AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity

Characterization of SlyA in *Shigella flexneri* Identifies a Novel Role in Virulence

Natasha Weatherspoon-Griffin, Helen J. Wing

School of Life Sciences, University of Nevada Las Vegas, Las Vegas, Nevada, USA

The SlyA transcriptional regulator has important roles in the virulence and pathogenesis of several members of the *Enterobacteriaceae* family, including *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. Despite the identification of the *slyA* gene in *Shigella flexneri* nearly 2 decades ago, as well as the significant conservation of SlyA among enteric bacteria, the role of SlyA in *Shigella* remains unknown. The genes regulated by SlyA in closely related organisms often are absent from or mutated in *S. flexneri*, and consequently many described SlyA-dependent phenotypes are not present. By characterizing the expression of *slyA* and determining its ultimate effect in this highly virulent organism, we postulated that novel SlyA-regulated virulence phenotypes would be identified. In this study, we report the first analysis of SlyA in *Shigella* and show that (i) the *slyA* gene is transcribed and ultimately translated into protein, (ii) *slyA* promoter activity is maximal during stationary phase and is negatively autoregulated and positively regulated by the PhoP response regulator, (iii) the exogenous expression of *slyA* rescues transcription and virulence-associated deficiencies during virulence-repressed conditions, and (iv) the absence of *slyA* significantly decreases acid resistance, demonstrating a novel and important role in *Shigella* virulence. Cumulatively, our study illustrates unexpected parallels between the less conserved *S. flexneri* and *S.* Typhimurium *slyA* promoters as well as a unique role for SlyA in *Shigella* virulence that has not been described previously in any closely related organism.

The MarR/SlyA family of transcription factors controls an array of biological functions critical to bacterial physiology and survival (1–5). In the *Enterobacteriaceae* family, the DNA binding protein SlyA regulates diverse aspects of virulence (reviewed in reference 6). SlyA originally was identified in *Salmonella enterica* serovar Typhimurium for its ability to induce hemolytic and cytotoxic phenotypes when overexpressed in *Escherichia coli* (7, 8). Since then, in *S.* Typhimurium, SlyA has been implicated in facilitating intracellular survival within professional macrophages (7, 9), contributing to cell envelope modification (10), and conferring resistance to antimicrobial peptides and oxidative stress (11, 12). Meanwhile, in *E. coli*, SlyA contributes to virulence differently by inducing a hemolytic phenotype (13), facilitating the synthesis of a virulence antigen (14, 15), and contributing to type 1 fimbriation (16). The role of SlyA in the human pathogen *Shigella*, however, has not been described despite the identification of a *slyA* gene (8) and the high amino acid identity that SlyA shares with SlyA proteins found in closely related organisms (Table 1).

Shigella flexneri is closely related to both *Salmonella* spp. and *E. coli*. It carries a large (~220-kb) virulence plasmid responsible for the invasive and virulent nature of this organism. Encoded on the large virulence plasmid is VirB, a transcriptional regulator essential to *Shigella* virulence. VirB functions to counteract the repression of virulence gene promoters mediated by H-NS (17–19), a histone-like nucleoid structuring protein that prevents the inappropriate expression of horizontally acquired genes (reviewed in reference 20). As a derepressor, VirB binds to promoters to facilitate the rearrangement of the H-NS–DNA nucleoprotein complex to ultimately upregulate transcription. This regulatory activity is a common feature of an emerging group of proteins that do not behave as traditional transcriptional activators; instead, they function solely to alleviate H-NS-mediated repression by remodeling the nucleoprotein complex (20). SlyA is included in this group of proteins, because for most SlyA-regulated promoters characterized so far, SlyA binds to target promoters to counteract repression mediated by H-NS to facilitate the transcription of virulence genes (11, 16, 21–25).

Included in the SlyA regulon is *slyA* itself, the promoter of which is positively autoregulated in *E. coli* (14) and negatively autoregulated in *S.* Typhimurium (26). This promoter also has been shown to be positively regulated by the PhoP response regulator of the PhoP/PhoQ two-component system in *S.* Typhimurium (10, 11). Meanwhile, H-NS negatively regulates the orthologous *rovA* promoter in *Yersinia pseudotuberculosis* (27) but has no effect on the *slyA* promoter in *E. coli* (14). The intricate regulation of *slyA* and its downstream targets is critical to many virulence features identified thus far in *E. coli* and *S.* Typhimurium; however, the regulation of *slyA* in *Shigella* and its role in virulence remain undetermined.

Here, we provide the first characterization of SlyA in *Shigella*. We describe three major aspects of *slyA*: the regulation and activity of the *slyA* promoter, the effect of exogenous *slyA* expression on *Shigella* virulence phenotypes and gene regulation, and a novel SlyA-dependent role in acid resistance. Overall, our study characterizes an important transcriptional regulator in *S. flexneri* and uncovers a novel role for SlyA in acid resistance, a virulence feature that is essential for the successful pathogenesis of the bacterium.

Received 9 July 2015 **Returned for modification** 11 August 2015
Accepted 23 January 2016

Accepted manuscript posted online 1 February 2016

Citation Weatherspoon-Griffin N, Wing HJ. 2016. Characterization of SlyA in *Shigella flexneri* identifies a novel role in virulence. Infect Immun 84:1073–1082. doi:10.1128/IAI.00806-15.

Editor: S. M. Payne, University of Texas at Austin

Address correspondence to Helen J. Wing, helen.wing@unlv.edu.

