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# Overcoming H-NS-mediated Transcriptional Silencing of Horizontally Acquired Genes by the PhoP and SlyA Proteins in *Salmonella enterica*[*] [S]

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**The acquisition of new traits through horizontal gene transfer depends on the ability of the recipient organism to express the incorporated genes. However, foreign DNA appears to be silenced by the histone-like nucleoid-structuring protein (H-NS) in several enteric pathogens, raising the question of how this silencing is overcome and the acquired genes are expressed at the right time and place. To address this question, we investigated transcription of the horizontally acquired *ugtL* and *pagC* genes from *Salmonella enterica*, which is dependent on the regulatory DNA-binding proteins PhoP and SlyA. We reconstituted transcription of the *ugtL* and *pagC* genes *in vitro* and determined occupancy of their respective promoters by PhoP, H-NS, and RNA polymerase *in vivo*. The SlyA protein counteracted H-NS-promoted repression *in vitro* but could not promote gene transcription by itself. PhoP-promoted transcription required SlyA when H-NS was present but not in its absence. *In vivo*, H-NS remained bound to the *ugtL* and *pagC* promoters under inducing conditions that promoted RNA polymerase recruitment and transcription of the *ugtL* and *pagC* genes. Our results indicate that relief of H-NS repression and recruitment of RNA polymerase are controlled by different regulatory proteins that act in concert to express horizontally acquired genes.**

Horizontal gene transfer contributes significantly to the genetic diversity of bacteria. The importance of this process in bacterial evolution is underscored by the fact that it allows microorganisms to rapidly acquire new traits, such as those involved in virulence, resistance to antibiotics, or the ability to live in new niches (1). However, the inappropriate expression of newly acquired genes can be detrimental and even place a microorganism at a competitive disadvantage (2). Enteric bacteria have solved this problem, in part, by using the DNA-binding histone-like nucleoid structuring protein (H-NS)[2] to silence the expression of foreign genes in a process referred to as "xenogeneic silencing" (3). *In vivo*, H-NS preferentially binds to sequences that are AT-rich, which results in increased binding to horizontally acquired DNA sequences (4–6) and silencing of their transcription. Although silencing foreign DNA sequences may avoid potential negative effects, the acquired genes must be expressed if they are to contribute to an organism's lifestyle. This implies that bacteria must have the means to counteract the H-NS silencing effects and to transcribe the acquired genes when their products are needed.

Expression of a large number of horizontally acquired genes is controlled by the $Mg^{2+}$-responding PhoP/PhoQ two-component regulatory system in the Gram-negative pathogen *Salmonella enterica* serovar Typhimurium (7) (Fig. 1). The DNA-binding protein PhoP regulates gene expression directly by binding to its target promoters and indirectly by governing the production and/or activity of other regulatory proteins (7). One of the PhoP-regulated targets is the DNA-binding protein SlyA, which is required for expression of a subset of PhoP-regulated genes (8) that exhibit a restricted phylogenetic distribution (often with no BLAST matches outside *Salmonella* species), suggesting that they have been acquired recently by the *Salmonella* lineage through horizontal gene transfer from unidentified sources.

We analyzed the regions of the *Salmonella* genome that have been reported to be bound by the H-NS protein *in vivo* (4, 5) and found that a subset of these regions overlaps with the set of genes known to be co-regulated by the PhoP and SlyA proteins. This suggested that these two regulatory proteins may provide a means to overcome the H-NS-mediated silencing of horizontally acquired genes. Thus, to understand this process, we investigated the expression of the PhoP- and SlyA-dependent *ugtL* and *pagC* genes, which are normally bound by the H-NS protein (4, 5). BLAST searches with the UgtL and PagC protein sequences retrieved no homologs for the former and only low similarity sequences (<48% identity) for the latter, indicating that the respective genes have no orthologs outside of *Salmonella* spp. The *ugtL* gene encodes an inner membrane protein that promotes the formation of monophosphorylated lipid A in the lipopolysaccharide and is required for resistance to the antimicrobial peptides magainin 2 and polymyxin B (9). The *pagC* gene encodes an outer membrane protein implicated in serum resistance (10).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S6.

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[2] The abbreviations used are: H-NS, histone-like nucleoid structuring protein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; nt, nucleotide(s); HA, hemagglutinin; MES, 4-morpholineethanesulfonic acid.

Using a combination of *in vivo* expression and promoter occupancy assays with *in vitro* transcription and DNA binding experiments, we now report the roles that the regulatory proteins PhoP and SlyA play in promoting transcription of the *ugtL* and *pagC* genes. Our findings indicate that H-NS repression relief and RNA polymerase recruitment are events controlled by different regulatory proteins that act in distinct ways to allow regulated gene expression. This may be applicable to other horizontally acquired genes that require the PhoP and SlyA proteins for transcription.

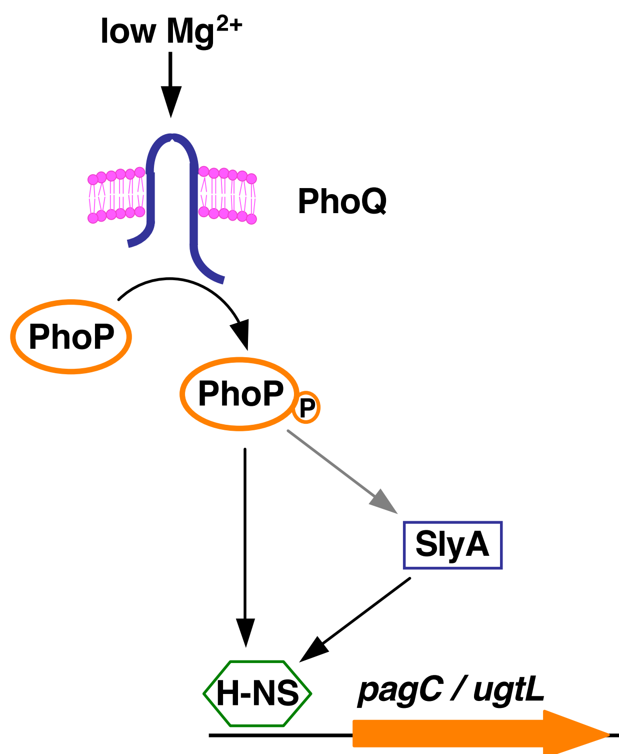


FIGURE 1. **Model depicting transcriptional control of the horizontally acquired *pagC* and *ugtL* genes by the regulatory proteins H-NS, PhoP, and SlyA.**

## EXPERIMENTAL PROCEDURES

*Bacterial Strains, Plasmids, and Growth Conditions*—Bacterial strains and plasmids used in this study are listed in Table 1. All *S. enterica* serovar Typhimurium strains are derived from wild-type strain 14028s and were constructed by phage P22-mediated transductions as described (11). Bacteria were grown at 37 °C in N-minimal medium (12) buffered in 50 mM BisTris, pH 7.7, supplemented with 0.1% casamino acids, 38 mM glycerol, and 10 μM or 10 mM $MgCl_2$. *Escherichia coli* strain DH5α was used as the host for the preparation of plasmid DNA. Ampicillin and kanamycin were used at 50 μg/ml, chloramphenicol at 20 μg/ml, and tetracycline at 10 μg/ml.

