A dual-signal regulatory circuit activates transcription of a set of divergent operons in *Salmonella typhimurium*

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We present a molecular mechanism for signal transduction that activates transcription of the SlyA regulon in *Salmonella typhimurium*. We demonstrate that SlyA mediates transcriptional activation in response to guanosine tetraphosphate, ppGpp, according to the following observations: (*i*) in vivo transcription of SlyA-dependent genes is repressed when ppGpp is absent; this transcription can be restored by overproducing SlyA; (*ii*) in vivo dimerization and binding of SlyA to the target promoter are facilitated in the presence of ppGpp; and (*iii*) in vitro SlyA binding to the target promoter is enhanced when ppGpp is supplemented. Thus, ppGpp must be the cytoplasmic component that stimulates SlyA regulatory function by interacting directly with this regulator in *Salmonella*. This signaling domain, integrated by the PhoP/PhoQ 2-component system that activates *slyA* transcription by sensing Mg^{2+}, forms feedforward loops that regulate chromosomal loci identified through a motif search over the *S. typhimurium* genome. Many such loci are divergent operons, each formed by 2 neighboring genes in which transcription of these 2 loci proceeds in opposite directions. Both genes, however, are controlled by PhoP and SlyA through a single shared PhoP box and SlyA box present in their intergenic regions. A substitution in either box sequence causes a simultaneous cessation of transcription of a divergent operon, *pagD-pagC*, equivalent to the phenotype in a *phoP* or *slyA* mutant. We also identified several chromosomal loci that possess *pagC*-type genes without the cognate *pagD*-type genes. Therefore, our results provide a molecular basis for the understanding of SlyA-dependent phenotypes associated with *Salmonella* virulence.

feedforward loop | ppGpp | SlyA | divergent operon | PhoP/PhoQ system

The MarR family member SlyA and its homologs are present in members of the family *Enterobacteriaceae*. In *Salmonella enterica* serovar Typhimurium, this transcriptional regulator modulates the intracellular expression of genes required for growth in macrophages (1) as well as bacterial resistance to cationic antimicrobial peptides (2, 3) and to oxidative stress (4). Transcription of the *slyA* locus is activated by the PhoP/PhoQ 2-component system (2, 5), which responds to different environmental signals including low-Mg^{2+}, host-derived antimicrobial peptides, and acidic pH (6–8). Consistently, results from different laboratories showed that SlyA regulates a subset of PhoP-dependent genes, including *ugtL* and *pagC*, in *Salmonella* (2, 3, 9–11). To activate transcription of *ugtL*, both PhoP and SlyA must bind to its promoter simultaneously (2). A direct repeat sequence, TAAAT-(6 nt)-TAAAC, was identified as the PhoP binding site, which resembles a typical PhoP box (12) but is located on the reverse strand in the *ugtL* promoter and also is conserved in the *pagC* promoter (2, 13, 14). On the other hand, DNA sequences for SlyA binding varied from footprinting analyses conducted by different laboratories (2, 3, 11, 13). The active form of SlyA is a dimer (15), which antagonizes a DNA-binding protein, H-NS, that seems to occupy AT-rich DNA regions throughout chromosomes of enteric bacteria (reviewed in ref. 16). Therefore, we reason that SlyA should bind to an AT-rich inverted or direct repeat sequence in the target promoter.

The feedforward regulatory loop is one of the most significant network motifs in which a transcription factor regulates a second transcription factor and both coordinately regulate a specific gene by responding to 2 different signals (17). In the arabinose utilization system, cAMP receptor protein (CRP) is the transcriptional regulator responding to cAMP induced by the primary signal (i.e., low environmental glucose levels), whereas CRP-activated arabinose-responsive protein AraC is the regulator directly binding to the secondary signal (i.e., *L*-arabinose) (reviewed in ref. 18). The arabinose catabolism operon *araBAD* can be activated only in a growth condition when glucose is depleted and arabinose is supplemented. As shown in our previous study, SlyA and PhoP can form a feedforward loop to regulate the *ugtL* gene in *Salmonella* (2).

Small molecules play important roles in cellular signaling circuits that modulate bacterial functions. The nucleotide guanosine tetraphosphate (ppGpp) is a major regulator of stringent control that mediates the adaptive response of bacteria to amino acid starvation by interacting with the RNA polymerase complex. It redirects transcription from growth-related genes to genes involved in stress resistance and starvation survival (reviewed in refs. 19 and 20). ppGpp exerts its effect by interacting with the β and β' subunits of the RNA polymerase complex (20). An accessory protein, DksA, binds the RNA polymerase complex and decreases open complex stability, therefore facilitating the negative effects of ppGpp on rRNA promoters (19). In addition, depletion of other nutrients, such as iron, also can induce accumulation of ppGpp in *Escherichia coli* (21). Recent studies showed that ppGpp stimulates *Salmonella* pathogenicity islands by enhancing the expression of their transcriptional regulators, HilA, InvF, and SsrA (3, 15, 22), suggesting that ppGpp might accumulate and play a role during bacterial infection. ppGpp has no influence on the PhoP/PhoQ system (22), but transcription of several SlyA- and PhoP-dependent genes, including *ugtL* and *pagC*, was repressed greatly in a *relA spoT* ($ppGpp^0$) mutant (23). This finding suggests that a ppGpp-stimulated regulatory circuit should govern their transcriptional activation through SlyA.