TABLE 1 *S. flexneri* SlyA conservation relative to members of the *Enterobacteriaceae* family

Organism	Rep strain[a]	Locus name	DNA accession no.[b]	DNA identity (%)	DNA length (bp)	Protein accession no.[b]	Amino acid identity (%)	Amino acid length (aa)
Escherichia coli	K-12 substr. MG1665	*b1642*	U00096.3	99	435	NP_416159.2	100	144
Escherichia coli (EIEC)[c]	53638	ND[d]	ND	ND	ND	EDU63848	100	144
Escherichia coli (EHEC)[e]	O157:H7 Sakai chromosome	ECs2351	BA000007.2	99	435	NP_310378.2	100	144
Shigella boydii	Sb227	SBO _1492	CP000036.1	100	435	YP_407937.2	100	144
Shigella dysenteriae	Sd197	SDY _1865	CP000034.1	99	435	YP_403465.2	100	144
Shigella flexneri	2457T	*S1801*	AE014073.1	100	435	NP_837330.2	100	144
Shigella sonnei	Ss046	SSON _1514	CP000038.1	100	435	YP_310449.2	100	144
Salmonella Typhimurium	14028s	STM14 _1742	CP001363.1	82	435	YP_005237314.1	95	144
Salmonella Typhi	Ty2	*t1312*	AE014613.1	81	435	NP_805113.1	90	144
Klebsiella pneumoniae	NTUH- K2044	KP1 _3054	AP006725.1	75	441	YP_002919758.1	87	146
Yersinia pestis	KIM10	*y1961*	AE009952.1	77	432	NP_669276.1	77	143
Yersinia pseudotuberculosis	YPIII	BZ22 _2437	CP009792	77	432	ACA68167	77	143
Dickeya dadantii	3937	Dda3937 _00475	CP002038.1	72	438	ADM98879	75	145

[a] Representative strain.
[b] Accession numbers are from the National Center for Biotechnology Information (NCBI).
[c] Enteroinvasive *Escherichia coli*.
[d] Not determined. Sequence unavailable.
[e] Enterohemorrhagic *Escherichia coli*.

MATERIALS AND METHODS

Bacterial growth. Bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* and *S. flexneri* strains were grown routinely in Trypticase soy broth (TSB) or Luria broth (LB) overnight at 30°C and then subcultured and grown at 37°C (unless otherwise noted) with aeration at 325 rpm. When necessary, antibiotics were added at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 25 or 50 μg/ml; tetracycline, 12.5 μg/ml; and spectinomycin, 100 μg/ml. To ensure that *S. flexneri* strains maintained the virulence plasmid, Congo red binding was tested routinely on TSB agar (TSA) plates (1.5% agar [wt/vol]) containing 0.01% (wt/vol) Congo red (referred to as Congo red plates).

Strain construction. NWG1 (Δ*slyA*::Kn) was constructed via P1-mediated transduction of the *E. coli* Δ*slyA*::Kn locus (which contains a deletion of the *slyA* gene between the start and stop codons generated using lambda red recombination; JW5267 from the Keio collection [28]) into 2457T. Transductants were selected on 100 μg/ml kanamycin. The *slyA* deletion in 2457T was confirmed via PCR with primers W424/W425 (Table 3). NWG11 (Δ*slyA*::*lacZ*) was constructed using FLP-mediated site-specific recombination and with the subsequent integration of pKG136 into the FLP recombination target site generated in strain NWG1, as previously described (29). The inserted *lacZ* and its endogenous start and stop codons create a transcriptional fusion directly controlled by the *slyA* promoter. NWG13 (Δ*phoP*::Kn^s) was constructed by using the FLP recombinase (30) to remove the kanamycin cassette from the *S. flexneri* 2457T Δ*phoP*::Kn mutant, which was constructed via P1-mediated transduction of the *E. coli* Δ*phoP*::Kn locus (which contains a deletion of the *phoP* gene between the start and stop codons generated using lambda red

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant description	Source or reference
Strains		
E. coli		
K-12 (MC4100)		62
JW5267	(BW25113) Δ*slyA*::Kn	28
S. flexneri		
2457T	*S. flexneri* serotype 2a	63
AWY3	(2457T) *virB*::Tn*5*	36
BS103	2457T cured of the virulence plasmid	49
BS184	(2457T) *mxiC-lacZ*	31
NWG1	(2457T) Δ*slyA*::Kn	This study
NWG11	(2457T) Δ*slyA*::*lacZ*	This study
NWG13	(2457T) Δ*phoP*::Kn^s	This study
NWG14	(2457T) Δ*slyA*::*lacZ hns*::Tn*10*	This study
NWG15	(2457T) Δ*slyA*::*lacZ* Δ*phoP*::Kn^s	This study
Plasmids		
pBAD18	Arabinose inducible, pBR*ori*; Amp^r	64
pATM324	pBAD18-*virB*; Amp^r	65
pNWG03	pBAD18-*his-slyA*; Amp^r	This study
pAFW04	pACYC184-P*icsP-lacZ*; Cm^r	19
pSRG58	pACYC184-P$_{phoP}$-*phoPQ*; Spc^r	33
pNWG07	pACYC184; Spc^r	This study
pBR322	pMB1*ori*; Amp^r	66
pNWG13	pBR322-P$_{slyA}$-*slyA*; Amp^r	This study

TABLE 3 Primers used in this study

Primer name	Sequence 5′→3′
W69	TGAGACGTCGACGGGCACCTCACTTTAGCACTGAAGCC
W70	GTGTCAGTCGACGCGTTTTCAAGGATTAGTCCTTAATCGG
W401	AACGTCAATGAGCAAAGGTATTAA
W402	TACGGGAGGCAGCAGTGG
W403	GGACTAATAGCAGGTTAC
W404	GCAGTTAATCCAATATAAGG
W408	CACTAGGTTCTGATCTGGCACGG
W409	CTCGATGCCAATCGCTTTTGCC
W410	CTTCTGGGAATAGTCCTGACAACC
W411	GAGTTGACTGACTTTTCGGCCTCC
W424	AAAGTAGATTCCTTTACGACCG
W425	AATGAACAAAACGCAGGGTGTC
W537	TCATTCAAGCTTCAAGAACCGCAACTATCGCTACAAT
W556	TCATTCGGATCCCCCCCTTCATTTCACCCTTTGGCCTG
W565	CAAATAGGGGTTCCGCGCACATTTC
W566	GGGGCCTGCCACCATACCCACGCCG

recombination; JW1116 from the Keio collection [28]) into 2457T. NWG14 (Δ*slyA*::*lacZ hns*::Tn*10*) was constructed via P1-mediated transduction of the *hns*::Tn*10* locus of BS185 (31) into NWG11. Transductants were selected on 12.5 μg/ml tetracycline. NWG15 (Δ*slyA*::*lacZ phoP*::Kn^s) was constructed via P1-mediated transduction of Δ*slyA*::*lacZ* (NWG11) into NWG13. Transductants were selected on 100 μg/ml kanamycin.