*Construction of Strains with Nucleotide Substitutions in Regulatory Sites and/or Deletions in the Chromosome*—Point mutations in the PhoP binding site in the *pagC* promoter were introduced in the *Salmonella* chromosome as follows. First, we introduced a $Km^R$ cassette immediately downstream of the stop codon of the *pagD* gene, which is divergently transcribed from *pagC*, using a PCR product generated with primers 7885 and 7886 (primer sequences are described in Table S1) and plasmid pKD4 as template (13). Chromosomal DNA from the resulting strain was used as template to generate a PCR product with primers 7886 and 7977. A second PCR product was generated with primers 2896 and 7978 and 14028s chromosomal DNA as template. These two DNA fragments were combined in a third PCR reaction with primers 2896 and 7886. The resulting product was introduced into wild-type *Salmonella* cells carrying plasmid pKD46 as previously described (13). The structure of the generated mutant was verified by colony PCR and DNA sequencing; the $Km^R$ cassette was removed using plasmid pCP20 as described (13). The presence of the "scar" sequence

**TABLE 1**
**Bacterial strains and plasmids used in this study**

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| *S. enterica* | | |
| 14028s | Wild type | ATCC |
| EG14078 | Δ*slyA*::$Cm^R$ | Ref. 9 |
| EG15598 | Δ*phoP/phoQ*::$Cm^R$ | Ref. 14 |
| EG13918 | *phoP*-HA | Ref. 14 |
| EG18825 | *phoP*-HA Δ*slyA*::$Cm^R$ | This work |
| EG18603 | *phoP*-HA PhoP box mut in *pagC* pr | This work |
| EG18817 | *phoP*-HA SlyA/H-NS box mut in *pagC* pr | This work |
| EG17828 | Δ*rpoS*::$Cm^R$ Δ*hns*::$Km^R$ | This work |
| EG18482 | Δ*rpoS phoP*7953::Tn*10* Δ*hns*::$Km^R$ | This work |
| EG18483 | Δ*rpoS* Δ*slyA*::$Cm^R$ Δ*hns*::$Km^R$ | This work |
| *E. coli* | | |
| DH5α | $F^-$ *sup*E44 Δ*lac*U169 (φ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 | Ref. 46 |
| ER2566 | *fhuA2* [*lon*] *ompT lacZ*::T7 *gene1 gal sulA11* Δ(*mcrC-mrr*)*114*::IS*10* R(*mcr-73*::miniTn*10*-$Tet^S$)2 R(*zgb-210*::Tn*10*-$Tet^S$) *endA1* [*dcm*] | New England Biolabs |
| EG17025 | ER2566 Δ*phoPQ*::$Km^R$ | This work |
| EG17246 | ER2566 Δ*slyA*::$Km^R$ | This work |
| Plasmids | | |
| pT7-7 | $rep_{PMB1}$ $Ap^R$ $p_{T7}$ | Ref. 47 |
| pT7.7-$His_6$-H-NS | $rep_{PMB1}$ $Ap^R$ $p_{T7}$ His6-*hns* | This work |
| pT7.7-$His_6$-SlyA | $rep_{PMB1}$ $Ap^R$ $p_{T7}$ His6-*slyA* | This work |
| pT7.7-PhoP-$His_6$ | $rep_{PMB1}$ $Ap^R$ $p_{T7}$ *phoP*-His6 | Ref. 48 |
| pKD3 | $rep_{R6K\gamma}$ $Ap^R$ FRT $Cm^R$ FRT | Ref. 13 |
| pKD4 | $rep_{R6K\gamma}$ $Ap^R$ FRT $Km^R$ FRT | Ref. 13 |
| pKD46 | $rep_{pSC101}$ts $Ap^R$ $p_{araBAD}$ γ β exo | Ref. 13 |
| pCP20 | $rep_{pSC101}$ts $Ap^R$ $Cm^R$ *cI*857 λ$P_R$ *flp* | Ref. 49 |

downstream of the stop codon of the *pagD* gene had no effect on the levels of *pagC* or *pagD* transcripts as determined by real time PCR experiments (data not shown).

The deletion of 12 nucleotides corresponding to the SlyA/H-NS binding site ~100 nt upstream the *pagC* transcription start site was carried out as follows. First, we replaced the region upstream of the *pagC* promoter, including the *pagD* gene, with a $Tet^R$ cassette generated with primers 7984 and 7888 and genomic DNA of *Salmonella* strain (EG16459) harboring a Tn*10* insertion as template. Second, a PCR product containing a $Km^R$ cassette was generated with primers 7886 and 8293 and template DNA from a *Salmonella* strain harboring a $Km^R$ cassette immediately downstream of the stop codon of the *pagD* gene (described above). This product was introduced into the $Tet^R$ strain carrying plasmid pKD46, and $Km^R$ transformants were selected and then screened for being $Tet^S$. The structure of the generated mutant was verified by DNA sequencing; the $Km^R$ cassette was removed using pCP20 as described (13).

Strain EG17828, which has a deletion of both the *rpoS* and *hns* genes was constructed by the one-step gene inactivation method (13) as follows. A $Km^R$ cassette was amplified using primers 7068 and 7069 and pKD4 as template and recombined into the *hns* region in a strain with deletion of the *rpoS* gene (EG14749). The structure of the generated mutant was verified by colony PCR as described (13).

*Plasmid Constructs*—Plasmid pT7.7-$His_6$-H-NS was constructed by cloning between the NdeI and HindIII sites of plasmid pT7.7 a DNA fragment generated by PCR with primers 7239 and 7252 and genomic DNA from wild-type *Salmonella* as template.

Plasmid pT7.7-$His_6$-SlyA was constructed by cloning between the NdeI and HindIII sites of pT7.7, a DNA fragment generated by PCR with primers 7070 and 7065, and genomic DNA from wild-type *Salmonella* as template.

PCRs were carried out with AccuPrime™ TaqDNA Polymerase High Fidelity (Invitrogen), and the correct sequence of the constructs was verified by DNA sequencing.

*RNA Isolation and Real Time PCR to Determine Transcript Levels*—Cells were grown in N-minimal medium containing 10 mM $MgCl_2$ to $A_{600}$ ~ 0.7. 3 ml of cell culture were washed with $Mg^{2+}$-free medium and inoculated into 10 ml of fresh medium containing either 10 μM or 10 mM $MgCl_2$. Cells were grown with vigorous shaking at 37 °C for 30 min. 1-ml samples were collected and used to prepare total RNA using the SV Total RNA Isolation System (Promega). cDNA was synthesized using TaqMan (Applied Biosystems) and random hexamers following the manufacturer's instructions. Quantification of transcripts was performed by real time PCR using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7000 sequence detection system (Applied Biosystems). The *pagC* and *ugtL* transcripts were each detected with two sets of primers (which gave similar results): primers 6684 and 6685 and primers 6492 and 6493 were used to quantify the *pagC* transcript. Primers 7108 and 7114 and primers 6494 and 6495 were used to quantify the *ugtL* transcript. The *mgtA* transcript was detected with primers 4443 and 4446. Results were normalized to the levels of 16 S ribosomal RNA, which were estimated using primers 6970 and 6971. The amount of each PCR product was calculated from standard curves obtained from PCRs with the same primers and serially diluted DNA.

*Chromatin Immunoprecipitation Assay*—Cells were grown in N-minimal medium containing 10 mM $MgCl_2$ to $A_{600}$ ~0.7. 6 ml of cell culture were washed with $Mg^{2+}$-free medium and inoculated into 20 ml of fresh medium containing either 10 mM or 10 μM $MgCl_2$. Cells were then grown with vigorous shaking at 37 °C for 30 min. Chromatin immunoprecipitation assays were carried out as described (14) with the following modifications. PhoP-HA-, H-NS-, and RpoB-cross-linked DNA was immunoprecipitated with monoclonal anti-HA sc-7392X (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-H-NS H113 (15), and anti-RpoB WP023 (Neoclone) antibodies, respectively. The anti-HA antibodies were captured with Protein A-agarose beads (Pierce), whereas Protein G-Sepharose (GE Healthcare) was used to capture anti-H-NS and anti-RpoB antibodies. After reversal of cross-linking, the immunoprecipitated and input DNA were purified using QIAquick columns (Qiagen) following the manufacturer's instructions.

Quantification of the immunoprecipitated and input DNA was performed by real time PCR using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7000 sequence detection system (Applied Biosystems). For amplification of the *rpoD*, *mgtA*, *pagC*, and *ugtL* promoter regions, primers 4149 and 4150, primers 5852 and 5853, primers 7857 and 7858, and primers 7855 and 7856 were used, respectively. The amount of each PCR product was calculated from standard curves obtained from PCRs with the same primers and serially diluted DNA. The level of protein binding to a particular promoter under each condition was calculated as follows,

$$\text{Relative protein bound to promoter } x = \frac{\text{promoter } x \text{ IP/promoter } x \text{ input}}{\textit{rpoD} \text{ IP}/\textit{rpoD} \text{ input}}$$

where IP represents immunoprecipitate. The *rpoD* promoter neither binds to nor is regulated by the PhoP protein.

*Immunoblotting Analysis*—Cells were grown in 15 ml of N-minimal medium to $A_{600}$ ~0.4, washed with Tris-buffered saline twice, resuspended in 400 μl of Tris-buffered saline, and opened by sonication. Whole-cell lysates were electrophoresed on 10% NuPAGE BisTris gels (Invitrogen) with MES running buffer, transferred to nitrocellulose membranes, and analyzed by immunoblotting with anti-HA or anti-RpoB (Neoclone) monoclonal antibodies or an anti-PagC polyclonal antibody (10). Blots were developed by using anti-mouse or anti-rabbit IgG horseradish peroxidase-linked antibodies (Amersham Biosciences) and the ECL detection system (Amersham Biosciences).