Genes can be organized into divergent operons in arginine utilization in *E. coli* (24), in tetracycline resistance in transposon Tn*10* (25), in base plate synthesis of bacteriophage T4 (26), and so forth. In *Agrobacterium tumefaciens*, TraR is a transcriptional activator required for Ti plasmid-mediated conjugation, which

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senses autoinducer 3-oxo-octanoyl-homoserine lactone, subsequently activating a set of loci including *traAFBH* and *traCDG* operons during bacterial conjugation (27). These 2 operons are transcribed divergently and share a single TraR binding site required for TraR-activated transcription (28).

In this study, we present a molecular mechanism for signal transduction in the feedforward loop formed by PhoP and SlyA. We provide evidence demonstrating that the regulatory activity of SlyA depends on ppGpp, which is the cytoplasmic component that interacts directly with this regulator, facilitates its dimerization, and thus stimulates its binding to target promoters. We identified intergenic regions throughout the *S. typhimurium* genome that are similar to the consensus SlyA box and PhoP box sequences. Our results show that several neighboring chromosomal loci form divergent operons whose transcription is activated by their intergenic region and contain only 1 SlyA box and 1 PhoP box. We demonstrate that the interaction of PhoP and PhoP-activated SlyA with these DNA motifs allows transcription of the adjacent genes to be activated simultaneously and to proceed in opposite directions. We propose that a dual signal, pH/CAMP/Mg^{2+} and ppGpp, modulates regulatory activity of the PhoP/PhoQ system and SlyA, respectively, and subsequently stimulates target genes by antagonizing H-NS function.

Results

SlyA and PhoP Control Transcriptional Regulation Independently. A direct repeat, TAAAT-(6 nt)-TCAAC, referred to as "the reverse PhoP box," was identified in the *ugtL* promoter (2), which is conserved in another PhoP and SlyA-dependent gene, *pagC* (shown as TAAAT-(6 nt)-TAAAC in Fig. 1*A*) (13, 14). After comparing the promoter regions of *ugtL* and *pagC*, we found that a repeat sequence, ATTATT-(10 nt)-ATTATT, located 11 bp upstream of the PhoP box, is highly conserved in these 2 promoters (Fig. 1*A*). The hexamers (ATTATT) proximal to the transcription start +1 of *pagC* and *ugtL* are identical, and each distal hexamer has a single nucleotide different from ATTATT (Fig. 1*A*). A footprinting result shows that both these sequences are centered in a 54-bp region protected by SlyA in the shared *pagD-pagC* promoter region [supporting information (SI) Fig. S1*A*], which partly overlaps the previously suggested SlyA-interacting regions (13, 14). Furthermore, substitutions of this repeat sequence (Fig. 2*A*) completely abolished the interaction of *pagC* promoter DNA and *C*-terminal His_6-tagged SlyA protein in an electrophoretic mobility shift assay (EMSA; Fig. 1*B*). Although the previous result suggested that SlyA could bind to 6 different DNA sequences located both upstream and downstream of the transcription start of *pagC* (13), the ATTATT direct repeat we identified should represent the SlyA recognition site (namely, the SlyA box). Thus, we conclude that PhoP and SlyA control transcription of a target promoter by interacting with a DNA region residing in the consensus sequence, ATTATT-(10 nt)-ATTATT-(11 nt)-TAAAT-(6 nt)-TAAAC.

We compared *lacZ* transcription from 2 plasmids carrying DNA fragments including 129 bp and 83 bp upstream of the *pagC* transcription start, that is, the *up-52* and the *up-1*, respectively (Fig. 1 *A* and *C*). We determined β-galactosidase activity from transformants of wild type and the isogenic *slyA* and *phoP* mutants after culture in N minimal medium (for details, see SI Text) supplemented with 0.01 mM (low) Mg^{2+}, a condition activating the PhoP/PhoQ system in vitro (6). The *up-52* fragment in pYS1033 controls *lacZ* expression in a SlyA-dependent manner because β-galactosidase activity is 4.7-fold lower in the *slyA* mutant than in wild type (Fig. 1*C*). The *up-1* fragment in pYS1035, having a deleted 5′ terminal 46-bp sequence of the *up-52* fragment in which the SlyA box is overlapped by the H-NS binding site identified previously (ref. 14; Fig. 1*C*), confers transcriptional activation independent of SlyA because β-galactosidase activity from the *slyA* mutant harboring pYS1035 is just slightly lower (1.4-fold) than that from the wild type harboring the same plasmid (Fig. 1*C*). Similar to our previous study in which SlyA enhanced PhoP binding to the promoter of *phoP* (29), *lacZ* expression controlled by *up-52* is about twice that controlled by *up-1* in wild-type cells, indicating that SlyA also facilitates PhoP binding to the *pagC* promoter (Fig. 1*C*). Thus, SlyA should antagonize H-NS, which controls transcriptional repression of *pagC* (3, 13, 14), by acting on the *up-52* fragment. Both *up-52* and *up-1* harbor the reverse PhoP box (Fig. 1 *A* and *C*) and therefore should retain PhoP-dependent regulation. Indeed, the β-galactosidase activity from cells harboring each plasmid was 8.1-fold and 6.1-fold lower, respectively, in the *phoP* mutant than in wild type (Fig. 1*C*). Taken together, these observations suggest that SlyA and PhoP should function independently.