Plasmid construction. pNWG03 (pBAD-*slyA*) was constructed by ligating fragments from EcoRI- and SalI-digested pBAD18 and pCA24N-*his-slyA* (JW5267 from the ASKA collection [32]), which generated 4,580-bp and 527-bp fragments, respectively. pNWG03 contains the *slyA* gene from the second codon onwards. pNWG07 (pACYC184-Spc^r), an empty vector control to pSRG58, was constructed by self-ligating the SphI-digested pSRG58 (pACYC184-P$_{phoP}$-*phoPQ* [33]), which removed the entire *phoP* locus and the majority of the *phoQ* locus. The remaining 3.2-kb fragment contains the *ori* and *aadA* gene. pNWG13 (pBR322-P$_{slyA}$-*slyA*) was constructed by ligating fragments from the HindIII- and BamHI-digested pBR322, which released a 3,739-bp fragment, and pNWG12 (a high-copy-number vector containing the entire *slyA* coding region and putative promoter [500 bp upstream of the start codon] that was PCR generated using W537/W556 [Table 3]), which released a 946-bp fragment. The sequence of the inserted DNA was verified by Sanger sequencing using primers W565/W566 (Table 3).

Reverse transcription-PCR (RT-PCR). Wild-type *S. flexneri* 2457T and *E. coli* MC4100 cells were grown in TSB at 37°C to late exponential phase. Total RNA was isolated using the TRIzol reagent (Life Technologies) using the manufacturer's specifications. Two micrograms of DNase I-treated RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). PCR was carried out using 100 ng of cDNA as the template. Specific primer sets (described in Table 3) were used to amplify the following regions of interest: *slyA* (primers W408/409), *rrsA* (primers W401/402), *icsP* (primers W403/404), *ompT* (primers W410/411), and *icsP* promoter region (P*icsP*; primers W69/70). In addition, genomic DNA from *S. flexneri* 2457T and *E. coli* MC4100 was used as a positive PCR control for each primer set.

Western blot analysis. *E. coli* MC4100 and *S. flexneri* 2457T wild-type and Δ*slyA*::Kn cells were cultured in 20 ml LB at 37°C to late exponential phase. Cells were harvested, washed twice with phosphate-buffered saline (PBS), resuspended in 0.5 ml PBS, and lysed via sonication. Total cell protein concentration was determined using a Bradford assay. Equal amounts of total protein preparations were separated on 17.5% SDS-acrylamide gels, and SlyA was detected using a polyclonal anti-SlyA antibody obtained from the Blomfield laboratory as previously described (16) and enhanced using chemiluminescence (ECL; Pierce).

β-Galactosidase assays. The activity of the *slyA* promoter was determined by measuring β-galactosidase activity in the *S. flexneri* 2457T and *E. coli* MC4100 Δ*slyA*::*lacZ* strains using the Miller protocol (34). Promoter activity was routinely analyzed in three independent assays with at least two independent transformations. To measure *slyA* promoter activity throughout a time course, β-galactosidase activity was determined in the Δ*slyA*::*lacZ* strain harboring pBR322 or pBR322-P$_{slyA}$-*slyA*. Overnight cultures were subcultured 1:100 in LB and grown at 37°C with aeration to various time points.

To measure the PhoP- and Mg^{2+}-dependent regulation of the *slyA* promoter, β-galactosidase activity was determined in the Δ*slyA*::*lacZ* strain harboring pACYC184 or pACYC184-P$_{phoP}$-*phoPQ* (33) using conditions similar to those used previously (24). Cultures were grown in N minimal buffer (35) supplemented with 0.4% glucose, 0.4% Casamino Acids, 0.01 mg/ml nicotinic acid, 0.01 mg/ml tryptophan, 0.01 mg/ml thiamine, and 0.1 mM $CaCl_2$ (collectively termed N minimal medium). Cells were grown overnight in N minimal medium supplemented with 10 mM $MgCl_2$, washed twice with N minimal medium, and subcultured 1:10 into fresh N minimal medium supplemented with 0.01 mM (low) or 10 mM (high) $MgCl_2$. Cells were grown at 37°C to late exponential phase and then harvested to measure β-galactosidase activity.

To measure the SlyA- and VirB-dependent regulation of the promoters controlling *mxiC* and *icsP*, β-galactosidase activity was measured in cells bearing either the *mxiC-lacZ* reporter (BS184 [31]) or P*icsP-lacZ* (pAFW04a [19]) and carrying either pBAD, pBAD-*virB*, or pBAD-*slyA*. Overnight cultures were subcultured 1:100 in LB and grown under derepressed (37°C or wild-type background, respectively) or repressed (30°C or *virB*::Tn*5* mutant background, respectively) conditions. As previously described (36), mid-exponential-phase cultures were supplemented with 0.2% L-arabinose to induce the pBAD promoter. Cells were grown to late exponential phase and then harvested to measure β-galactosidase activity.

Congo red binding assay. *S. flexneri* 2457T wild-type and *virB*::Tn*5* cells harboring pBAD, pBAD-*virB*, or pBAD-*slyA* were grown overnight in LB supplemented with 0.2% (wt/vol) D-glucose. The next day, cultures were serially diluted (40-fold dilutions) and ~6 μl of each dilution was dropped onto TSA Congo red plates supplemented with 0.2% (wt/vol) L-arabinose or D-glucose. Plates were incubated overnight at 37°C to determine the Congo red phenotype. To quantify the relative amount of Congo red dye bound by cells, a fixed number of cells (1.3×10^9 cells/ml) was evaluated. Two culture spots for each sample were scraped off the agar plate and resuspended in 0.75 ml 25% ethanol to remove the Congo red bound to cells. The optical density at 600 nm (OD_{600}) of the cell suspension was measured to normalize samples to cell number. Cell suspensions then were centrifuged to pellet cells. The OD_{498} of the supernatant was measured to quantify the amount of Congo red dye released from the cells. Relative Congo red binding was determined with the following equation: $[(OD_{498}/OD_{600})/(\text{average } (OD_{498}/OD_{600})_{2457T\ pBAD})] \times 100$.

