concentrations. When necessary, antibiotics are added at final concentrations of 50 μg/ml for ampicillin, 20 μg/ml for chloramphenicol, 50 μg/ml for kanamycin, and 12.5 μg/ml for tetracycline. E. coli DH5α is used as a host for the preparation of plasmid DNA.

Construction of Chromosomal Mutations, lac Fusions, and Epitope-tagged Proteins—Oligonucleotides used as probes for the construction of strains and plasmids are described in Table 2. PCR products were used to generate coding region deletions, introduce FLAG/HA epitope sequence, or introduce a scar sequence for the lacZ fusion in bacterial chromosome as described previously (21). Primers were listed as pairs for individual genes in Table 2, and plasmid pKD3 was used as a template. All resulting strains were confirmed using colony PCR and DNA sequencing. A lac gene was integrated behind a coding region, in chromosome, using plasmid pKG137, into the FLP recombination target sequence generated after the CmR cassette was removed using plasmid pCP20 (22). The up-scarr-phoP-HA strain (YS11591) was constructed as follows. CmR cassette was introduced upstream of the phoP promoter using a PCR fragment synthesized with primers 395 and 396 from pKD3. DNA amplification was then carried out using chromosomal DNA from the above CmR strain as template and primers 28 and 395. This DNA product was electroporated into wild type harboring pKD46, and CmR colonies were selected. The HA fusion was confirmed using colony PCR and DNA sequencing. The CmR cassette was removed using plasmid pCP20 (22). Phage P1-mediated transductions in E. coli and phage P2-mediated transductions in Salmonella were performed as described previously (23, 24).

Construction of Plasmids—Plasmids pYS1109 and pYS1177 were constructed by digesting PCR fragments containing 472 bp of the wild-type slyA gene, generated with primers 45 and 46, and 675 bp of the wild-type phoP gene, generated with primers 88 and 89, and strain 14028s chromosomal DNA as template, with BamHI and HindIII and cloning between the EcoRI and HindIII sites of pUHE21. Plasmid pYS1055 was constructed by digesting a PCR fragment containing 437 bp of the wild-type slyA gene, generated with primers 478 and 479 and strain 14028s chromosomal DNA as template, with EcoRI and BamHI and cloning between the EcoRI and BamHI sites of pET11a. Inserted DNA sequences in plasmids were confirmed by DNA sequencing.

Analysis of slyA Gene Profile Using SILAC—We conducted SILAC analysis using the following steps modified from an original reference (25). (i) For preparation of bacterial samples, the growth conditions were 37 °C for 4 h aerobically in N minimal medium (pH 7.4; no casamino acids added) supplemented with 0.01 mM MgCl2 and 40 μg/ml L-[13C6]arginine and L-[13C6]lysine (Cambridge Isotope Laboratories, Inc.) for wild type or 40 μg/ml normal L-arginine and L-lysine (Sigma) for the slyA mutant. Salmonella cells were collected and opened, and the supernatant was separated from the cell debris. Samples from the wild-type strain and the slyA mutant with equal...
amounts of protein were mixed and subjected to SDS-PAGE. (ii) For excision of protein bands after staining the gel with Coomassie Brilliant Blue R-250, a whole lane was cut into 10 equal pieces with a clean scalpel, cutting as close to the edges of the bands as possible. The excised gel was then chopped into small pieces, roughly $1 \times 1$ mm, and transferred into 1.5-ml Eppendorf tubes. (iii) For in gel trypsin digestion, gel pieces were washed twice with 500 $\mu$l of an equal volume mixture of 25 mM NH$_4$HCO$_3$ and acetonitrile for 20 min, dehydrated with 100% acetonitrile for 20 min, and then dried with Speed vacuuming at room temperature. (iv) For reduction of proteins with dithiothreitol, protein samples in dried gel pieces were treated with 500 $\mu$l of 10 mM dithiothreitol in 25 mM NH$_4$HCO$_3$ for 60 min at 56 °C. (v) For alkylation of protein samples with iodoacetamide and after removing dithiothreitol solution by spinning, gel pieces were treated with 100 $\mu$l of 55 mM iodoacetamide in 25 mM NH$_4$HCO$_3$ in the dark at room temperature for 60 min and then centrifuged to remove the iodoacetamide solution. Gel pieces were washed with 200 $\mu$l of 25 mM NH$_4$HCO$_3$ for 20 min, dehydrated with 100% acetonitrile for 20 min, and then dried by Speed vacuuming. (vi) For digesting protein samples in gel with trypsin, gel pieces were first treated with 300 $\mu$l of trypsin (10 ng/lin 25 mM NH$_4$HCO$_3$ solution) by standing at room temperature for 10 min, and then 50 $\mu$l of 25 mM NH$_4$HCO$_3$ solution was added to keep the gel wet during digestion. Samples were then digested at 37 °C for 6 h or overnight. (vii) For peptide extraction from gel after trypsin digestion, the suspension from trypsin treatment was transferred to a new Eppendorf tube, and a 200-$\mu$l solution of 5% trifluoroacetic acid, 50% acetonitrile was added to the tube with gel pieces and incubated with gentle oscillation at room temperature for 60 min. The supernatant was combined with previous fractions. This extraction was repeated again by adding another 200 $\mu$l of 5% trifluoroacetic acid, 50% acetonitrile to the gel pieces for 60 min; the supernatant was then combined with the above fractions. The volume of supernatant was reduced to 30 $\mu$l using Speed vacuuming at room temperature, and then an equal amount (30 $\mu$l) of buffer containing 3% acetonitrile and 0.1% formic acid in water was added. (viii) For peptide high pressure liquid chromatography separation and liquid chromatography/electrospray ionization/MS/MS mass spectrometer analyses, samples (0.2–1.4 $\mu$l) were injected into a Micromass CapLC liquid chromatography system (Micromass, Manchester, UK) and concentrated in a PepMap C18 precolumn (300 mm x 5 mm). The precolumn was washed (3 min, 0.1% formic acid, flow rate of 30 $\mu$l/min), and then the peptide mixture was eluted into an analytical C18 column (150 mm x 17 mm) and analyzed using a solvent gradient from solution A (3% acetonitrile) to solution B (95% acetonitrile) containing 0.1% formic acid in water over 50 min at flow rates gradually reduced from 5 $\mu$l/min to 200 nl/min by stream splitting. Liquid chromatography eluent was applied into the nanoflow source of a Q-TOF micromass spectrometer (Micromass, Manchester, UK). Sample running conditions were set as follows: the source temperature was 80 °C; and the cone gas flow was 50 liters/h. A voltage of 3.2 kV was applied to the nanoflow probe tip, and data were acquired in positive ion mode. Survey scans were integrated over 1 s, and MS/MS scans were integrated over 3 s. Switching from survey