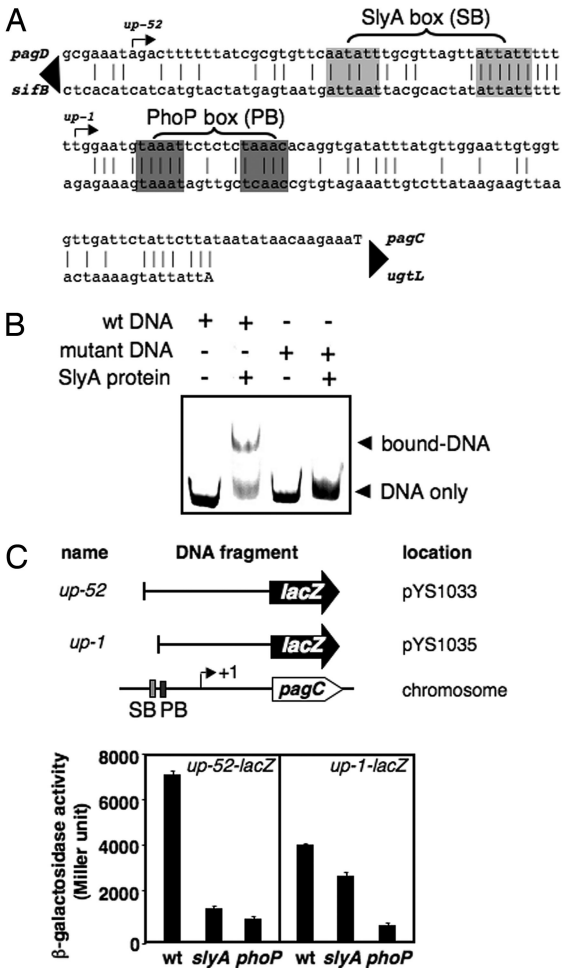

Fig. 1. Study of the regulatory function of the SlyA box in *Salmonella*. (*A*) Sequence alignment of the chromosomal regions from *pagD-pagC* and *sifB-ugtL* intergenic regions in *Salmonella typhimurium*. The arrowheads indicate the transcription direction. The right-pointing arrows correspond to the 5′ ends of cloned DNA fragments present in plasmids (pYS1033 and pYS1035 in *C*). The light and dark boxes correspond to the SlyA and PhoP boxes, respectively. (*B*) EMSA. A 20-nM DNA fragment with wild-type or substituted SlyA box sequence was incubated with 200 nM SlyA-His_6 protein, separated in a 4% polyacrylamide gel, and visualized with ethidium bromide. (*C*) β-Galactosidase activity was determined in wild type (14028s), a *phoP* mutant (YS11590), and a *slyA* mutant (YS11068) harboring pYS1033 (*up-52-lacZ*) or pYS1035 (*up-1-lacZ*).

A Single PhoP Box and a Single SlyA Box Contribute to Divergent Transcription. The *pagD* and *pagC* genes are PhoP-activated genes (9) located next to each other in the *Salmonella* chromosome but transcribed in opposite directions from a shared intergenic region. A previous result suggested that the *pagD* gene might be SlyA dependent, like the *pagC* gene (3). However, when we searched the

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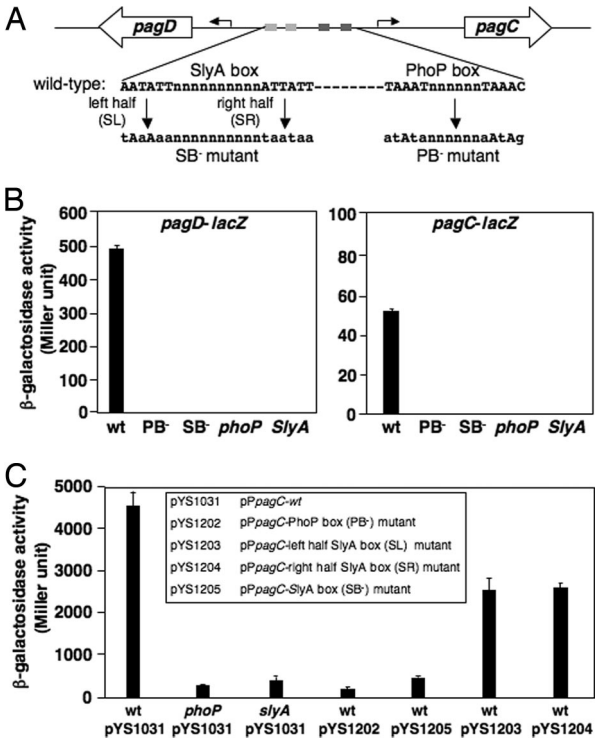

Fig. 2. A single SlyA box and a single PhoP box in the *pagD-pagC* intergenic region are essential for transcription of this divergent operon. (*A*) Substituted sequences (*lowercase letters*) in a half SlyA box (*Left*, SL^-; *Right*, SR^-), an entire SlyA box (SB^-); and a PhoP box (PB^-) of the *pagD-pagC* intergenic region. (*B*) β-Galactosidase activity from the *pagD-lacZ* and *pagC-lacZ* strains (see Table S2) with wild-type, chromosomal PhoP box (PB^-), and SlyA box (SB^-) mutated sequences. Expression in *phoP* mutant and *slyA* mutant was negative control. (*C*) β-Galactosidase activity was determined in wild-type (14028s) and in *phoP* (YS11590) and *slyA* (YS11068) mutant strains that harbor pYS1031 constructed with the intergenic region from the *pagC* direction and wild-type strains harboring a pYS1031-derived plasmid with substituted sequence at the SlyA box or PhoP box in *A*. Bacteria in *B* and *C* were grown for 4 h in N medium, 0.01 mM Mg^{2+}.