Acid resistance assay. Acid resistance assays were adapted from previously described assays (37, 38). *S. flexneri* 2457T wild-type cells harboring pBAD and Δ*slyA* cells harboring pBAD or pBAD-*slyA* were grown overnight in LB at 30°C. The next day, cultures were subcultured 1:100 into 5 ml of LB, pH 7.0 (buffered in 50 mM morpholinepropanesulfonic acid), or LB, pH 5.5 (buffered in 50 mM morpholineethanesulfonic acid), and grown overnight at 37°C. Before acid challenge, 0.2% (wt/vol) L-arabinose was added to cultures to induce the pBAD promoter for 2 h at 37°C. After induction, cultures were diluted 1:1,000 into 5 ml LB, pH 2.5 (acidified with HCl), for the acid challenge. Cultures were challenged in the acidic medium at 37°C for 0, 30, 60, and 120 min. The challenge was stopped by diluting cells (1:40) into fresh LB. Cell viability was determined by (i) dropping ~6 μl of serially diluted ($\sim 10^{-3}$ and 10^{-5}) cultures onto Congo red plates and observing growth after overnight incubation at 37°C and (ii) spreading 100 μl of an appropriate dilution and determining CFU after overnight incubation at 37°C. Percent normalized survival was determined by the following equation: [CFU after challenge/CFU of 2457T pBAD (pH 5.5) before challenge (i.e., 0 min)] × 100.

Statistical analyses. Two-tailed, two-sample Student's *t* tests, assuming equal variance, were used throughout this work to determine confidence levels.

RESULTS

***slyA* is transcribed and translated in *S. flexneri*.** To begin the characterization of SlyA, we chose to determine if its chromosomally encoded gene is expressed in *S. flexneri*, since this had not been established when this genetic locus was identified (8). The transcription of *slyA* was determined by reverse transcription-PCR (RT-PCR) using total RNA isolated from *S. flexneri* 2457T. *E. coli* MC4100 was used as a positive control for this study, because *slyA*, which is 99% similar to the *slyA* gene in *S. flexneri* (Table 1), is known to be expressed in *E. coli* (14). Specific primer sets (described in Materials and Methods) were used to amplify the following regions of interest: (i) *slyA*, gene of interest; (ii) *rrsA*, positive control; (iii) *icsP*, *S. flexneri* positive control and *E. coli* negative control; (iv) *ompT*, *S. flexneri* negative control and *E. coli* positive control; and (v) *icsP* promoter region (P*icsP*), negative nontranscribed control. In addition, each primer set also was used with genomic DNA from *S. flexneri* 2457T and *E. coli* MC4100 as a

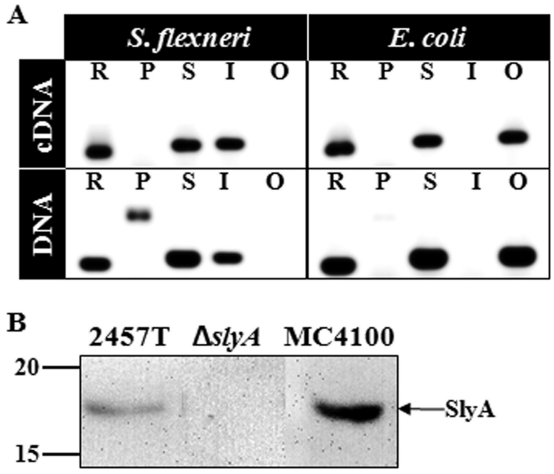

FIG 1 *slyA* is transcribed and translated in *S. flexneri* 2457T. (A) *slyA* is expressed. PCR used cDNA (upper) and genomic DNA (lower) from *S. flexneri* 2457T and *E. coli* MC4100 grown to late exponential phase in TSB at 37°C. Transcribed sequences were amplified using primers that bind to the coding regions of *rrsA* (R), *slyA* (S), *icsP* (I), and *ompT* (O), while primers within the *icsP* promoter (P*icsP*; P) region were used to amplify nontranscribed sequences. (B) SlyA is produced. Western blot analysis of whole-cell extracts from *S. flexneri* 2457T, *S. flexneri* Δ*slyA*::Kn, and *E. coli* MC4100 cells grown to late exponential phase using a polyclonal anti-SlyA antibody obtained from the Blomfield laboratory (16).

positive control for PCR. As shown in Fig. 1A, *slyA* was amplified in both the *S. flexneri* and *E. coli* cDNA samples. The positive controls were present and the negative controls were absent, as expected. Taken together, these data demonstrate that *slyA* is transcribed in *S. flexneri* 2457T.

Since transcription is not always indicative of translation, we determined if SlyA protein is produced in *S. flexneri* using Western blot analysis. Protein samples obtained from cultures of *S. flexneri* 2457T wild-type and Δ*slyA* mutant cultures, as well as *E. coli* MC4100 wild-type culture, were electrophoresed and probed for the presence of SlyA (which is 100% identical in *S. flexneri* and *E. coli*; Table 1) using an affinity-purified polyclonal anti-SlyA antibody (gift from the Blomfield laboratory). The SlyA protein (17.4 kDa) was detected in both wild-type backgrounds but not in the Δ*slyA* mutant (Fig. 1B), demonstrating SlyA is produced in *S. flexneri* 2457T.