| TABLE 2 | Primers used in this study (classified) |
|---------|----------------------------------------|
| **A. Construction of deletion mutation** | |
| E. coli SlyA | ata ata tct tag caa ggt aat tat gag gat atg aat atc ctc ctt ag |
| | lgc ggt lac tga eca ggc gcc ctc tle atg ttg tag gga ggt get tc |
| **B. Construction of chromosomal epitope** | |
| upA-phoP-MA | gca gaa aat ggc gga caa att ttt cta tga ata aac aac aca aca |
| | ega aca aca gca ccc aac aac aac aac aac aac |
| slyA-FLAG | coc aat att agg gaa ttc ctc taa gat ggc gcc gac gat gca |
| | taa cca gct gac taa tct ctc ctc |
| corA-FLAG | ggc gct taa gca gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| | 100 $\mu$M acetonitrile, 25 mM NH$_4$HCO$_3$ |
| ammon-HA | lgc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| pmrA-FLAG | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| cpxR-HA | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| mgtB-FLAG | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| archA-HA | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| **C. Construction of chromosomal lacZ fusion** | |
| STM35655-behind | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| | 100 $\mu$M acetonitrile, 25 mM NH$_4$HCO$_3$ |
| **D. Construction of plasmids** | |
| pYS1109 | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| | 100 $\mu$M acetonitrile, 25 mM NH$_4$HCO$_3$ |
| pYS1177 | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| pYS1155 | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| **E. ChiP assay** | |
| phoP promoter | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| | 100 $\mu$M acetonitrile, 25 mM NH$_4$HCO$_3$ |
| **F. Footprinting for phoP promoter** | |
| 469 | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| 470 | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| **G. Reverse transcription PCR** | |
| Salmonella typhimurium | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| phoP | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| roPD | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| corA-FLAG | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| E. coli | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| phoP | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |
| corA-FLAG | ggc gct taa ggc gca gta gaa ttc ctc taa aag aac aac aac aac aac |

All oligonucleotides were purchased from Integrated DNA Technologies.
SlyA Feedback Activates the PhoP/PhoQ Two-component System

FIGURE 1. SILAC analysis demonstrates that SlyA mediates fine-tuning of two-component systems in Salmonella. A, experimental design for identification of the SlyA regulon using SILAC analysis (details see “Experimental Procedures”). B, Western blot analysis of cell extracts prepared from chromosomal pagC-FLAG, phoP-FLAG, ompR-FLAG, cpxR-FLAG, pmrA-FLAG, arcA-FLAG, mgtB-FLAG, and corA-FLAG in wild type (YS11644, YS11591, YS10848, YS10170, YS10666, YS14033, YS15021, and YS11477) and slyA mutant (YS11664, YS11592, YS10849, YS10173, YS10665, YS14034, YS15022, and YS14099). Bacteria were grown for 4 h in N medium (pH 7.4) containing 0.01 mM MgCl2. Expression of the phoP gene and the phoP mutant (YS11664, YS11592, YS10849, YS10173, YS10665, YS14034, YS15022, and YS14099) was induced from strains harboring pYS1109 and pYS1177 by adding 0.2 mM IPTG under the same growth conditions. The tagged proteins from whole-cell lysates were separated in 12.5% SDS-polyacrylamide gels and detected using the immunoblot analysis (ECL, Pierce). Quantification was conducted using software Quantity One (Bio-Rad).