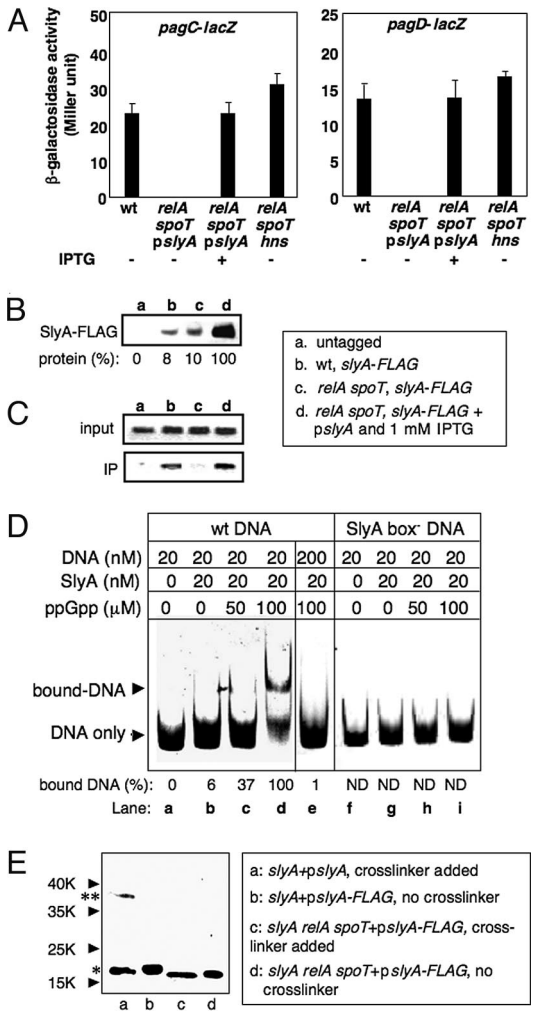

Fig. 3. ppGpp interacts with SlyA. (*A*) β-Galactosidase activity from *pagC-lacZ* and *pagD-lacZ* strains was determined in wild type (YS11865 and YS12000), the *relA spoT* mutants (YS12167*a* and YS12166*a*) harboring pYS1109 (p*slyA*), and *relA spoT hns* mutants (YS12167*b* and YS12166*b*). When required, 1 mM IPTG was supplemented. (*B*) Western blot analysis of SlyA-FLAG protein. Samples from strains in *C* were separated in 15% SDS/PAGE, and signals were monitored using M2 anti-FLAG antibodies. Relative protein amount (%) = (a given amount ÷ amount of d) × 100. Bacteria in *A* and *B* were grown in LB for 8 h. (*C*) In vivo SlyA binding to the *pagD-pagC* intergenic region was determined in YS11125 and YS11372*b*; wild-type 14028s was used as an untagged control. (*D*) EMSA. A DNA fragment with wild-type or substituted SlyA box sequence was incubated with SlyA-His_6 protein. Equal amounts of DNA were separated in a 4% polyacrylamide gel and visualized with ethidium bromide. ppGpp was supplemented when required. Relative bound DNA amount (%) = (a given amount ÷ amount of d) × 100. (*E*) Crosslinking of the SlyA dimer. Cell extracts prepared from *slyA* (YS11068) and *slyA relA spoT* mutants (YS14682) harboring pYS1109 (p*slyA*). Bacteria were grown for 8 h in LB. Anti-FLAG M2 antibodies (Sigma) were used for signal monitoring. The asterisk indicates the SlyA monomer; the double asterisk indicates the dimer. The arrowheads indicate a protein ladder marked with molecular weights.

pagD-pagC intergenic region between their transcription starts (Fig. S1*B*), we could not find another sequence resembling the PhoP box or SlyA box, raising the possibility that the PhoP box and the SlyA box that control *pagC* expression also may control *pagD* expression. We conducted site-directed mutagenesis to generate chromosomal substitutions at the PhoP box or the SlyA box in strains harboring the *pagD-lacZ* or *pagC-lacZ* fusion (Fig. 2*A*). β-Galactosidase activity from these strains with either a substituted PhoP box (PB^-) or SlyA box (SB^-) is zero when bacteria are grown in low-Mg^{2+} conditions (Fig. 2*B*), suggesting that a single PhoP box and a single SlyA box control transcriptional activation of both *pagD* and *pagC*. Deficient *lacZ* expression is the result of a mutation at the SlyA box or the PhoP box, because β-galactosidase activity from wild-type strains harboring plasmids carrying a *lacZ* fusion with *pagD-pagC* intergenic region (the *pagC* direction) is reduced considerably when the SlyA box or the PhoP box is substituted (Fig. 2*C*). Substitutions at either half of the SlyA box still could retain some SlyA-dependent regulation (Fig. 2*C*).