*Overproduction and Purification of Proteins*—Histidine-tagged H-NS, SlyA, and PhoP proteins were overproduced in *E. coli* strain EG17246 harboring pT7-7-$His_6$-H-NS or pT7.7-$His_6$-SlyA and in *E. coli* strain EG17025 harboring pT7-7-PhoP-$His_6$, respectively. Protein purification was performed as described (16) with the following modifications. After purification, the buffer of the eluate was exchanged with 10 mM Hepes (pH 8.0), 10% (v/v) glycerol (for the H-NS protein, the buffer contained 200 mM NaCl), and the proteins were concentrated using an Amicon Ultra-15 column

($M_r$ 10,000; Millipore). Proteins were stored at −80 °C. Protein concentration was determined with a BCA protein assay (Pierce) using bovine serum albumin as a standard. Protein preparations were >99% pure as determined by SDS-PAGE followed by Coomassie Blue staining (Fig. S1).

*In Vitro Single Round Transcription Assays*—Linear DNA templates for *in vitro* transcription assays were generated by PCR using primers 7756 and 7758 for *pagC* and 7193 and 7194 for *ugtL* and genomic DNA of wild-type *Salmonella* as template. The DNA fragments were then gel-purified with QIAquick columns (Qiagen). The *in vitro* transcription reactions were carried out under standard conditions as described (17, 18). Briefly, a mixture of template DNA (9 nM), purified His-tagged proteins, and RNA polymerase holoenzyme (Epicentre) were incubated in 15 μl of transcription buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 25 μg/ml bovine serum albumin) for 30 min at 37 °C to form open complexes. A 5-μl mixture of substrate and heparin was then added to make a final concentration of 160 μM each of ATP, GTP, and CTP; 50 μM UTP; 2 μCi of [α-$^{32}$P]UTP; and 200 μg/ml heparin. After 10 min of incubation at 37 °C, reactions were stopped by adding TBE-urea loading buffer (Invitrogen) and resolved in 10% TBE-urea gels (Invitrogen).

*DNase I Footprinting and Gel Mobility Shift Assays*—DNase I footprinting with the PhoP protein was performed as reported (19). The *pagC* promoter region was amplified with primers 7756 and 7758 and genomic DNA of wild-type *Salmonella* as template. Primer 7756 was labeled for the coding strand, and primer 7758 was labeled for the noncoding strand. Footprinting with the H-NS and SlyA proteins was carried out as described (20) with several modifications as follows. Labeled primer 7756 and unlabeled primer 7758 were used to generate the DNA fragment containing the *pagC* promoter. After purification with QIAquick columns (Qiagen), the labeled fragment (9 nM) was incubated with the indicated amount of H-NS and/or SlyA proteins for 20 min at room temperature in 20 μl of 40 mM Hepes (pH 8.0), 8 mM $MgCl_2$, 60 mM potassium glutamate, 5 mM dithiothreitol, 0.05% Nonidet P-40, and 0.1 mg/ml bovine serum albumin (Ambion). DNase I (Epicentre) (0.02 units), 10 mM $CaCl_2$, and 10 mM $MgCl_2$ were added and incubated for 3 min or 2.5 min (in the absence of protein). The reaction was stopped by the addition of 100 μl of phenol (pH 8.0), and the aqueous phase was precipitated. Samples were analyzed by electrophoresis on a 6% polyacrylamide, 7.5 M urea gel and compared with a Maxam-Gilbert A + G DNA ladder generated from the same DNA probe.

The *pagC* DNA fragments for gel mobility shift assays were generated by PCR using primers 7756 and 7758 and genomic DNA of wild-type or mutant (EG18603) *Salmonella* as template. The DNA fragments were then gel-purified with QIAquick columns (Qiagen) and 100 ng of DNA labeled with T4 polynucleotide kinase and [γ-$^{32}$P]ATP. Unincorporated [γ-$^{32}$P]ATP was removed using G-50 microcolumns (Amersham Biosciences). $1 \times 10^4$ cpm of labeled probe (~6 fmol), 200 ng of poly(dI-dC)·poly(dI-dC) (Amersham Biosciences), and purified His-tagged PhoP were mixed with binding buffer (20 mM Hepes (pH 8.0), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 10% glycerol) in a total volume of 20 μl and incubated for 20 min at room temperature. Samples were then electrophoresed on 4–20% TBE gels (Invitrogen), and the gels were dried and autoradiographed.

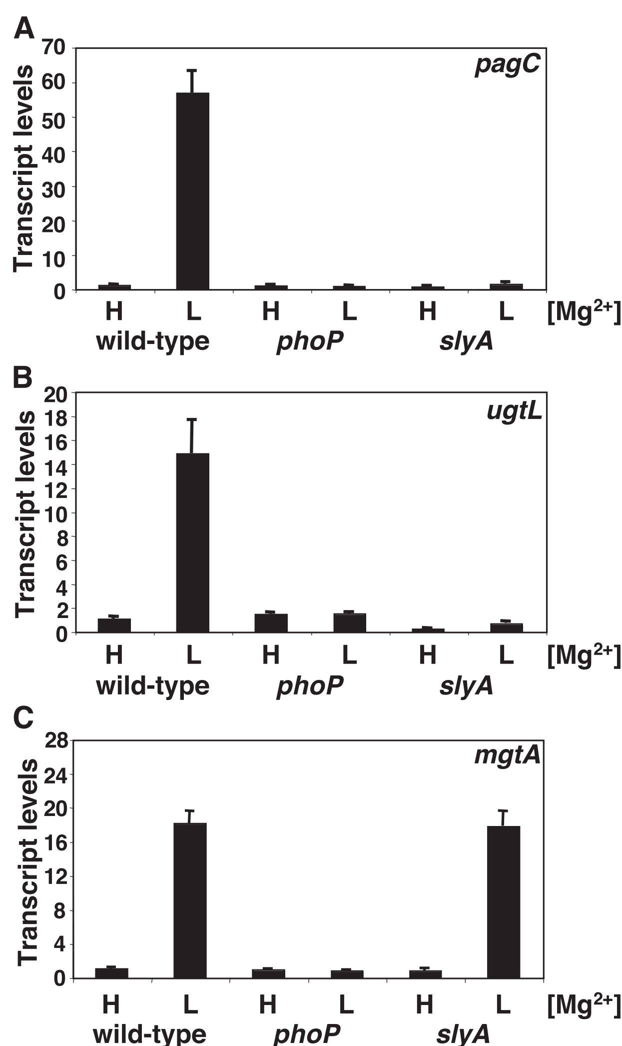


FIGURE 2. **The *slyA* gene is necessary to promote transcription of the horizontally acquired PhoP-regulated *pagC* and *ugtL* genes but not the ancestral PhoP-regulated *mgtA* gene.** *A–C*, transcript levels corresponding to the *pagC* (*A*), *ugtL* (*B*), and *mgtA* (*C*) genes as determined by quantitative real time PCR in wild-type (14028s), *phoPQ* (EG15598) and *slyA* (EG14078) cells grown in N-minimal medium containing 10 mM (*H*) or 10 μM (*L*) $MgCl_2$. Shown are the mean and S.D. values of at least three independent experiments.

## RESULTS

*Transcription of the Horizontally Acquired pagC and ugtL Genes Is PhoP- and SlyA-dependent*—We examined the mRNA levels of the PhoP-activated *pagC*, *ugtL*, *mgtA*, *pagP*, *rstA*, and *slyB* genes following bacterial growth under inducing (10 μM) and repressing (10 mM) $Mg^{2+}$ concentrations in isogenic wild-type, *phoP*, and *slyA Salmonella* strains. Transcription of all six genes was detected in the wild-type strain following growth in low $Mg^{2+}$ but not in high $Mg^{2+}$, and it was absent from the *phoP* mutant regardless of the $Mg^{2+}$ concentration (Figs. 2 and S2), which is in agreement with previous results (21, 22). A functional *slyA* gene was necessary for expression of the horizontally acquired *pagC* (Fig. 2*A*) and *ugtL* (Fig. 2*B*) genes but not of the ancestral *mgtA*, *pagP*, *rstA*, and *slyB* genes (Figs. 2*C* and S2).