β-Galactosidase Assays—Galactosidase assays were carried out in triplicate, and the activity was determined as described previously (23). Data correspond to three independent assays conducted in duplicate. Percentage of β-galactosidase activity in Fig. 5B was calculated by the following: (β-galactosidase activity in y mm) ÷ β-galactosidase activity in 0.01 mM) × 100. y mm means a given Mg2+ concentration we tested. C1/2 represents the experimentally determined value of Mg2+ concentration, which allows percentage of β-galactosidase activity to reach 50% maximum level.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described previously (27). 0.2 mM IPTG was supplemented in N medium for pYS1109-carrying and pYS1177-carrying bacteria. The phoP promoter region was detected by PCR using primers 409 and 410 (Table 2).

Electrophoretic Mobility Shift Assay (EMSA)—1 pmol of 32P-labeled phoP DNA fragment amplified with primers in Table 2 was incubated at room temperature for 30 min with 50 pmol of SlyA-FLAG protein 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. The “cold” DNA was added in excess of the “hot DNA” amount when required. Monoclonal anti-FLAG M2 (−1 μg, Sigma), as well as FLAG octapeptide (Sigma), was used when required. After the addition of the DNA dye solution (40% glycerol, 10% SDS, 0.1 mg/ml bromophenol blue), DNA was denatured by boiling and run on an 8% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography with a MS50C phosphor screen reader (Molecular Dynamics).

Western blot analysis of cell extracts prepared from chromosomal pagC-FLAG, phoP-FLAG, ompR-FLAG, cpxR-FLAG, pmrA-FLAG, arcA-FLAG, mgtB-FLAG, and corA-FLAG in wild type (YS11644, YS11591, YS10848, YS10170, YS10666, YS14033, YS15021, and YS11477) and slyA mutant (YS11664, YS11592, YS10849, YS10173, YS10665, YS14034, YS15022, and YS14099). Bacteria were grown for 4 h in N medium (pH 7.4) containing 0.01 mM MgCl2. Expression of the phoP gene and the phoP mutant (YS11664, YS11592, YS10849, YS10173, YS10665, YS14034, YS15022, and YS14099) was induced from strains harboring pYS1109 and pYS1177 by adding 0.2 mM IPTG under the same growth conditions. The tagged proteins from whole-cell lysates were separated in 12.5% SDS-polyacrylamide gels and detected using the immunoblot analysis (ECL, Pierce). Quantification was conducted using software Quantity One (Bio-Rad).

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Enhanced Expression of the PhoP/PhoQ System by SlyA—A cDNA microarray showed that SlyA influences many PhoP-regulated loci (16). However, a SlyA-FLAG fusion protein was unable to interact with every promoter of these genes because not all intergenic regions can be gel-shifted by this protein (data not shown). This observation suggests that SlyA should regulate these genes indirectly by modulating regulatory activity of another transcriptional regulator. To find this possible factor not yet identified in the previous studies, we employed a new approach by conducting a quantitative proteomic analysis (i.e. SILAC, see Ref. 25) to profile protein levels affected by SlyA in Salmonella under a growth condition in which transcription of the slyA gene is activated (11, 12). The experiment design is illustrated in Fig. 1A, in which wild-type culture was supplemented with stable isotope [13C]-labeled arginine and lysine (shown as Arg-[13C]6 and Lys-[13C]6, respectively), whereas the slyA mutant was supplemented with normal arginine and lysine (Arg-[12C]6 and Lys-[12C]6, respectively). Comparison of a peptide synthesized in wild type and the slyA mutant from constitutively expressed genes yields a ratio of [13C]-labeled peptide versus normal [12C]-labeled peptide (shown as 13C/12C in this work) ~0.7 (results of the arcA and ompA gene products in Table 3, and data not shown). This is because Salmonella possesses de novo synthesis pathways for arginine and lysine (for review see Ref. 28); therefore, both normal [12C]-embedded and exogenous [13C]-labeled amino acids will be incorporated into proteins of wild-type cells. Genes that produced proteins with ratios above 0.7 were regarded as SlyA-activated loci. Consistent with this definition, five peptides from SlyA-activated PagC (16) gave an average 13C/12C ratio of 13.39 (Table 3). In this study, only selected chromosomal loci responding to SlyA regulation are listed in Table 3 from our ongoing SILAC analysis.

Three peptides derived from PhoP have an average 13C/12C ratio of 3.34 (Table 3), indicating that this regulator should be up-regulated by SlyA. Consequently, SlyA may facilitate the overall expression of PhoP-activated genes by raising the level of the PhoP/PhoQ system. Indeed, results from SILAC in Table 3 revealed that SlyA activates several loci dependent directly or indirectly on PhoP (10, 29, 30), including mgtB (average 13C/12C = 3.00), ulgD (2.90), pmrF (1.43), yaiB (2.41), pmrA (2.50), and rtaA (1.66). However, a gel-shift result showed that the SlyA-FLAG protein could not interact with promoter regions of the mgtCB and yaiB loci (data not shown). Taken together, these results demonstrate that SlyA enhances the expression of PhoP-activated genes by a feedback up-regulation of the PhoP/PhoQ system.