ppGpp Interacts Directly with SlyA, Facilitates Its Dimerization, and Thus Enhances SlyA Binding to Target Promoters. Because simply altering the Mg^{2+} concentration is sufficient to modulate SlyA and PhoP-dependent regulation in vitro (2, 3, 10), we reasoned that SlyA should respond to an endogenously generated signal. A previous result suggested that such a molecule, ppGpp, mediates transcriptional regulation of the invasion and intracellular virulence genes including *pagC* and *ugtL* in *Salmonella* (23). Our results show that transcription of chromosomal *pagD-lacZ* and *pagC-lacZ* fusions are turned off in a *relA spoT* ($ppGpp^0$) mutant (Fig. 3*A*). β-Galactosidase activity in the *relA spoT* mutant could be restored to wild-type levels when it was grown in LB medium supplemented with isopropyl 1-thio-β-D-galactoside (IPTG) (Fig. 3*A*), in which SlyA synthesis was induced from a plasmid pYS1109 harboring a *slyA-FLAG* fusion (p*slyA*) 10- to12-fold higher than the noninduced strains (Fig. 3*B*). Transcriptional activation of *pagD* and *pagC* is a

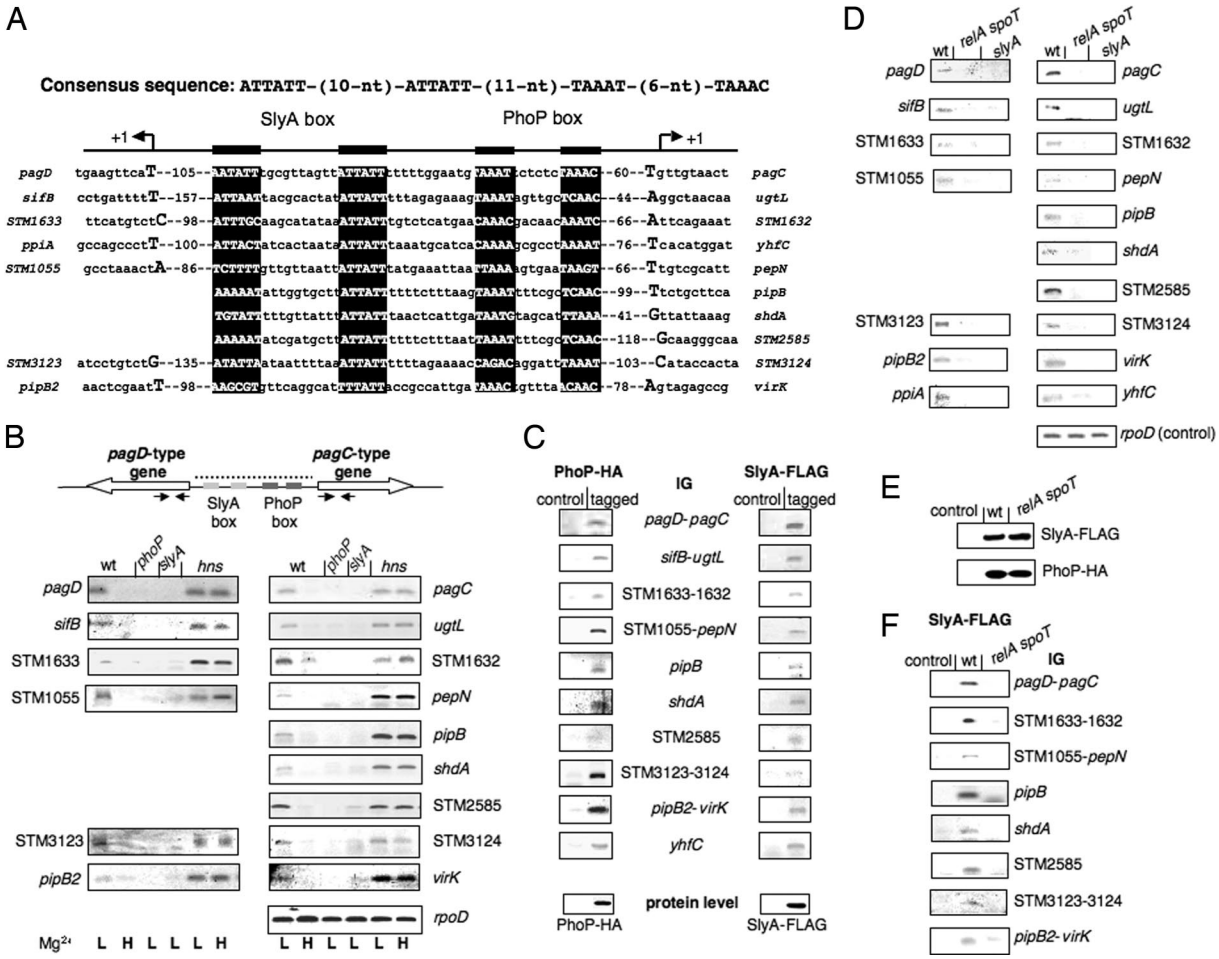

Fig. 4. SlyA and PhoP-activated chromosomal loci in *Salmonella typhimurium*. (*A*) Sequence alignment of the intergenic regions of SlyA and PhoP-dependent genes. White letters are sequences of the SlyA and PhoP boxes. Large bold letters are transcription starts (+1) demonstrated in Fig. S2. Numbering is the distance of a transcription start site to proximal SlyA box (for a *pagD*-type gene) and PhoP box (for *pagC*-type gene). (*B*) The mRNA level of transcripts was determined using RT-PCR analysis in wild type, *phoP* mutant, *slyA* mutant, and *hns* mutant. Constitutively transcribed *rpoD* gene indicated that similar amounts of total RNA were used. The DNA fragment was amplified using the primers (*horizontal arrows* in schematic diagram) listed in Table S3 and separated in an agarose gel. The dotted line indicates the intergenic regions. (*C*) In vivo binding of PhoP and SlyA to the intergenic regions was determined in strains harboring a chromosomal *phoP-HA* (YS11796) or *slyA-FLAG* (YS11125). Wild-type 14028s was used as an untagged control. Results from the immunoprecipitated DNA sample (IP) are shown. PCR was performed to amplify the intergenic regions (IG, *dotted line* in *B*), which were separated in an agarose gel. Amounts of input DNA were similar (data not shown). Bacteria were grown for 4 h in N medium (pH 7.4) containing 0.01 mM (L, in *B* and *C*) or 10 mM (H, in *B*) Mg^{2+}. The PhoP-HA and SlyA-FLAG proteins shown in *C* and *E* were analyzed by Western blot using anti-HA and anti-FLAG M2 antibodies, respectively. (*D*) The mRNA level of transcripts was determined using RT-PCR analysis in wild type, *relA spoT* mutant, and *slyA* mutant. Signal was monitored as in *B*. (*E*) Western blot analysis of the amount of SlyA and PhoP in wild type (YS11125 and YS11796) and *relA spoT* mutant (YS11372*b* and YS14449). (*F*) In vivo SlyA binding to the intergenic regions (IG) was determined in wild type and *relA spoT* mutant as in *C*. Bacteria in *D–F* were grown for 8 h in LB medium.