Transcriptional profile and autoregulation of *slyA* in *S. flexneri*. Since we had determined that *slyA* is expressed in *S. flexneri* (Fig. 1), we next monitored *slyA* promoter activity throughout a 24-h growth period using a chromosomal *slyA* transcriptional *lacZ* fusion (Δ*slyA*::*lacZ*) constructed in the *S. flexneri* 2457T background, as well as in the *E. coli* MC4100 background as a control. To approximate physiological levels of SlyA in this reporter strain, a low-copy-number plasmid harboring the *slyA* gene under the control of its own promoter (pBR322-P_{slyA}-*slyA*) was introduced. In the presence of SlyA (pBR322-P_{slyA}-*slyA*), *S. flexneri slyA* promoter activity peaked during early stationary phase (Fig. 2A). This regulatory pattern is similar to that exhibited by the *slyA* promoter in *S.* Typhimurium, whose activity peaks during stationary phase (12), but contrasts with that of the *slyA* promoter in *E. coli*, whose activity peaks during late exponential phase and declines upon entry into stationary phase (Fig. 2A) (14). Although it is possible that the LacZ protein is more stably maintained in the *S. flexneri* background than in *E. coli*, leading to higher β-galactosidase activity, this seems unlikely because LacZ activity has been shown to decline during stationary phase, when *lacZ* is expressed from another promoter in *S. flexneri* (39).

Moreover, starting at the 3-h time point, *slyA* promoter activity was found to decrease 2-fold ($P < 0.01$) in the absence (pBR322) versus the presence of SlyA (Fig. 2A). This suggests that SlyA negatively autoregulates its promoter in *S. flexneri*, which is similar to the regulation in *S.* Typhimurium (26) but differs from the regulation in *E. coli*, where SlyA positively autoregulates the *slyA* promoter (14). Cumulatively, these observations demonstrate that the regulation of the *slyA* promoter in *S. flexneri* is more similar to that found in the more distantly related *S.* Typhimurium than in its closest relative, *E. coli*.

Since SlyA has been shown to specifically bind to the *slyA* promoter in *E. coli* (14) and *S.* Typhimurium (11, 26), we searched the *S. flexneri slyA* promoter sequence for putative SlyA binding sites. Five putative SlyA binding sites (bearing at least a 7/12 match to the consensus sequence [26]) (Fig. 2B) were found within the predicted *S. flexneri slyA* promoter region (500 bp upstream of the *slyA* start codon), three of which overlap SlyA binding sites identified in *E. coli* or *S.* Typhimurium (Fig. 2B). Taken together, these findings strongly suggest that SlyA directly negatively autoregulates its promoter in *S. flexneri*.

PhoP-dependent regulation of the *slyA* promoter in *S. flexneri*. PhoP, the response regulator of the PhoP/PhoQ two-component system, has been shown to positively regulate the *slyA* promoter in *S.* Typhimurium (10, 11) and to play a role in *Shigella* virulence (40). To further characterize the regulation of the *S. flexneri slyA* promoter, we evaluated the effects of PhoP on *slyA* promoter activity. β-Galactosidase activity was determined using the Δ*slyA*::*lacZ* construct in the wild-type background and an isogenic Δ*phoP* mutant background under PhoP-activating (i.e., low Mg^{2+}) and PhoP-repressing (i.e., high Mg^{2+}) conditions. In wild-type *S. flexneri*, a 3-fold ($P = 0.006$) Mg^{2+}-dependent response was observed between low Mg^{2+} and high Mg^{2+} (Fig. 3A). Furthermore, when *phoP* was absent, *slyA* promoter activity was abolished (17-fold decrease [$P = 0.003$] under low-Mg^{2+} condition) but could be restored to wild-type levels when a wild-type copy of the *phoP* locus was introduced in *trans* (pACYC-*phoPQ*) (Fig. 3A). These data demonstrate that, similar to the *slyA* promoter in *S.* Typhimurium, the *S. flexneri slyA* promoter responds to Mg^{2+} and is positively regulated by PhoP.

Three putative PhoP binding sites (minimal 6/10 match to consensus sequence [41–43]) (Fig. 3B) were identified in the *S. flexneri slyA* promoter region (500 bp upstream of the *slyA* start codon). One of these sites overlaps a sequence previously shown to be bound by PhoP in *S.* Typhimurium (11). The presence of these putative sites provides strong evidence that PhoP directly regulates the *slyA* promoter in *S. flexneri*.

H-NS does not regulate the *slyA* promoter in *S. flexneri*. The global transcriptional repressor H-NS has been shown to negatively regulate the promoter of the *slyA* ortholog *rovA* in *Yersinia pseudotuberculosis* (27) but has no effect on the *slyA* promoter in *E. coli* K-12 (14) (currently, the effect of H-NS on the *slyA* promoter in *S.* Typhimurium has not been reported). H-NS is well characterized in *S. flexneri* as a transcriptional repressor of virulence genes (31, 44). So, to conclude our characterization of the *slyA* promoter in *S. flexneri*, the role of H-NS was examined. In *Shigella*, H-NS-mediated repression is observed at a nonphysiological