In addition, SILAC results show that several two-component systems are modulated by SlyA (Table 3), indicating that SlyA might enhance expression of these systems that were not yet associated with the PhoP/PhoQ system. The OmpR/EnvZ system (31) appears to be activated by SlyA because the average 13C/12C ratio of two peptides from regulator OmpR was 3.76. Meanwhile, HtrA, a protease activated by the CpxR/CpxA sys-

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### TABLE 3

**MS analysis of the SlyA-activated loci from SILAC assay**

| Gene number | Gene symbol | Function | Peptide identified | 13C/12C | Average 13C/12C |
|-------------|-------------|----------|-------------------|--------|-----------------|
| STM1246     | pgcC        | unknown  | 2QGQSNPVEPGYH1DR  | 20.00  | 13.39           |
|             |             |          | YSLMLKPGYRR      | 13.62  |                 |
|             |             |          | 6LDNFLXALVGYCR   | 8.13   |                 |
|             |             |          | 6LDNFLXALVGYCR   | 5.00   |                 |
|             |             |          | 6LDNFLXALVGYCR   | 20.00  |                 |
| STM2576     | mgtB        | Mg2+ transporter | ELQELLERYEDNYACQG   | 2.80   | 0.70           |
|             |             |          | 6LDNFLXALVGYCR   | 3.12   |                 |
| STM2818     | aldF        | dehydrogenase | 6LTENPSPIEUK      | 3.20   | 2.90           |
|             |             |          | 6FDAAUQEBAK      | 2.00   |                 |
| STM2898     | pmrF        | glycosyl transferase | 6LHD999KLSLNR     | 1.45   | 1.43           |
|             |             |          | 6LDNFLXALVGYCR   | 1.30   |                 |
|             |             |          | 6FDAAUQEBAK      | 1.55   |                 |
| STM3289     | htrA        | protease  | 6LHD999KLSLNR     | 1.22   | 1.27           |
|             |             |          | 6LDNFLXALVGYCR   | 0.82   |                 |
| STM3483     | yuhB        | acrosomal regulator | 6LNL322KLSLNR     | 2.51   | 2.41           |
|             |             |          | 6LDNFLXALVGYCR   | 2.30   |                 |
| STM3631     | phoP        | TCR      | VQVQSD99KLSLNR    | 3.25   | 3.34           |
|             |             |          | 6LDNFLXALVGYCR   | 3.68   |                 |
|             |             |          | 6LDNFLXALVGYCR   | 3.05   |                 |
| STM3650     | ompR        | TCR      | VQVQSD99KLSLNR    | 3.32   | 3.76           |
|             |             |          | 6LDNFLXALVGYCR   | 4.20   |                 |
| STM3692     | pmrA        | TCR      | VQVQSD99KLSLNR    | 1.70   | 2.50           |
|             |             |          | 6LDNFLXALVGYCR   | 3.29   |                 |
| STM4175     | rtaA        | TCR      | VQVQSD99KLSLNR    | 1.66   | 1.66           |
|             |             |          | 6LDNFLXALVGYCR   | 1.66   |                 |
| STM4520     | arcA        | TCR      | VQVQSD99KLSLNR    | 0.70   | 0.70           |
|             |             |          | 6LDNFLXALVGYCR   | 0.70   |                 |

### Notes

*1* Calculated using the most abundant peak from mass spectrum.
*2* TCR is an abbreviation for regulator protein in a two-component system.

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erol, 0.05% bromphenol blue, 0.05% xylene cyanol), the mixture was directly subjected to 5% PAGE. Signals were detected by autoradiography.

**DNase I Protection Assays**—DNase I protection assays were carried out using DNA fragments amplified by PCR using Salmonella chromosomal DNA as template. Prior to the PCR, primers 469 and 470 (Table 2) were labeled with T4 polynucleotide kinase and [γ-32P]ATP. The phoP promoter region was amplified with primers 32P-labeled 469 and 470 for the coding strand or with 469 and 32P-labeled 470 for the noncoding strand. Approximately 25 pmol of labeled DNA and 0, 50, 100, or 200 pmol of the SlyA-FLAG protein were used in a 100-μl reaction. DNase I digestion was carried out as described previously (8). DNase I was purchased from Invitrogen, and 0.05 μg/ml of DNase I was added, and bacteria were incubated for another 2 h. Cells were harvested, washed with PBS once, resuspended in 10 ml of PBS, and opened by sonication. The whole-cell lysate was used for SlyA-FLAG purification by mixing with EZview Red Anti-FLAG M2 affinity Gel (Sigma) following the instructions from the manufacturer. Pure SlyA-FLAG sample was tested using silver staining (Pierce) following the instructions from the manufacturer.
SlyA Feedback Activates the PhoP/PhoQ Two-component System

Immunoblot analyses were performed to evaluate the SILAC results using strains harboring HA or FLAG epitope immediately upstream of the stop codon of their chromosomal loci for two-component regulator genes and other genes. Consistent with previous results (16), the PagC protein level was dramatically reduced in a slyA mutant when compared with wild type (Fig. 1B). Meanwhile, we found that protein levels of PhoP, OmpR, CpxR, PmrA, and MgtB were lower in slyA mutants than those levels in wild-type strains, whereas protein levels of ArcA and the control CorA were similar in wild-type and the slyA mutant (Fig. 1B). Peptides representing components from other two-component systems were unable to be identified in the current SILAC assays. One possible reason was that synthesized proteins in the current growth condition were below detectable levels.