result of SlyA binding to the intergenic region in this condition, because a ChIP analysis shows that enrichment of the intergenic DNA by SlyA-FLAG reached levels similar to that in the wild-type strain in the *relA spoT* mutant harboring pYS1109 (Fig. 3*C*). Thus, the DNA affinity of SlyA is much lower in the absence of ppGpp in the presence of ppGpp. An EMSA result showed that ppGpp is sufficient to facilitate in vitro SlyA binding to the *pagD-pagC* intergenic region in a concentration-dependent manner (Fig. 3*D*, *Left*), indicating that ppGpp can interact directly with the SlyA protein. This facilitated binding of SlyA occurred in the SlyA box sequence because the DNA fragment substituted in the SlyA box sequence (see Fig. 2*A*) was not retarded (Fig. 3*D*, *Right*). Bacterial cells producing SlyA-FLAG fusion from heterologous promoter P_{lac} in plasmid pYS1109 were treated with crosslinking reagent formaldehyde. We found that SlyA-FLAG appeared as 2 bands migrated within an SDS polyacrylamide gel with estimated molecular weights of 17.7 kDa (monomer) and 37 kDa, respectively. Because the molecular weight of this band is approximately double that of the SlyA monomer, and its sequence is identical to that of the SlyA protein (data not shown), we conclude that it represents an SlyA dimer. When induced at a similar level, SlyA tends to form more dimers in the wild-type strain than in a *relA spoT* mutant (Fig. 3*E*), suggesting that ppGpp probably induces a conformational change in SlyA structure that preferentially forms a dimer.

Identification and Genetic Analysis of Chromosomal Loci Similar to the *pagD-pagC* Gene Cluster in *Salmonella typhimurium*. We searched the genome of *S. typhimurium* LT2 and the virulence plasmid pSLT (30) to identify more SlyA and PhoP-dependent genetic loci (hereinafter called "target genes") using the consensus sequence, ATTATT-(10 nt)-ATTATT-(11 nt)-TAAAT-(6 nt)-TAAAC (Figs. 1*A* and 4*A*). We demonstrated that 7 divergent operons and 3 "unpaired" *pagC*-type genes without cognate *pagD*-type partners, all localized in the bacterial chromosome, share homologous se-

quences in their promoter regions (Fig. 4*A*). The nucleotide sequence of the left half in the SlyA box is variable but mostly comprises a T-rich hexamer. Examination of their cellular localizations revealed that proteins encoded from the *pagD*-type genes are all soluble polypeptides putatively located in the cytoplasm or secreted to the extracytoplasmic space, 2 of which, SifB and PipB2, have been demonstrated to be secreted effectors (see Table S1 and *SI Text*). Many *pagC*-type gene products putatively are located in bacterial membranes as summarized in Table S1. Three proteins encoded by the *ppiA-yhfC* pair and by the *pepN* gene share 95.8%, 90.6%, and 94.4% identity to their *E. coli* counterparts, whereas other genes are present only in *Salmonella*. The peptidase *pepN* gene forms a gene pair with *STM1055* in *Salmonella* but pairs with an unrelated *pncB* encoding nicotinate phosphoribosyltransferase in *E. coli*. However, no SlyA box or PhoP box sequence could be found in *E. coli ppiA/yhfC* and *pepN* loci (data not shown).