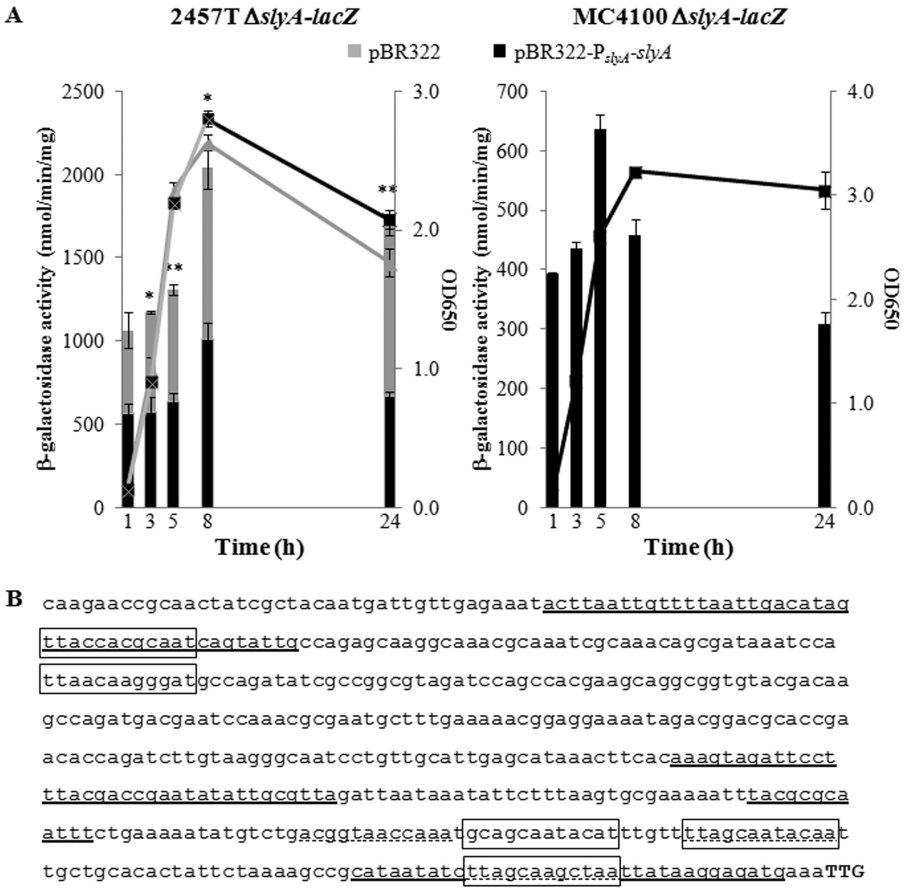

FIG 2 Transcriptional profile and autoregulation of the *slyA* promoter in *S. flexneri*. (A) Growth-dependent activity of the *slyA* promoter. β-Galactosidase activity of the Δ*slyA*::*lacZ* reporter strain in *S. flexneri* 2457T harboring pBR322 (vector-only control) or pBR322-P$_{slyA}$-*slyA* and in *E. coli* MC4100 harboring pBR322-P$_{slyA}$-*slyA* over a 24-h time course. Bars represent *slyA* promoter activity, and lines represent optical density (650 nm) readings of cultures over time. Standard deviations are shown. Data are representative of three independent trials. A two-tailed Student's *t* test (assuming equal variance) was used for statistical analysis. *, $P < 0.01$; **, $P < 0.001$. (B) Putative SlyA binding sites within the *slyA* promoter. The *slyA* promoter sequence (500 bp upstream of the start codon, TTG) with the following sites identified: (i) solid underline, SlyA binding to the *E. coli slyA* promoter (14); (ii) dashed underline, SlyA binding to the *S.* Typhimurium *slyA* promoter (26); (iii) solid box, putative SlyA binding to the *S. flexneri slyA* promoter.

temperature of 30°C and alleviated at 37°C either directly or indirectly through the production of other thermally responsive transcriptional regulators (31, 44–47). Thus, we used the Δ*slyA*::*lacZ* reporter strain to measure *slyA* promoter activity in isogenic wild-type and *hns* mutant backgrounds at both 30°C and 37°C. Neither H-NS nor temperature had a significant effect on β-galactosidase activity (data not shown), suggesting that the *S. flexneri slyA* promoter, similar to the *E. coli slyA* promoter, is not regulated by H-NS.

Exogenous expression of *slyA* rescues a virulence-associated phenotype in *S. flexneri*. Having characterized the expression of *slyA* in *Shigella*, we next focused our attention on the role that SlyA plays in the modulation of *Shigella* virulence traits. To do this, we initially monitored virulence phenotypes in wild-type *S. flexneri* and an avirulent *virB* mutant when *slyA* was expressed from a high-copy-number plasmid (i.e., pBAD-*slyA*). We reasoned that this strategy would uncover SlyA-associated virulence phenotypes that may not be so easily detected with physiological levels of SlyA or in the presence of the essential virulence regulator VirB.

The ability of *S. flexneri* to bind the organic dye Congo red from agar medium correlates well with the virulence properties of this bacterium, in part due to the presence of a type three secretion system (48, 49). The expression of genes encoding the type three secretion system is regulated by VirB, so the absence of *virB S. flexneri* produces a Congo red-negative (CR$^-$) phenotype (50, 51). Surprisingly, our initial phenotypic evaluation revealed that the exogenous expression of *slyA* in a *virB* mutant background restored Congo red binding. To determine the significance of this restoration, we developed an assay to quantify Congo red binding to cells grown on agar medium (described in Materials and Methods). As expected, under inducing and noninducing conditions, wild-type cells maintained their CR$^+$ phenotype (~100% Congo red binding) and *virB* mutant cells harboring pBAD maintained their CR$^-$ phenotype (~28% relative Congo red binding) (Fig. 4). Not surprisingly, under inducing conditions, pBAD-*virB* restored the CR$^+$ phenotype of the *virB* mutant (70% relative Congo red binding; $P = 0.0004$), but strikingly, pBAD-*slyA* also could restore the CR$^+$ phenotype (68% relative Congo red binding; $P = 0.002$) in the *virB* mutant (Fig. 4). These data indicate that the exogenous expression of *slyA* can restore a CR$^+$ phenotype in the absence of *virB*.