SlyA Facilitates Transcription of the phoPQ Operon—SlyA seems unlikely to control a post-transcriptional modification of PhoP because the level of this protein became similar when it was expressed from heterologous promoter \( \text{Pr}_{\text{lac}} \) (Fig. 2A). To see if SlyA could exert its effect directly on phoP transcription, we determined mRNA levels of the phoP transcript corresponding to the first 100 nucleotides of the coding region. When bacterial cells were grown in 0.01 mM Mg\(^{2+}\), the phoP mRNA level was \( \sim 2.5\)-fold higher in wild type than the isogenic slyA mutant (Fig. 2B). Consistently, the PhoP protein level was \( \sim 3\)-fold higher in wild type than the slyA mutant (Fig. 2B). SlyA does not influence overall mRNA levels because the mRNA level of a constitutive gene, \( \text{rpoD} \), was similar in both wild type and slyA mutant, nor does it influence overall protein levels because the level of CorA protein was similar in both strains (Fig. 2B).

**FIGURE 2.** SlyA facilitates transcriptional activity of the PhoP/PhoQ system in \textit{Salmonella}. Bacteria were grown for 4 h in N medium (pH 7.4) containing 0.01 mM Mg\(^{2+}\) in the following assays. 0.2 mM IPTG was supplemented to growth of the bacterial cells harboring pUHE21–2lac\(^{4C}\) with a slyA-FLAG fusion (pYS1109, pslya) or pUHE21–2lac\(^{4C}\) with a phoP-HA fusion (pYS1177, phoP). A, Western blot analysis of cell extracts prepared from wild type (14028s) and the slyA mutant (YS11068) harboring plasmid pYS1177. Monoclonal anti-HA antibodies (Sigma) were used. Percentage of the protein (Pr) amount was calculated by formula: \( \% \text{Pr} = \frac{\text{density of test strain}}{\text{density of wild type}} \times 100 \). B, mRNA levels of phoP were determined using RT-PCR analysis in wild type (14028s), slyA mutant (YS11068), and slyA mutant harboring pYS1109. Constitutively transcribed \( \text{rpoD} \) gene indicated that similar amounts of total RNA were used. The PCR products were separated in an agarose gel. Meanwhile, the protein levels of PhoP-HA, CorA-FLAG, and SlyA-FLAG were determined, respectively, using Western blot analysis in wild type (YS11591, YS11477, and YS10075), slyA mutant (YS11592, YS14099, and YS11068), and slyA mutant harboring pYS1109 (pystyla). Monoclonal anti-HA antibodies (Sigma) were used for HA-tagged protein and anti-FLAG M2 (Sigma) for FLAG-tagged protein. Constitutively produced CorA indicated that similar amounts of total protein were used. Percentage of the protein (Pr) amount was calculated by formula: \( \% \text{Pr} = \frac{\text{density of test strain}}{\text{density of wild type}} \times 100 \). C, \( \beta \)-galactosidase activity from bacteria harboring pYS1100 (\( \text{Pr}_{\text{phoP}} \)), WT, with wild-type sequence, pYS1115 (\( \text{Pr}_{\text{phoP}} \) up-6, with substituted actatt sequence), and pYS1244 (\( \text{Pr}_{\text{phoP}} \) up-far, with substituted trttttct sequence) was determined in wild type (14028s), slyA mutant (YS11068), and slyA mutant harboring pYS1109 (pystyla). Data in C and D correspond to three independent assays, and all graphed values are means \( \pm \) S.D.

The reduced phoP mRNA level or PhoP protein level in the slyA mutant could be recovered by a plasmid (pYS1109, pslya) carrying a wild-type copy of the slyA-FLAG fusion, indicating that this phenotype resulted solely from an absence of the SlyA protein that was also demonstrated by a Western blot analysis (Fig. 2A).
SlyA Feedback Activates the PhoP/PhoQ Two-component System

![SlyA-FLAG DNA fragment](image)