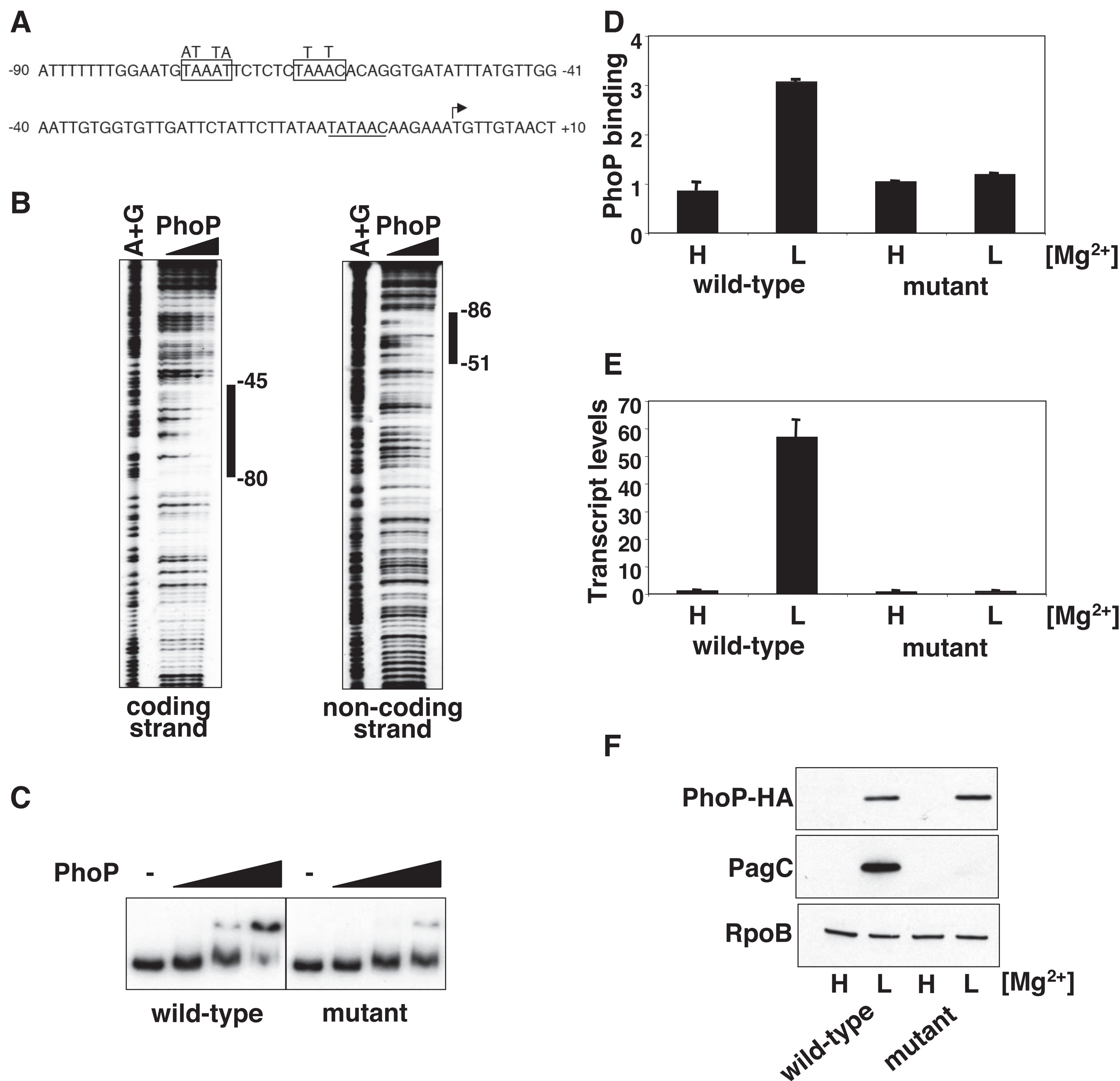


FIGURE 3. **The PhoP protein promotes *pagC* transcription through direct interaction with the *pagC* promoter region.** *A*, DNA sequence of the *pagC* promoter region. The transcription start site is indicated by the *bent arrow*; the PhoP binding site is *boxed*, and the putative −10 sequence is *underlined*. Nucleotide substitutions introduced in the PhoP binding site are indicated *above* the *boxes*. *B*, DNase I footprinting analysis of the *pagC* promoter region performed with probes for the coding and noncoding strands and increasing amounts of PhoP protein (0, 0.1, 0.2, 0.4, and 0.8 μM). The *bars* indicate protected regions at lower PhoP concentrations. *C*, electrophoretic mobility shift assay analysis of the *pagC* promoter region (−178 to +122 nt with respect to the transcription start site) using fragments harboring the wild-type sequence (wild type) or containing point mutations in the PhoP box (mutant). The amounts of PhoP protein used were 0, 1, 2, and 3 μM. *D*, *in vivo* PhoP binding to the *pagC* promoter in a strain with a wild-type *pagC* promoter (EG13918) or an isogenic strain containing point mutations in the PhoP box (EG18603). Cells were grown in N-minimal medium containing 10 mM (*H*) or 10 μM (*L*) $MgCl_2$. PhoP binding was determined by chromatin immunoprecipitation. Shown are the mean and S.D. values of at least three independent experiments. *E*, *pagC* transcription in strains EG13918 and EG18603 grown as described in *D*. Transcript levels were determined by quantitative real time PCR. Shown are the mean and S.D. values of at least three independent experiments. *F*, Western blot analysis of crude extracts prepared from strains EG13918 and EG18603 grown as described under "Experimental Procedures" to detect the PhoP-HA, PagC, and RpoB proteins.

*The PhoP Protein Promotes pagC Transcription through Direct Interaction with the pagC Promoter Region*—A requirement for both the PhoP and SlyA proteins to activate *pagC* and *ugtL* transcription could be due to these two regulatory proteins directly interacting with the promoter regions of the *pagC* and *ugtL* genes. Alternatively, PhoP and SlyA could be part of a regulatory cascade whereby one of these proteins controls the expression and/or activity of the other. It appears that both of these mechanisms may be operating because the PhoP and SlyA proteins have been shown to footprint the *ugtL* promoter *in*

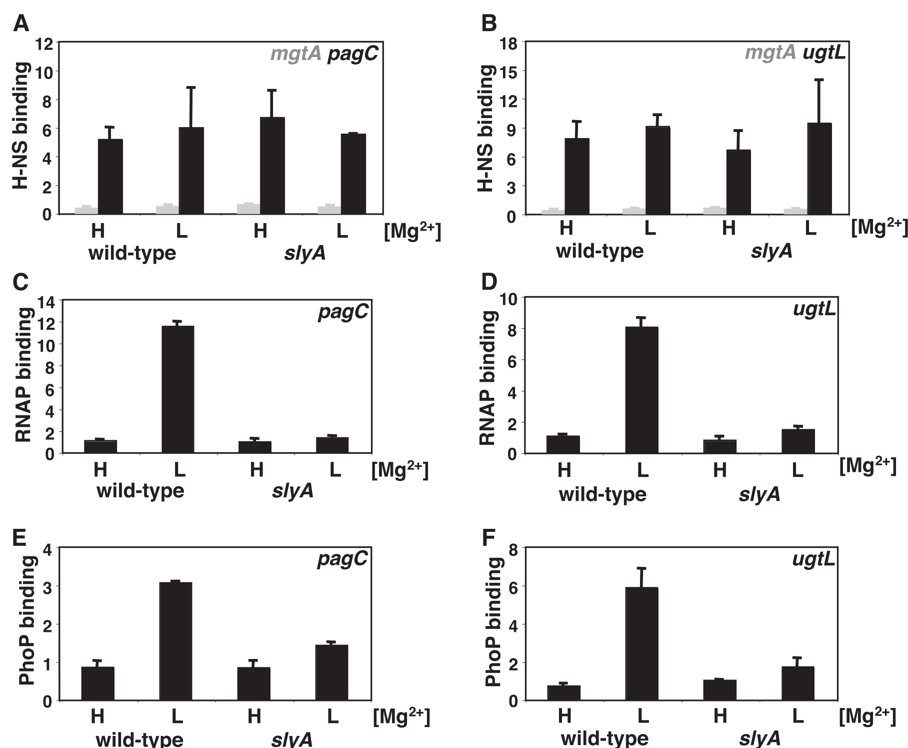


FIGURE 4. **Occupancy of the *pagC* and *ugtL* promoter regions by H-NS, PhoP, and RNA polymerase *in vivo*.** *A–F*, occupancy of the *pagC* (*A*, *C*, and *E*) and *ugtL* (*B*, *D*, and *F*) promoters by H-NS (*A* and *B*), PhoP (*C* and *D*), and RNA polymerase (*E* and *F*) was determined by chromatin immunoprecipitation in wild type (EG13918) or an isogenic strain lacking the *slyA* gene (EG18825). Cells were grown in N-minimal medium containing 10 mM (*H*) or 10 $\mu$M (*L*) $MgCl_2$ as described under "Experimental Procedures." H-NS occupancy of the *mgtA* promoter (*A* and *B*) represents background levels of nonspecifically immunoprecipitated DNA. Shown are the mean and S.D. values of at least three independent experiments.