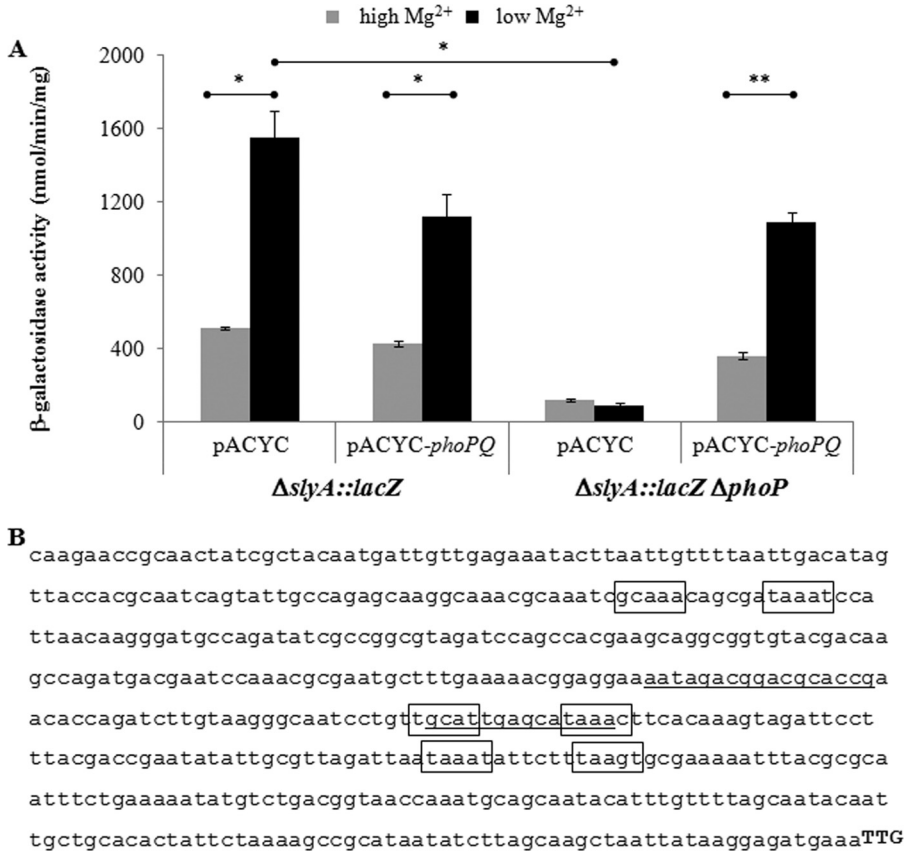

FIG 3 PhoP-dependent regulation of the *slyA* promoter in *S. flexneri*. (A) PhoP- and Mg^{2+}-dependent activity of the *slyA* promoter. β-Galactosidase activity of a Δ*slyA*::*lacZ* reporter strain in *S. flexneri* 2457T wild-type and isogenic Δ*phoP* backgrounds harboring pACYC (vector only control) or pACYC-P_{phoP}-*phoPQ* (pSRG58 [33]). Strains were grown in N minimal medium supplemented with 10 mM Mg^{2+} (high; gray bars) or 0.01 mM Mg^{2+} (low; black bars). Each data set is representative of three independent trials. A two-tailed Student's *t* test (assuming equal variance) was used for statistical analysis. *, $P < 0.01$; **, $P < 0.001$. (B) Putative PhoP binding sites within the *slyA* promoter. The *slyA* promoter sequence (500 bp upstream of the start codon, TTG) is shown with the following sites identified: (i) solid underline, PhoP binding to the *S.* Typhimurium *slyA* promoter (11); (ii) solid box, putative PhoP binding to the *S. flexneri slyA* promoter.

Exogenous expression of *slyA* upregulates virulence gene promoters in *S. flexneri*. Since the exogenous expression of *slyA* can compensate for a lack of *virB* in Congo red binding assays (Fig. 4), we next chose to determine if this SlyA-dependent effect was caused by the upregulation of genes encoding the type three secretion system, which reside in the *mxi-spa* locus. To test this, β-galactosidase activity was measured in cells carrying the virulence plasmid-encoded *mxiC* promoter reporter, *mxiC-lacZ* (BS184 [31]), and harboring pBAD, pBAD-*virB*, or pBAD-*slyA* under both repressing conditions (i.e., 30°C) and derepressing conditions (i.e., 37°C). The *mxiC* promoter activity increased 5-fold ($P = 0.0006$) when *slyA* was exogenously expressed (Fig. 5A) and, consistent with previous reports (52), increased 10-fold ($P = 0.002$) when *virB* was exogenously expressed at 30°C (considered repressing conditions, because VirB levels are low and cannot counter H-NS). The SlyA-dependent upregulation of this promoter likely explains the restoration of Congo red binding that was observed when *slyA* was exogenously expressed in a *virB* mutant (Fig. 4).

To determine if SlyA-dependent upregulation can extend beyond the *mxi-spa* locus, we measured the β-galactosidase activity of the *icsP* promoter, which resides outside the *mxi-spa* locus and also is regulated by VirB, using the plasmid-borne *icsP* promoter reporter, P*icsP-lacZ* (pAFW04a [19]). The *icsP* promoter activity increased 3-fold ($P = 0.003$) when *slyA* was exogenously expressed (Fig. 5B) and, consistent with previous reports (36), increased 7-fold ($P = 0.0008$) when *virB* was exogenously expressed in the *virB* mutant background (considered repressed conditions, because VirB is absent and cannot counter H-NS). This demonstrates that the exogenous expression of *slyA* also can lead to the upregulation of a gene outside the *mxi-spa* locus in the absence of *virB*.

Cumulatively, these data show that superphysiological levels of SlyA can regulate VirB-dependent promoters when VirB is absent. Interestingly, under derepressing conditions where VirB is present and able to counter H-NS-mediated repression, the exogenous expression of *slyA* led to a modest 2-fold decrease in the activity of both the *mxiC* (at 37°C; $P = 0.004$) and *icsP* (in the wild-type background; $P = 0.03$) promoters (Fig. 5). This raises the possibility that SlyA does not solely compensate for the lack of VirB but has some additional regulatory role when present at superphysiological levels. Regardless, the effect of SlyA observed in our assays is specific to VirB-dependent promoters, because the exogenous expression of *slyA* does not increase the activity of the VirB-independent *icsA* promoter irrespective of whether or not its native regulator, VirF, is present (data not shown).