**FIGURE 3. SlyA binds to the promoter region of Salmonella phoP gene in vitro.** A, EMSA. 32P-Labeled phoP DNA fragment was incubated with SlyA-FLAG protein. The 5th lane is the same as 3rd lane but supplemented with nonlabeled phoP DNA fragment (or cold). Monoclonal anti-FLAG M2 antibodies (Ab) were applied in 2nd and 4th lanes and 7th to 9th lanes. Increasing amounts of FLAG octapeptide (0.01 and 0.1 μg, respectively) were applied in 8th and 9th lanes. SlyA/DNA mixtures were subjected to 5% PAGE. Binding of the SlyA-FLAG protein from result in the coding and noncoding strands (see “Experimental Procedures”) and increasing amounts of SlyA-FLAG (0, 50, 100, and 200 pmol) proteins. Solid vertical lines correspond to regions protected by the SlyA protein. Lane AG corresponds to DNA ladder derived from Maxam and Gilbert A+G reaction. Numbering is from the putative start codon of the phoP open reading frame. Dotted lines correspond to the phoP box region. C, sequence of chromosomal purR phoP intergenic region in S. typhimurium. Black lines indicate the DNA region protected by the SlyA-FLAG protein from result in B. The dark gray boxes correspond to the PhoP box. The light gray box and open box correspond to the sequences for substitutions in plasmids pYS1115 carrying P\text{phoP} up-6 and pYS1100 carrying P\text{phoP} up-far. **D, mRNA levels from PhoP expression in the** E. coli mutant (Fig. 2D), in which this peptide competed for anti-FLAG antibody with SlyA-FLAG. Similar to PhoP transcription, this plasmid failed to rescue expression of these loci in a phoP mutant (Fig. 2C), indicating that SlyA-dependent activation requires a functional PhoP/PhoQ system. These results demonstrate a new mechanism that modulates regulatory activity of the PhoP/PhoQ system through SlyA-mediated feedback transcriptional activation of the phoP operon.

**SlyA Binds to the Promoter Region of the phoPQ Loci—SlyA binds to the promoter region of ugtL and pagC (12, 16) to compete with H-NS, subsequently activating these type II PhoP-dependent genes (17). We hypothesized that SlyA mediates autoregulation of the phoPQ operon by interacting with its promoter. We carried out an EMSA using SlyA-FLAG protein, and found that this fusion protein alone gel-shifted the P\text{phoP1} DNA fragment present in plasmid pYS1100 (Fig. 3A). Binding of the SlyA protein is further confirmed by a supershift assay using anti-FLAG M2 antibodies (Sigma) that inhibited SlyA-DNA interaction (Fig. 3A). This is probably because the antibody bound to the FLAG epitope and subsequently blocked the DNA recognition domain in the SlyA protein. Consistent with this notion, we found that phoP promoter DNA was shifted more by SlyA-FLAG in the presence of antibody when FLAG octapeptide (DYKDDDDK) was supplemented to reaction systems (Fig. 3A), in which this peptide competes for anti-FLAG antibody with SlyA-FLAG.

Next, we determined the DNA sequence in the phoP promoter recognized by SlyA protein using DNase I footprinting assays. We show that SlyA interacts with the AT-rich regions in the phoP promoter because the SlyA-FLAG protein protected the −106 to −101 and −90 to −72 region (numbering from the first ATG in the PhoP coding region) in the coding strand and the −109 to −102 and −88 to −76 region in the noncoding strand (Fig. 3B). The ACTATT sequence, which is located 6 nucleotides upstream of the PhoP box (Fig. 3C) and is identified as the SlyA box, corresponds to the sequences for substitutions in plasmids pYS1115 carrying P\text{phoP} up-6 and pYS1100 carrying P\text{phoP} up-far. P1 is the PhoP-dependent transcription start site and the 3’ end of P\text{phoP1} fragment in pYS1100. The black box is the corresponding sequence of the phoP promoter from E. coli. The dots correspond to the conserved nucleotides in phoP promoters of Salmonella and E. coli. Numbering is from the A (as +1) in the predicted phoP start codon (shown as uppercase letters). D, mRNA levels from E. coli were determined using RT-PCR in wild type (MC4100), and SlyA mutant (YS14200) grown for 4 h in N medium (pH 7.4) containing 0.01 or 10 mM Mg\textsuperscript{2+}. Percentage of the mRNA amount was calculated by formula: %mRNA = (ratio of the individual phoP/mrpD ÷ ratio of wild type from 0.01 mM Mg\textsuperscript{2+}) × 100.

2B). We studied the in vivo phoP transcription using a plasmid (pYS1100) carrying a lacZ transcriptional fusion to a phoP promoter fragment (P\text{phoP1}), in which lacZ expression was dependent on the PhoP/PhoQ system (17) (Fig. 2C). β-Galactosidase activity was 2.4-fold lower in a phoP mutant than in the wild-type strain, which further demonstrated SlyA feedback activating transcription of the phoPQ operon. The compatible plasmid harboring the slyA gene (pYS1109) complemented the deficient phenotype of the lacZ gene in the slyA mutant, but not in the phoP mutant (Fig. 2C), indicating that SlyA modulates transcription of the phoPQ operon through PhoP (i.e. autoregulation).