We carried out primer extension analysis to determine transcription starts using mRNA transcripts isolated from bacterial cells grown in low-Mg^{2+} conditions (Fig. S2). We found that the SlyA box is located ≥ 86 nt upstream of 7 *pagD*-type transcription starts, and the PhoP box is located ≥ 41 nt upstream of 10 *pagC*-type transcription starts (summarized in Fig. 4*A*), suggesting that the SlyA box and the PhoP box are located upstream of the -35 region of target promoter regions. Interestingly, identified loci are evenly distributed into 2 replication fork regions (Fig. S3). Our results from primer extension demonstrate that transcriptions of the identified loci are activated by PhoP and SlyA because the mRNA level of transcripts is reduced significantly in the *phoP* and *slyA* mutants grown in low-Mg^{2+} conditions (Fig. S2). As with typical PhoP-dependent genes (31), both transcriptions from a divergent operon (except low expression in the *ppiA-yhfC* loci) are dependent on the environmental Mg^{2+} level, because RT-PCR analysis shows that the mRNA level is higher in 0.01-mM (low) Mg^{2+} than in 10-mM (high) Mg^{2+} (Fig. 4*B*). As suggested by our previous study (14), establishing the Mg^{2+}-responsive regulation of these loci requires the transcriptional repressor H-NS, which antagonizes the functions of SlyA and PhoP; thus the mRNA level is reduced greatly in the *phoP* or *slyA* mutants in PhoP-activating (low-Mg^{2+}) conditions but remains high in PhoP-repressing (high-Mg^{2+}) conditions in the *hns* mutant (Fig. 4*B*).

ChIP assay using strains expressing a PhoP-HA fusion and SlyA-FLAG fusion from their chromosomal location showed that both PhoP and SlyA proteins can enrich DNA fragments containing each target promoter region from cell cultures grown in low-Mg^{2+} conditions (Fig. 4*C*). This analysis was specific, because there was no significant enrichment of the SlyA-bound or PhoP-bound DNA fragment when the same number of input cells from the control strain (i.e., the untagged wild-type) was used (Fig. 4*C* and data not shown). Thus, our results demonstrate that, in vivo, PhoP and SlyA proteins bind to these intergenic regions we identified.

RT-PCR results show that mRNA levels from target genes are reduced greatly in the *relA spoT* mutant or the *slyA* mutant grown in LB (Fig. 4*D*). ppGpp does not exert its effect by controlling transcription of *slyA* and *phoP*, because the protein levels of these 2 regulators expressed from their chromosomal loci are similar in wild-type and *relA spoT* mutant strains (Fig. 4*E*). ChIP analysis using the same strains shows that in vivo SlyA binding to the target promoters (except *sifB-ugtL* and *ppiA-yhfC*) is reduced significantly in the absence of ppGpp because their promoter fragments are enriched from the wild-type strain but not from the *relA spoT* mutant (Fig. 4*F*). We conclude that ppGpp facilitates SlyA interaction with, and subsequently activation of, the target promoters (illustrated in Fig. S4).

Discussion

We have demonstrated that in *Salmonella* SlyA and ppGpp form a regulatory domain in which ppGpp stimulates SlyA to form a dimer in vivo (Fig. 3*E*) and bind the target promoter in vivo and in vitro (Fig. 3*C* and *D*). We have identified the SlyA box, a direct repeat from target promoters, which is sufficient to function as the *cis*-acting factor for SlyA-dependent regulation (Figs. 1*B*, 2*B*, and 3*D*). By analyzing identified genetic loci through the *S. typhimurium* genome in this study (Fig. 4*A*), we confirmed our previous observation (2) that SlyA and PhoP comprise a signaling circuit, namely a feedforward loop, illustrated in Fig. S4. These findings indicate that SlyA not only specifies a subset of genes regulated by the PhoP/PhoQ system that respond to pH/CAMP/Mg^{2+} but also mediates the integration of an alternative signal, ppGpp, in the transcriptional regulation of these genes.

SlyA Regulates Transcription in Response to ppGpp. We now have shown that the SlyA and PhoP-dependent transcription is reduced severely overall when *Salmonella* fail to produce alarmone ppGpp (Fig. 3*A*). This phenotype is not associated with the PhoP/PhoQ system because transcriptional levels of PhoP-dependent but SlyA-independent genes, such as *pcgL*, are reduced 2-fold overall in a *relA spoT* mutant (data not shown). In vitro, the SlyA protein gel retards more of the DNA fragments present in the *pagD-pagC* intergenic region when ppGpp is supplemented, indicating that it interacts directly with ppGpp. The interaction of ppGpp and SlyA still requires the SlyA box in vitro, because the mutated sequence abolishes SlyA binding to the intergenic DNA even in the presence of ppGpp (Fig. 3*D*). SlyA functions independently when it forms a feedforward loop with the PhoP/PhoQ system, because the *up-1* sequence that harbors only the PhoP box results in PhoP-dependent but SlyA-independent transcription of *pagC* (Fig. 1*A*). SlyA-dependent but PhoP-independent transcription of the *Salmonella* pathogenicity island II gene, *ssrA*, (3) is repressed significantly in a *relA spoT* mutant (32), further suggesting that ppGpp stimulates SlyA-dependent gene regulation. ppGpp should exert its effect on SlyA but not on other transcriptional components, because overproduction of SlyA is sufficient to restore SlyA binding (Fig. 3*B*) and thus *pagC* transcription (Fig. 3*A*) and because the *pagC* expression is similar in wild-type cells and a *dksA* mutant (unpublished data). Interestingly, ppGpp stimulates transcriptional activation of *Salmonella* pathogenicity island I and II (23), suggesting a possible role of SlyA in regulating transcription of their regulators. It remains to be determined whether ppGpp concentration is changed in different Mg^{2+} conditions.