*vitro* (23) and because expression of the *slyA* gene is PhoP-dependent under certain conditions (23, 24).

It has been reported that the SlyA protein, but not the PhoP protein, binds to the *pagC* promoter (8). However, using a recently developed method to analyze bacterial promoter sequences (25), we could identify a putative PhoP binding site ~60 nt upstream of the transcription start site of the *pagC* gene (Fig. 3*A*), at a position and orientation relative to the −10 region that is shared with other PhoP-regulated promoters (26). We determined that the predicted PhoP box in the *pagC* promoter is a *bona fide* PhoP-binding site, because the PhoP protein footprinted this region (Fig. 3*B*).

To test whether binding of the PhoP protein to the PhoP box in the *pagC* promoter was required for *pagC* transcription, we constructed a strain harboring nucleotide substitutions in the PhoP box of the *pagC* promoter in the *Salmonella* chromosome (Fig. 3*A*). First, we established that the nucleotide substitutions impaired the ability of the PhoP protein to gel-shift a DNA fragment corresponding to the *pagC* promoter *in vitro* (Fig. 3*C*). Accordingly, PhoP could not bind to the *pagC* promoter in the mutant strain *in vivo* when assayed by chromatin immunoprecipitation (Fig. 3*D*). Moreover, the *pagC* promoter mutant strain failed to express the *pagC* gene, since neither the *pagC* transcript (Fig. 3*E*) nor the PagC protein (Fig. 3*F*) was detected under conditions promoting their production in the wild-type strain. *In vitro*, the nucleotide substitutions also completely abolished the ability of the PhoP protein to promote *pagC* transcription (Fig. S3). Taken together with previous findings (23, 24), these results demonstrate that transcriptional activation of the *pagC* and *ugtL* genes entails binding of both the PhoP and SlyA proteins to the promoters of these two genes.

*H-NS Remains Associated with the pagC and ugtL Promoters under Inducing Conditions*—The PhoP and SlyA proteins could activate transcription of the *pagC* and *ugtL* genes by either displacing H-NS from their respective promoters or by counteracting its repressing effects in a manner not involving removal of H-NS from the promoters. To distinguish these two possibilities, we probed the association of the H-NS protein with the *pagC* and *ugtL* promoters *in vivo* using chromatin immunoprecipitation (which produces DNA fragments mostly 200–500 bp in size). H-NS displayed significantly higher (>10-fold) association with the *pagC* and *ugtL* promoter regions compared with regions known not to associate with H-NS, such as the *rpoD* promoter (which was used to normalized the data) or the ancestral *mgtA* promoter (Figs. 4, *A* and *B*). Interestingly, H-NS displayed similar levels of promoter occupancy under repressing and inducing conditions for the PhoP/PhoQ system (*i.e.* in cells grown in either high or low $Mg^{2+}$, respectively). These results indicate that the H-NS protein remains associated with the promoter regions of the *pagC* and *ugtL* genes even under conditions in which these genes are transcribed.

*RNA Polymerase Recruitment to the pagC and ugtL Promoters*—The H-NS protein has been shown to impede transcriptional activation by at least two mechanisms: 1) H-NS binding to a promoter may hinder recruitment of RNA polymerase, and 2) H-NS may prevent mRNA elongation by trapping RNA polymerase in the promoter (27, 28). To determine whether RNA polymerase is recruited to the *pagC* and *ugtL* promoters, we examined the *in vivo* promoter occupancy by RNA polymerase using chromatin immunoprecipitation with an antibody directed against the $\beta$ subunit of the enzyme. The DNA fragments corresponding to the *pagC* and *ugtL* promoter regions were recovered ~11- or ~8-fold less in bacteria grown in high $Mg^{2+}$, which are nonactivating conditions, compared with those grown in low $Mg^{2+}$ (Fig. 4, *C* and *D*), when these genes are transcribed (Fig. 2). These results argue that H-NS does not trap RNA polymerase in the *pagC* and *ugtL* promoter regions under noninducing conditions.

*SlyA Is Required for PhoP Binding and RNA Polymerase Recruitment to the pagC and ugtL Promoters in Vivo*—The PhoP protein binds to its activated promoters and recruits RNA polymerase during growth in low $Mg^{2+}$ (29), which is a condi-

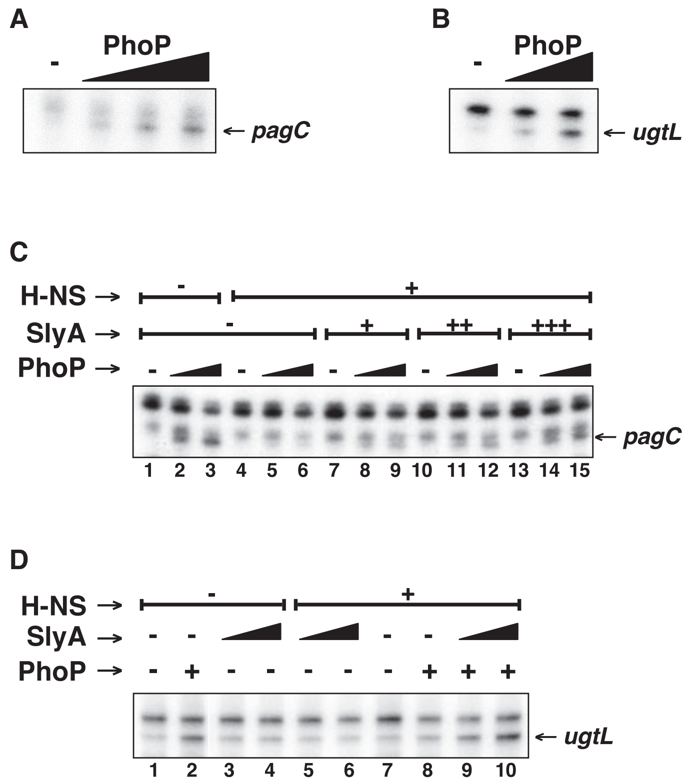


FIGURE 5. **SlyA counteracts H-NS-promoted repression but does not activate gene transcription by itself.** *A–D*, run-off *in vitro* transcription assays with linear templates corresponding to the *pagC* promoter region (−178 to +122 nt with respect to the transcription start site) (*A* and *C*) or the *ugtL* promoter region (−180 to +125 nt with respect to the transcription start site) (*B* and *D*), RNA polymerase, and increasing amounts of PhoP protein only (0, 1, 2, and 3 μM in *A*; 0, 1, and 2 μM in *B*) or PhoP (0, 1, and 2 μM in *C*; 0 and 2 μM in *D*) in combination with H-NS (0 and 1.25 μM) and SlyA (0, 100, 200, and 300 nM in *C*; 0, 100, and 200 nM in *D*). The *pagC* and *ugtL* transcripts are indicated with *arrows*. The uppermost band in *C* corresponds to a ∼150-nt run-off transcript resulting from spurious transcription going in the reverse orientation, whereas the band just on top of the *pagC* transcript originates ∼5 nt upstream of the transcription start site indicated in Fig. 3. (A transcript originating at this position has not been observed *in vivo*.)