To see if changed PhoP levels could influence transcription of PhoP-activated genes, we constructed strains carrying a lacZ fusion in PhoP-activated chromosomal loci STM3595, pcgL, and many others demonstrated previously from different studies (29, 33, 34). Expression of lacZ from these strains was PhoP-dependent because no β-galactosidase activity was detected when the phoP locus was mutated (Fig. 2D), and data not shown). Analysis of the lacZ expression indicates that SlyA facilitates expression of these selected genes in 0.01 mM Mg\textsuperscript{2+} (Fig. 2D, and data not shown). Different from the results of pagC and ugtL activation (12, 16), expression of these PhoP-activated genes decreased ≥2-fold, but were not turned off in slyA mutants (Fig. 2D and data not shown). This phenotype resulted solely from an absence of the SlyA protein because it could be complemented by pSlyA plasmid (Fig. 2D). Similar to phoP transcription, this plasmid failed to rescue expression of these loci in a phoP mutant (Fig. 2C), indicating that SlyA-dependent activation requires a functional PhoP/PhoQ system. These results demonstrate a new mechanism that modulates regulatory activity of the PhoP/PhoQ system through SlyA-mediated feedback transcriptional activation of the phoPQ operon.
SlyA Feedback Activates the PhoP/PhoQ Two-component System

SlyA facilitated phoP transcription occurs in Salmonella but not in E. coli. The mRNA levels of the phoP transcript were similar in wild type and slyA mutant (Fig. 3D), indicating that SlyA is unlikely to be integrated in the PhoP/PhoQ regulatory circuit in E. coli. Currently, we are elucidating the role of SlyA in fine-tuning of two-component signaling by systematically comparing the binding ability of SlyA and H-NS to promoter regions of the two-component systems in Salmonella and E. coli. |  

SlyA Facilitates the Interaction of the PhoP Protein to Its Own Promoter—To examine the in vivo binding of SlyA and PhoP to the phoP promoter by implementing ChIP assays, we constructed the following strains producing SlyA and PhoP from heterologous promoter $\text{P}_{\text{lac}}$: slyA mutant and slyA phoP double mutant harboring plasmid pYS1109, which directs synthesis of SlyA-FLAG protein; phoP mutant and slyA phoP double mutant harboring plasmid pYS1109, which directs synthesis of PhoP-HA protein. We ruled out different binding abilities of these regulators to the phoP promoter caused by varied levels of the protein because bacterial cultures supplemented with 0.2 mM IPTG produced similar amounts of SlyA-FLAG or PhoP-HA proteins, regardless of their genetic background (Figs. 4, A and B).

The PhoP promoter DNA was enriched equally by the SlyA-FLAG protein from both strains producing SlyA and PhoP when it has been binding to its target promoters. We are elucidating the role of SlyA in fine-tuning of two-component signaling by systematically comparing the binding ability of SlyA and H-NS to promoter regions of the two-component systems in Salmonella and E. coli. |  

SlyA activates phoP regulation in a PhoP-

as an H-NS-binding site (i.e. up-6, see Ref. 17), overlaps the -90 to -72 sequence and resembles the ATTATT repeat (the SlyA box) from the pagC and ugtL promoters. We compared lacZ expression from strains harboring pYS1100 (wild-type $P_{\text{phoP}}$) and pYS1100-derived plasmids with substitutions at the $P_{\text{phoP}}$ sequence. Surprisingly, $\beta$-galactosidase activity was similarly activated in wild type and slyA mutant harboring plasmid $\text{pup-6}$ with substitution at ACTATT (Fig. 2C). Because mutation of this sequence also abolished H-NS-mediated transcriptional repression in the phoPQ operon (17), we believe that SlyA is an effector that antagonizes the H-NS function in phoP regulation. Therefore, phenotype of a slyA mutant could be recessive when H-NS is absent (Fig. 2C). On the other hand, lacZ expression from wild type harboring another pYS1100-derived plasmid with a heptamer substitution at TTTTCTTT within the -109 to -101 sequence (i.e. $\text{pup-far}$ in Ref. 17), similar to pYS1100, is higher than that from the slyA mutant (Fig. 2C). These results indicate that binding of the SlyA protein to the region adjacent to the PhoP box in the phoP promoter is required for its transcriptional activation in wild-type bacteria. The promoter region corresponding to the Salmonella SlyA-binding site is a GC-rich DNA fragment in the E. coli phoP promoter (letters in white, Fig. 3C). When the in vivo binding of the SlyA-FLAG protein to the E. coli phoP promoter was approached using ChIP assays, no significant enrichment of the phoP DNA was observed (data not shown). These observations suggest that

\[ \text{Percentage of mRNA amount} = \frac{\text{mRNA} \times 100}{\text{Input DNA} \times \text{Input Protein}} \]