The SlyA and PhoP-Regulated Gene Profile. We have identified SlyA and PhoP-dependent genetic loci throughout the *S. typhimurium* genome. Architecture, identified as the SlyA box and the PhoP box, in many intergenic regions highlights a singular example of the regulatory mechanism for these gene clusters. The status of the PhoP box is differentiated between a PhoP-regulated gene and an SlyA and PhoP-regulated gene (including *pagD*-type and *pagC*-type), and therefore different levels of PhoP are required to antagonize the inhibitory activity of H-NS (14). PhoP is a transcriptional activator required for expression of PhoP-regulated genes carrying a PhoP box that overlaps the -35 region (12, 33). This motif in such promoters depends functionally on its orientation, probably because the reverse sequence in this box would change the interaction of PhoP and RNA polymerase, thereby nullifying PhoP-regulated *mgtA* transcription (unpublished data). However, the PhoP box is located upstream of the -35 region in the SlyA and PhoP-regulated promoters. We reason that a change in the location of this motif could provide this group of PhoP-dependent genes with many new features: (*i*) tolerance of its orientation (both PhoP box directions are functional); (*ii*) tolerance of its location (the distance from the PhoP box to a transcription start is changeable); (*iii*) integration of an additional motif (i.e., the SlyA box, which is located upstream of the PhoP box in *pagC*-type promoters but is located downstream of the PhoP box in *pagD*-type promoters); and (*iv*) PhoP function as an anti-repressor, required only when H-NS

is present. PhoP and SlyA are dispensable for SlyA and PhoP-mediated transcription when H-NS is absent (ref. 14; Fig. 4*B*) and suggesting that these regulators are antagonizing factors against H-NS rather than interacting with RNA polymerase. The "hit-and-run" interactions between H-NS and these 2 regulators may occur independently but simultaneously in their own DNA-binding regions. Indeed, we found that regulation mediated by PhoP and SlyA could be exhibited separately with regard to a target gene (Fig. 1*C*).

Three "unpaired" loci, *pipB*, *shdA*, and *STM2585*, are all *pagC*-type genes (Fig. 3*A*). The regulatory region including the SlyA and PhoP boxes of the *pipB* gene is located within the coding region of a putative protein gene *STM1089*, whereas the farther left flanking region, *STM1090* (the *pipC* gene), is transcribed in the same direction as the *pipB* gene. The *ratB* and *gogB* genes also are transcribed in the same direction as their respective rightward genes *shdA* and *STM2585*. It remains to be investigated whether non-protein coding regions can be transcribed opposite to *pipB*, *shdA*, and *STM2585* as putative *pagD*-type genes.

Functions of the SlyA and PhoP-Regulated Genes. It is well established that PhoP and SlyA are essential for *Salmonella* virulence (1, 34). Our results bridged the gap, at least in part, between SlyA and PhoP-determined phenotypes and their target genes (Table S1). Because the biochemical functions of members within an operon usually are correlated with each other, the discovery of these divergent operons provides a putative functional link between individual members. For instance, one can ask whether UgtL is required for SifB function. On the other hand, searching similar regulatory units in other enteric bacteria, such as other *Salmonella* strains or *Yersinia* species, is becoming fruitful in revealing different regulations in their host responses (unpublished data).

In summary, we propose the following model depicted in Fig. S4. The environmental signals pH, CAMP, and Mg^{2+} activate PhoQ, which phosphorylates PhoP, which subsequently binds to the promoter regions of target genes, including *slyA* and the target genes shown in Table S1. The cytoplasmic ppGpp facilitates the regulatory activity of SlyA by enhancing its affinity to DNA. Cumulatively, PhoP and SlyA activate transcription of genetic loci including a group of divergent operons by binding to their intergenic regions simultaneously to antagonize the inhibitory effect of H-NS.

Materials and Methods

We describe construction of chromosomal mutants and plasmids, growth conditions, protein purification, gel retardation, and Western blot in *SI Text*. Strains used in this study are described in Table S2. Oligonucleotides used in this study are described in Tables S3 and S4.

Chromatin Immunoprecipitation Assay. Strains harboring chromosomally encoded PhoP and SlyA protein with a *C*-terminal HA or FLAG epitope were grown in 25 ml N medium as described previously in this paper for 4 h, washed once with PBS, and resuspended in 25 ml PBS. Proteins were crosslinked to promoter DNA by adding formaldehyde to a 1% final concentration. ChIP assays were performed as described previously (14). Enriched DNA fragments were detected by PCR using the primers listed in Table S3.

RT-PCR. Bacterial cells were grown for 4 h in N medium supplemented with 0.01 mM and 10 mM $MgCl_2$. Expression of the *slyA* gene was induced from strains harboring pYS1109 by adding 0.2 mM IPTG under the same growth conditions. Total RNA was isolated from bacterial culture using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry at 260 nm. RNA quality was confirmed by agarose gel electrophoresis. cDNA was synthesized using murine leukemia virus reverse transcriptase and random primers (BioLabs). DNA was amplified with primers in Table S3 using Taq polymerase (BioLabs) and performed in a thermocycler (Bio-Rad). Quantification was conducted using Quantity One software (Bio-Rad).

β-Galactosidase Assay. β-Galactosidase assays were carried out (35) in triplicate, and the activity (Miller unit) was determined using a VERSAmax plate reader (Molecular Device). Data correspond to 3 independent assays conducted in duplicate, and all values are mean ± standard deviation.

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