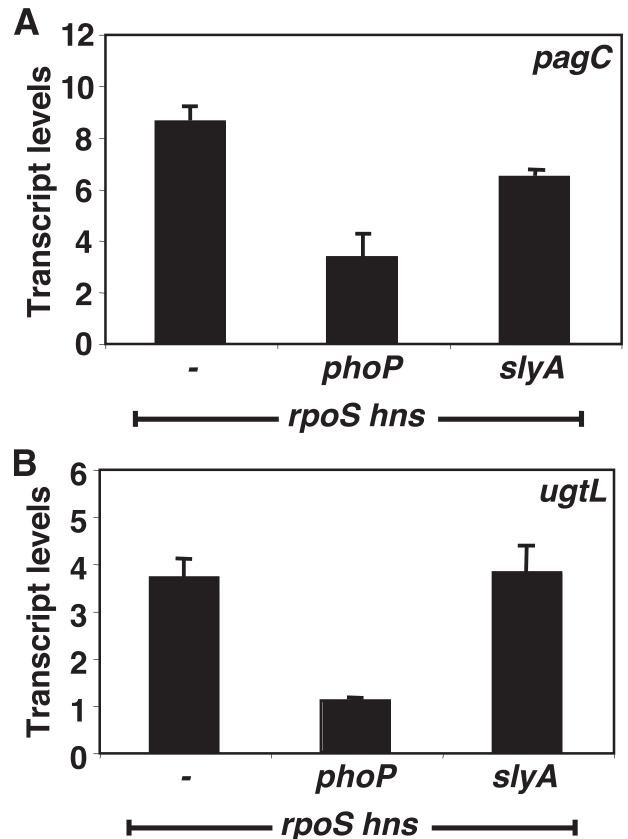


FIGURE 6. **The *slyA* gene is dispensable to promote transcription of the *pagC* and *ugtL* genes in the absence of *hns*.** *A* and *B*, transcript levels corresponding to the *pagC* (*A*) and *ugtL* (*B*) genes as determined by quantitative real time PCR in *hns rpoS* (EG17828), *hns rpoS phoP* (EG18482), and *hns rpoS slyA* (EG18483) *Salmonella* cells grown in LB medium to late log phase at 37 °C. Shown are the mean and S.D. values of three independent experiments.

tion that promotes synthesis (Fig. 3*F*) and activation (29) of the PhoP protein. Then what prevents PhoP from promoting transcription of the *pagC* and *ugtL* genes when *Salmonella* is missing the *slyA* gene? We investigated the possibility that PhoP may be unable to associate with H-NS-bound promoters in the absence of SlyA by comparing the *in vivo* promoter occupancy by the PhoP protein in isogenic wild-type and *slyA* strains. PhoP binding to the *pagC* and *ugtL* promoters was severely diminished in the *slyA* mutant under conditions that resulted in PhoP binding in the *slyA*$^+$ strain (Fig. 4, *E* and *F*). Consistent with the requirement for PhoP to recruit RNA polymerase to its activated promoters, there was no RNA polymerase binding to the *pagC* and *ugtL* promoters in the *slyA* mutant (Fig. 4, *C* and *D*). Thus, the SlyA protein is necessary for PhoP binding and RNA polymerase recruitment to the promoters of these horizontally acquired genes.

*SlyA Counteracts H-NS-promoted Repression in Vitro but Does Not Promote Transcription by Itself*—To further investigate the role that the PhoP and SlyA proteins play in expression of the *pagC* and *ugtL* genes, we carried out *in vitro* transcription assays with purified proteins and linear or supercoiled DNA templates. The PhoP protein was required to initiate transcription from the *pagC* (Fig. 5*A*) and *ugtL* (Figs. 5*B* and S4) promoters even in the absence of H-NS. By contrast, when H-NS was present, PhoP and RNA polymerase were no longer able to promote *pagC* (Fig. 5*C*, *lanes 4–6*) or *ugtL* (Fig. 5*D*, *lanes 7* and *8*, and Fig. S4) transcription, unless SlyA was added to the reaction (Fig. 5, *C* (*lanes 7–15*) and *D* (*lanes 9* and *10*), and Fig. S4). However, the same amounts of SlyA protein failed to promote *pagC* or *ugtL* transcription in the absence of the PhoP protein regardless of whether the H-NS protein was present or absent (Fig. 5, *C* (*lanes 7*, *10*, and *13*) and *D* (*lanes 3–6*)).

*The slyA Gene Is Dispensable to Promote Transcription of the pagC and ugtL Genes in the Absence of hns in Vivo*—Our findings suggest that the role of SlyA in promoting *pagC* and *ugtL* transcription is limited to counteracting the silencing effects of H-NS. This predicts that transcription of these genes should become *slyA*-independent in a strain lacking a functional *hns* gene. To test this notion, we compared the levels of *pagC* and *ugtL* transcripts in three isogenic strains: *hns rpoS*, *hns rpoS phoP*, and *hns rpoS slyA*. (These were carried out in an *rpoS* mutant background, because the *hns* gene is essential in *Salmonella* (5), and the *rpoS* mutation suppresses the lethality. Moreover, the experiments had to be performed with bacteria grown in LB broth, because the *hns rpoS* strain is unable to grow in N-minimal medium.) The *slyA* mutation had either only a small effect or no effect at all on *pagC* or *ugtL* transcription, respec-

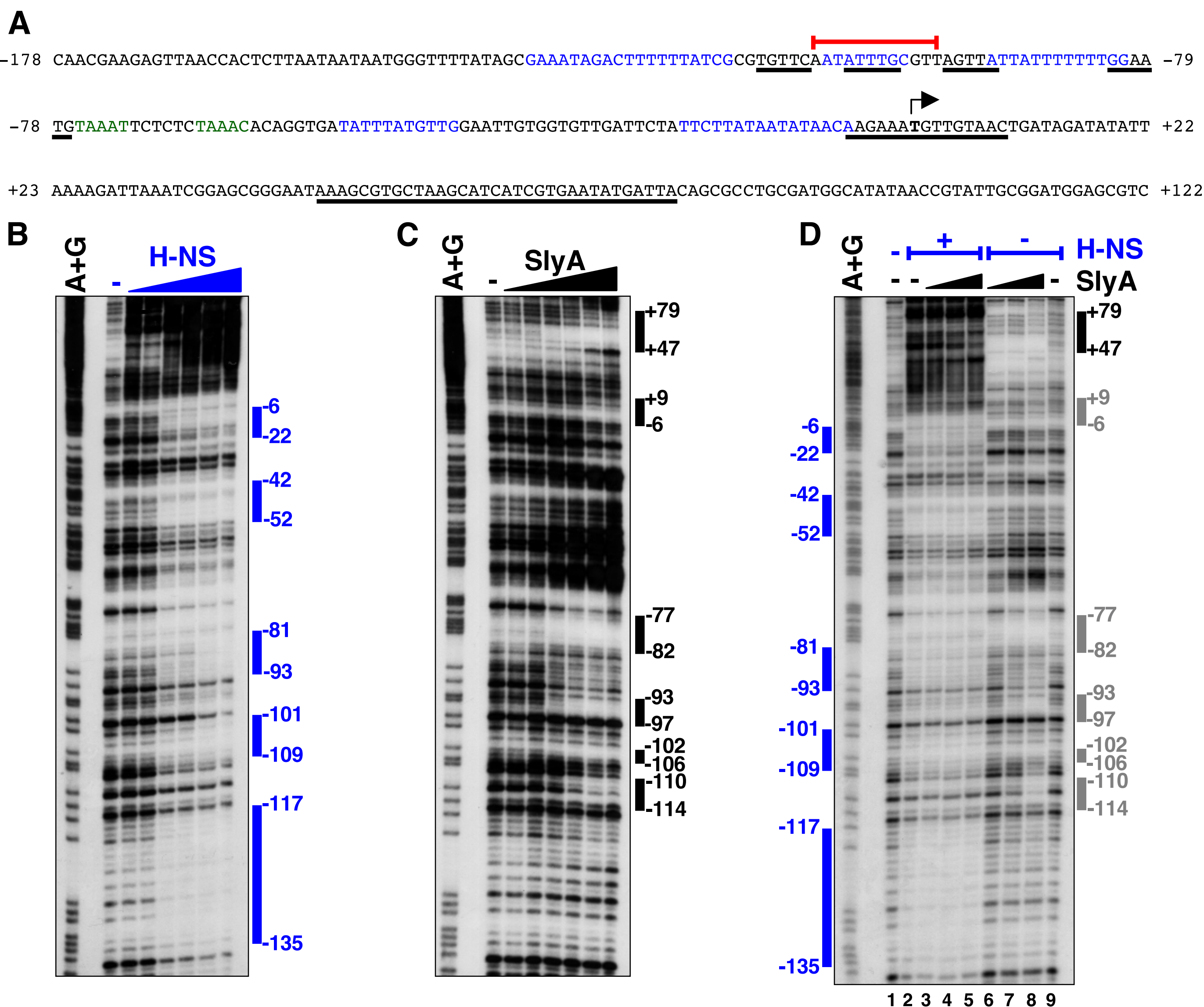


FIGURE 7. **The H-NS and SlyA proteins bind to several sites in the *pagC* promoter.** *A*, nucleotide sequence of the promoter region of the *pagC* gene (−178 to +122 nt with respect to the transcription start site) displaying regions bound by the H-NS (in *blue*) and SlyA (*underlined*) proteins. The transcription start site is indicated in *boldface type* and by the *bent arrow*, whereas the sequence in *green* highlights the PhoP box. The *red line* indicates the 12 nucleotides deleted in strain EG18817 (Fig. 8). *B–D*, DNase I footprinting analysis of the *pagC* promoter carried out as described under "Experimental Procedures" with increasing amounts of H-NS (0, 0.2, 0.4, 0.8, 1.2, 1.6, and 3.2 μM) (*B*) or SlyA (0, 4, 20, 80, 200, 400, and 600 nM) alone (*C* and *D*, *right side*) or H-NS (0.8 μM) in combination with SlyA (40, 80, and 400 nM) (*D*, *left side*).