FIGURE 4. SlyA facilitates PhoP binding to its own promoter in vivo. Bacteria were grown for 4 h in N medium (pH 7.4) containing 0.01 mM Mg\(^{2+}\) in the following assays. 0.2 mM IPTG was supplemented to growth of the bacterial cells harboring pYS1109 (pysA) or pYS1177 (pphoP). A, in vivo SlyA binding to the phoP promoter was determined in slyA mutant (YS11068) harboring pYS1109 (pysA) and slyA phoP double mutant (YS14047) harboring pYS1109 (pysA). % DNA = (individual density - individual input density) × 100. % protein (Pr) = (individual density - density in b) × 100. Value “0” indicates an actual value was below 1%. B, in vivo PhoP binding to the phoP promoter was determined in phoP mutant (YS111590) harboring pYS1177 (pphoP) and slyA phoP double mutant (YS14047) harboring pYS1177 (pphoP). Wild-type 14028s was used in A and B as untagged strain. Input is total DNA, and IP is immunoprecipitated DNA. PCR amplification was performed for 26 cycles, and DNA fragments were separated in an agarose gel and visualized by ethidium bromide. % DNA and % protein were calculated as in Ref. 49. As shown in Fig. 4, A, B, and C, mRNA levels were determined by RT-PCR in phoP–strain (pho-24, YS11249), and phoP–slyA-FLAG protein; pYS1109, which directs synthesis of SlyA-FLAG protein; phoP mutant and slyA phoP double mutant harboring plasmid pYS1109, which directs synthesis of PhoP-HA protein. 

| A | B |
|---|---|
| ChIP | ChIP |
| % DNA | % DNA |
| a | b |
| c | a |
| c | b |
| c | a |

4 G. Zhao, W. Kong, and Y. Shi, manuscript in preparation.
SlyA Feedback Activates the PhoP/PhoQ Two-component System

Figure 5. SlyA enhances PhoP-activated transcription but does not influence Mg\(^{2+}\) responsiveness. A, \(\beta\)-galactosidase activity from strains harboring pYS1100 (\(P_{\text{phoP}}\)) or chromosomal STM3595-lacZ fusion was determined in wild type (wt) (14028s and YS11620) and slyA mutant (YS11068 and YS11621) grown for 4 h in N medium (pH 7.4) supplemented with 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, and 10.0 mM Mg\(^{2+}\). Values are means ± S.D. B, percentage of \(\beta\)-galactosidase activity in A. \(C_{1/2}\) (wt) and \(C_{1/2}\) (slyA) represent the Mg\(^{2+}\) concentration that allows percentage of lacZ transcription from wild type and the slyA mutant to reach 50% maximum level, respectively.

Discussion

We have identified a regulatory mechanism that is responsible for activating transcription of the PhoP/PhoQ two-component signaling system of S. enterica. We establish that the PhoP regulon is up-regulated by a positive feedback controlled by the PhoP-activated SlyA. This model is supported by the following data. (i) The level of PhoP and PhoP-activated gene products was reduced in slyA mutants (Table 3 and Fig. 1B). (ii) Transcription directed by PhoP-dependent promoters decreased in slyA mutants (Fig. 2, B–D). (iii) Interaction of SlyA to the phoP promoter was observed in vitro (Fig. 3, A and B). (iv) The DNA-binding sites of SlyA and H-NS are overlapped (Fig. 3, B and C). (v) SlyA facilitates PhoP in binding to its own promoter in vivo (Fig. 4, A and B). (vi) SlyA up-regulates the PhoP regulon in varied Mg\(^{2+}\) conditions (Fig. 5A).

Transcription of a genetic locus may be more complicated than being turned on/off. It has been demonstrated that PhoP-activated PmrD functions as a regulator that enhances the PmrA/PmrB system in low Mg\(^{2+}\) condition (9). On the other hand, the PmrA regulator could inhibit the synthesis of PmrD by negative feedback because \(\beta\)-galactosidase activity from a pmrD-lacZ fusion in a pmrA or pmrB mutant became about 2-fold higher than that in wild type (37). Apparently, fine-tuning of the transcription process was designed as a strategy to control the pmrD mRNA level and therefore to control the PmrD protein level. Here, we unraveled novel regulatory mechanisms underlying the transcriptional activation of the PhoP/PhoQ system. We demonstrate that PhoP-activated SlyA functions as a positive feedback activator to facilitate transcription of the PhoP/PhoQ system, which therefore enhances PhoP-activated genes overall in Salmonella.

The SlyA protein seems to exert its effect as a positive regulator by antagonizing inhibitory action of the global regulator H-NS in the phoP expression. We proposed that two key components determine transcription of the phoPQ operon as follows: the PhoP protein that binds to the PhoP box and activates the ratio of phosphorylated PhoP at any given signal level. Our result indicates that the presence of SlyA simply increases transcriptional level but does not influence Mg\(^{2+}\) sensing of the PhoP/PhoQ system. Consistent with this notion, expression of several other PhoP-activated genes also gave similar \(C_{1/2}\) values from wild type and slyA mutant (data not shown).
SlyA Feedback Activates the PhoP/PhoQ Two-component System

PhoQ is modulated, which mediates phosphorylation of the cognate regulator PhoP. The activated regulator then enhances SlyA protein at a transcriptional level, which up-regulates transcription of phoPQ, as well as ompR/envZ that responds to osmotic stress (19) and cpxRA that responds to extracellular stress (32) in enteric bacteria. Therefore, depletion of Mg^{2+}, exposure to acidic pH, and antimicrobial peptides would generate stresses that require gene products from those loci controlled by other two-component systems. It remains to be investigated whether SlyA could activate other two-component systems by antagonizing the inhibitory effect of H-NS.

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SlyA Feedback Activates the PhoP/PhoQ Two-component System

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