tively, in the *hns rpoS* strain (Fig. 6), which is in contrast to what happens in an *hns*$^+$ background (Fig. 2). On the other hand, the *phoP* mutation clearly decreased the levels of both the *pagC* and *ugtL* transcripts under these conditions (Fig. 6). The lower levels for the *pagC* and *ugtL* mRNAs are probably due to the less efficient activation of the PhoP/PhoQ system taking place in LB broth, which contains >200 μM $Mg^{2+}$, a concentration >20 times higher than in the N-minimal medium with 10 μM $Mg^{2+}$, which induced higher mRNA levels (Fig. 2). In addition, this could reflect the fact that the strains were defective for *rpoS*, which can be highly pleiotropic on general cell physiology. Taken together with the results of the *in vitro* experiments (Fig. 5), these *in vivo* findings indicate that the PhoP and SlyA proteins are necessary and sufficient to promote *pagC* and *ugtL* transcription in the presence of H-NS and that they play different roles in transcriptional activation of these promoters.

*The SlyA and H-NS Proteins Simultaneously Occupy Sites in the pagC Promoter Region*—To explore how the SlyA protein relieves H-NS-promoted gene silencing, we first used DNase I footprinting to identify the regions of the *pagC* promoter that are bound by the H-NS and SlyA proteins. The H-NS protein protected multiple sites in this region (Fig. 7, *A* and *B*), which is a common feature of H-NS-regulated promoters (30). The SlyA protein also protected several sites (Fig. 7, *A* and *C*), one of which had been previously reported (8). The presence of multiple binding sites for the SlyA protein has been documented for other promoters as well (31). In addition, we identified several DNase I-hypersensitive sites (Fig. 7*C*), which could result from SlyA-promoted bending of the DNA or changes in the DNA topology (32).

Some of the regions protected by the H-NS and SlyA proteins overlap either completely or partially (Fig. 7*A*). This raises the

possibility that these two proteins may compete for a common binding site, and suggests that the role of SlyA may be to displace H-NS from its binding site to allow PhoP binding and RNA polymerase recruitment. We tested this possibility by analyzing whether H-NS binding to the *pagC* promoter was altered in the presence of SlyA. The H-NS footprint pattern in presence of an amount of SlyA protein that was enough to counteract H-NS repression (Fig. 5*C*) and to footprint the *pagC* promoter region (Fig. 7, *C* and *D*, *lanes 6–8*) *in vitro* looked similar to that generated by H-NS alone (Fig. 7*D*, compare *lanes 3*, *4*, and *5* with *lane 2*). The SlyA protein footprinted the *pagC* promoter region between nucleotides +47 and +79 either alone (Fig. 7*D*, compare *lanes 8* and *9*) or in the presence of H-NS (Fig. 7*D*, compare *lanes 2* and *5*), indicating that both proteins can bind to this promoter simultaneously. H-NS prevented SlyA binding at the site between −110 and −114 (Fig. 7*D*, compare *lanes 5* and *8*), but it is unclear whether SlyA binds at the other four sites (between +9 and −6, −77 and −82, −93 and −97, and −102 and −106) in the presence of H-NS (Fig. 7*D*, compare *lanes 5* and *8*). Interestingly, the SlyA protein led to the generation of a DNase I-hypersensitive site at position +5 in the presence of H-NS (Fig. 7*D*, *lane 5*). In agreement with the footprint data, the SlyA and H-NS proteins could simultaneously occupy the *pagC* promoter region as determined by gel shift analysis (Fig. S5), suggesting that derepression of the *pagC* promoter region by SlyA does not involve complete H-NS displacement from the promoter.

*A Site Bound by the SlyA and H-NS Proteins in the pagC Promoter Is Dispensable for H-NS-mediated Repression but Necessary for Derepression by the SlyA Protein*—If competition were the sole basis for relieving H-NS-promoted repression, elimination of a binding site shared by H-NS and SlyA should result in SlyA-independent *pagC* transcription. To test this notion, we constructed a strain with a 12-nt chromosomal deletion that removed the overlapping SlyA and H-NS binding site located ~100 nt upstream from the *pagC* transcription start site (Fig. 7*A*). Gel shift analysis of a *pagC* promoter fragment prepared from this mutant demonstrated that the SlyA protein retained the ability to bind to the mutated promoter, presumably at the other SlyA binding sites (Fig. S6). However, *pagC* transcription was abolished in the *pagC* promoter mutant *in vivo* (Fig. 8*A*). The *pagC* promoter mutation does not appear to affect PhoP binding and RNA polymerase recruitment because PhoP was still able to promote transcription in the presence of RNA polymerase *in vitro* (Fig. 8*B*). Moreover, it did not affect the ability of H-NS to repress transcription *in vitro*. By contrast, the SlyA protein was no longer capable of counteracting the H-NS silencing effect (Fig. 8*B*). These findings, which are consistent with the *in vivo* results (Fig. 8*A*), indicate that the investigated site is required for SlyA function but not essential for H-NS-promoted silencing.

## DISCUSSION

The acquisition of DNA sequences by horizontal gene transfer provides bacteria with the opportunity for "quantum leap evolution" in the development of new traits (33). For this to happen, bacteria must regulate the acquired genes so that they are expressed at the right time and place. It has been estimated

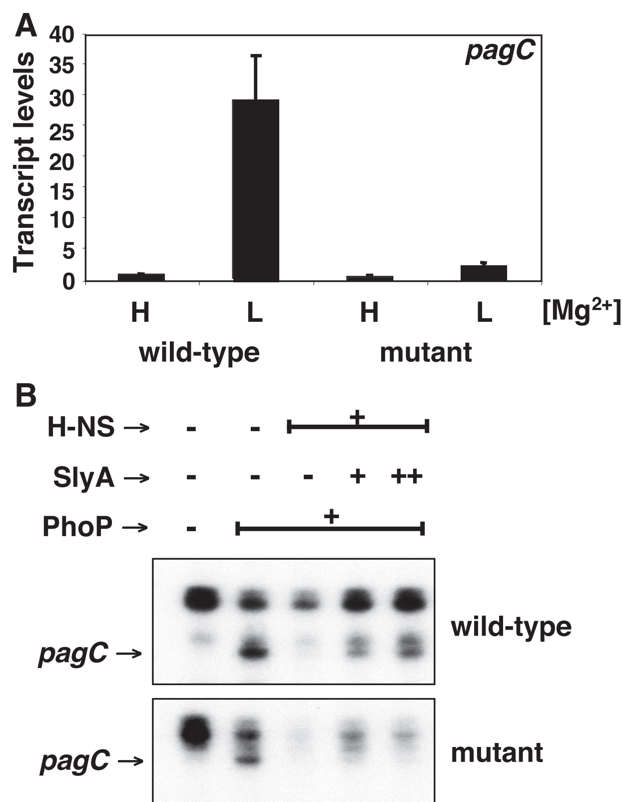


FIGURE 8. **An overlapping SlyA and H-NS binding site in the *pagC* promoter is dispensable for H-NS repression but necessary for derepression by SlyA.** *A*, transcript levels corresponding to the *pagC* gene as determined by quantitative real time PCR in wild type (EG13918) or the isogenic strain EG18817 with a 12-nt deletion in the SlyA/H-NS binding site located ~100 nt upstream of the *pagC* transcription start site (see Fig. 7*A*). Cells were grown in N-minimal medium containing 10 mM (*H*) or 10 μM (*L*) $MgCl_2$. Shown are the mean values and S.D. of at least three independent experiments. *B*, run-off *in vitro* transcription assays with linear templates corresponding to the wild-type *pagC* promoter region (−178 to +122 nt with respect to the transcription start site) (*top*) or an equivalent DNA fragment with a 12-nt deletion in the SlyA/H-NS binding site (*bottom*), RNA polymerase, PhoP (0 or 2 μM), H-NS (0 or 1.25 μM), and SlyA (0, 200, and 300 nM). The *pagC* transcript (*lower band*) is indicated by an *arrow*. The *uppermost band* in *both panels* corresponds to a ~150-nt run-off transcript resulting from spurious transcription going in the reverse orientation, whereas the band just on top of the *pagC* transcript originates ~5 nt upstream of the transcription start site indicated in Fig. 3. (A transcript originating at this position has not been observed *in vivo*.)

that a new sequence is acquired and stably maintained by enteric bacteria on an average of only once every several hundred thousand years (3), suggesting that only rarely is a new sequence favorably selected, and thus its expression has to be regulated. In the case of enteric bacteria, this regulation involves two distinct aspects; on the one hand, it entails counteracting the silencing effects that the H-NS protein imposes on newly acquired DNA; on the other hand, it requires reprogramming the expression of the foreign genes so that it is coordinated with that of the recipient bacterial genome. We have now established that, at least for a subset of horizontally acquired genes in *Salmonella*, these two aspects are governed by two different proteins, SlyA and PhoP, whose concerted action allows *Salmonella* to display some of its pathogenic properties, since these proteins govern the expression of a large number of virulence genes residing within pathogenicity islands and islets (7, 8).

The critical role played by the PhoP and SlyA proteins in promoting transcription of horizontally acquired genes, such as

*pagC* and *ugtL* investigated here, is supported by several lines of evidence. First, transcription of the *pagC* and *ugtL* genes was abolished in bacteria lacking the *phoP* or *slyA* genes (Fig. 2, *A* and *B*); second, both the PhoP and SlyA proteins footprinted and gel-shifted DNA fragments harboring the *pagC* and *ugtL* promoters (Fig. 3, *B* and *C*) (23); and third, the PhoP and SlyA proteins were necessary and sufficient to promote transcription from the *pagC* and *ugtL* promoters in the presence of H-NS *in vitro* (Fig. 5, *C* and *D*). The SlyA protein was required to promote transcription of the horizontally acquired *pagC* and *ugtL* genes but not of the ancestral *mgtA*, *pagP*, *rstA*, and *slyB* genes (Figs. 2*C* and S2) (8). Since the latter group of genes is also PhoP-dependent, our results suggest that the joint requirement for PhoP and SlyA in gene expression may be specific to foreign DNA sequences in *Salmonella*.

The role of SlyA in transcription of the *pagC* and *ugtL* genes appears to be limited to countering the silencing effects of the H-NS protein, because the purified SlyA protein was unable to promote *pagC* and *ugtL* transcription *in vitro* by itself (*i.e.* with RNA polymerase but no PhoP) (Fig. 5, *C* and *D*), even in the absence of H-NS (Fig. 5*D*, *lanes 3* and *4*). Indeed, we determined that the PhoP protein is directly involved in promoting *pagC* transcription, because PhoP bound to the *pagC* promoter *in vitro* (Fig. 3, *B* and *C*) and *in vivo* (Fig. 3*D*) and because point mutations in the PhoP box that prevented PhoP binding (Fig. 3, *C* and *D*) abolished *pagC* transcription (Fig. 3*E*). Therefore, the role of PhoP in *pagC* transcription is 2-fold: as a direct transcriptional activator of the *pagC* promoter and as a regulator of SlyA at transcriptional (23, 24) and/or post-transcriptional (8) levels. This form of regulation is in striking parallel to that controlling expression of the *ugtL* gene (23), and it may apply to other PhoP- and SlyA-dependent genes, such as *mig-14* and *virK* (which also seem to have been horizontally acquired), because the PhoP protein has been shown to bind to their respective promoters *in vitro* and *in vivo* (26).[3]

How does SlyA antagonize H-NS-promoted transcriptional silencing? One model posits that SlyA displaces H-NS from the promoters where it binds (34), suggesting that these two proteins compete for shared binding sites (35). In agreement with this model, *E. coli* SlyA and its ortholog in *Yersinia*, designated RovA, footprinted DNA segments that overlap with regions protected by H-NS *in vitro* (36, 37), although it is presently unclear whether SlyA or RovA can remove H-NS from a promoter *in vivo*. On the other hand, SlyA does not seem to function by displacing H-NS from the *Salmonella pagC* promoter, because both proteins could simultaneously bind to this promoter *in vitro* (Figs. 7*D* and S5), and removal of a binding site shared by the SlyA and H-NS proteins abolished the function of SlyA but not silencing by H-NS (Fig. 8). Furthermore, H-NS remained associated with the *pagC* and *ugtL* promoters *in vivo* (as determined by chromatin immunoprecipitation, which has a maximum resolution of ~500 bp) when these genes were expressed (Fig. 4, *A* and *B*).

We propose an alternative model, whereby SlyA remodels the local structure of the H-NS-DNA complex in ways that allow other DNA binding proteins to be recruited to a promoter. This remodeling would entail rearrangement of the H-NS molecules associated with a particular promoter region or partial disruption of H-NS-mediated DNA duplex bridges (38) but not H-NS removal from a promoter. The apparent simultaneous occupancy of the *pagC* and *ugtL* promoter regions by the H-NS, SlyA, and PhoP proteins and even RNA polymerase is not unprecedented, since many regions of the *E. coli* genome bound by H-NS can also associate with RNA polymerase (6, 39), and binding of H-NS and of SlyA to various promoters is not mutually exclusive, at least *in vitro* (40, 41) (Fig. S5). Recent findings with the *E. coli* SlyA and H-NS proteins acting on the *hlyE* promoter (41) also support this notion. The proposed model takes into account the role of H-NS in the formation and maintenance of topological domain barriers in the bacterial chromosome (42), because overcoming H-NS repression would not involve complete H-NS clearance from a given region, which would be detrimental for the organization of the bacterial nucleoid. Consistent with our proposal, the H-NS footprinting pattern of the *pagC* promoter was hardly modified in the presence of the SlyA protein, except for the appearance of hypersensitive sites (Fig. 7*D*), which may reflect changes in the local topology of the promoter DNA. Indeed, changes in temperature and osmolarity have been shown to facilitate derepression of certain H-NS-repressed genes *in vivo* by altering the degree of local DNA supercoiling (30). This mode of action may not be limited to SlyA because the VirB protein of *Shigella flexneri* seems to use a similar mechanism to antagonize H-NS (43). Finally, it is also possible that SlyA could mediate the reported chemical modification of H-NS (44).

The widespread distribution of PhoP and SlyA in enteric bacteria raises the possibility that these proteins may relieve H-NS-dependent repression of horizontally acquired genes in species other than *S. enterica*. For example, PhoP has been shown to regulate the expression of *E. coli*-specific genes (26) and of horizontally acquired genes in *Yersinia*.[3] However, the PhoP and SlyA homologs may behave in a fashion different from those from *Salmonella*. For example, the *Yersinia* RovA protein exhibits both anti-repressor and transcriptional activator functions (45), which might reflect a unique ability of this SlyA homolog. Alternatively or in addition, SlyA might also act as a transcriptional activator of yet-to-be-discovered *Salmonella* promoters.

In summary, the ability of *Salmonella* to express certain horizontally acquired genes requires the ordered and sequential participation of the ancestral DNA-binding proteins PhoP and SlyA. Upon experiencing inducing conditions for the PhoP/PhoQ system, the PhoP protein promotes expression (23) and/or activation of the SlyA protein (8). This results in SlyA binding to its target promoters, which, by overcoming H-NS repression, allows the PhoP protein to bind to the promoters and then to recruit RNA polymerase (Figs. 1 and 4). The role of SlyA appears to be limited to antagonizing H-NS silencing, because expression of the *pagC* and *ugtL* genes *in vivo* was rendered *slyA*-independent in a strain lacking *hns* (Fig. 6), and transcription of the *pagC* and *ugtL* promoters *in vitro* required the purified SlyA protein only when the H-NS protein was present in the reaction (Fig. 5). By contrast, the PhoP protein was

[3] J. C. Perez, T. Latifi, and E. A. Groisman, unpublished results.

required for transcription of these promoters even in the absence of H-NS both *in vivo* (Fig. 6) and *in vitro* (Fig. 5). Therefore, the PhoP and SlyA proteins carry out distinct jobs in the activation of promoters for horizontally acquired genes.

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