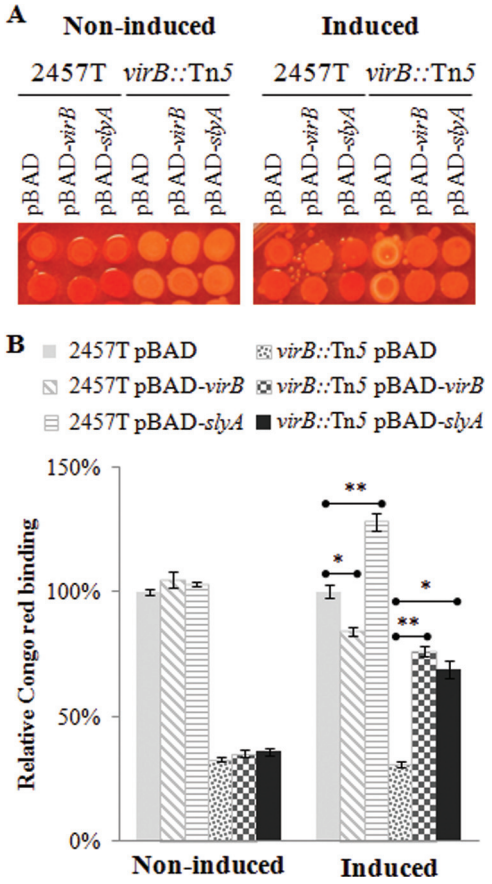

FIG 4 Exogenous expression of *slyA* rescues the Congo red binding deficiency in a *virB* mutant. (A) Qualitative analysis of Congo red binding. *S. flexneri* 2457T wild-type and *virB*::Tn*5* strains harboring pBAD, pBAD-*virB*, or pBAD-*slyA* were serially diluted (40-fold dilutions) and spotted (approximately 6 μl) onto TSA Congo red plates supplemented with 0.2% (wt/vol) D-glucose (non-induced) or 0.2% (wt/vol) L-arabinose (induced). (B) Quantitative analysis of Congo red binding. Two culture spots (shown in panel A) were scraped off the agar plate and resuspended in 0.75 ml 25% ethanol to remove the Congo red bound to cells. The OD_{600} was determined to normalize samples against cell number. Cell suspensions were centrifuged to pellet cells, and the OD_{498} of the supernatant was determined. Relative Congo red binding was calculated as $[(OD_{498}/OD_{600})/(\text{average } (OD_{498}/OD_{600})_{2457T\ pBAD})] \times 100$. Data shown are representative of three independent trials. A two-tailed Student's *t* test (assuming equal variance) was used for statistical analysis. *, $P < 0.01$; **, $P < 0.001$.

Acid resistance: a novel role for SlyA in *Shigella*. To gain greater insight into the role of SlyA at physiological levels, a literature search was conducted. This revealed that a proteomic analysis in enteroinvasive *E. coli* (EIEC) had identified over 30 putative SlyA-dependent gene products, several of which had been implicated previously in acid resistance (53). Although the authors did not formally test the role of SlyA in acid resistance in EIEC, we decided to examine the role that SlyA plays in acid resistance in *Shigella*. To test this, *S. flexneri* 2457T wild-type and isogenic Δ*slyA* mutant strains were evaluated using a traditional acid resistance assay (adapted from references 37 and 38).

As described previously (54), adaptive conditions "prime" cells to survive an extreme acid challenge at levels several orders of magnitude higher than nonadapted cells. Strikingly, when grown under adaptive conditions (pH 5.5), the survival of Δ*slyA* cells was decreased approximately 200-fold ($P = 0.002$) compared to that of wild-type cells after just 30 min of extreme acid challenge (Fig. 6). Importantly, the survival of Δ*slyA* cells could be restored to wild-type levels when *slyA* was expressed exogenously (pBAD-*slyA*) (Fig. 6). As expected, none of the cultures grown under non-adaptive conditions (pH 7.0) were able to survive extreme acid challenge. Consequently, these findings are the first to demonstrate that SlyA has a role in acid resistance in *S. flexneri*. Since acid resistance has been suggested to be a key factor in the low infectious dose of *Shigella* spp. (37, 55, 56), these findings elevate the importance of SlyA and SlyA-mediated regulation in *Shigella* virulence.

DISCUSSION

The SlyA transcriptional regulator has been demonstrated to be an important virulence factor in several enteric bacteria (reviewed in reference 6); however, prior to this study it had not been studied in the human pathogen *Shigella*. Here, we describe three significant features of *slyA* in *S. flexneri*: (i) the regulation of the *slyA* promoter, (ii) *Shigella* virulence phenotypes and promoters regulated by the exogenous expression of *slyA*, and (iii) the requirement of SlyA in acid resistance. Overall, our characterization of *slyA* and the regulator it encodes uncovers some interesting parallels between *E. coli*, *Salmonella*, and *Shigella* and also reveals a novel and unique role for SlyA in *Shigella* virulence.

Our characterization of the *S. flexneri slyA* promoter revealed that certain aspects of its regulation were more similar to the less conserved *S.* Typhimurium *slyA* promoter (68% identical) than the highly conserved *E. coli slyA* promoter (99% identical). Specifically, this was demonstrated by our observations that the *S. flexneri slyA* promoter activity is (i) maximal during stationary phase (Fig. 2A), (ii) negatively autoregulated (Fig. 2A), and (iii) positively regulated by PhoP (Fig. 3A). Our finding that PhoP regulates the *slyA* promoter in *S. flexneri* adds another member to the PhoP/Q regulon, which previously had consisted of a single virulence plasmid-carried operon (*shf-wabB-virK-msbB2*) that encodes enzymes involved in bacterial cell wall biosynthesis (33, 57). Interestingly, although the *slyA* promoter in both *S.* Typhimurium and *S. flexneri* has been shown to be regulated by PhoP (10 and 11 and Fig. 3A, respectively), the *slyA* transcript has not been identified in genomic approaches attempting to characterize the PhoP/Q regulons in *S.* Typhimurium (22, 58, 59). This apparent discrepancy may be explained by the different experimental conditions and approaches used in these studies (i.e., promoter activity versus mRNA levels) or the possibility that posttranscriptional regulation or modification of *slyA* mRNA in *S.* Typhimurium and possibly *S. flexneri* exists. This will be the subject of future investigations in our laboratory. The observation that the regulation of the *slyA* promoter in *S. flexneri* is similar to that in *S.* Typhimurium but different from that in *E. coli* is intriguing, since *Shigella* species diverged from *E. coli* after *E. coli* and *Salmonella* had diverged from their common ancestor. Pondering this evolutionary paradox, one can posit that the similar regulation of *slyA* in *S. flexneri* and *S.* Typhimurium proves advantageous because of their common intracellular lifestyle. Future examination of the *S. flexneri* SlyA regulon will reveal if there is any support for this idea.

SlyA is a well-characterized transcriptional regulator of virulence-associated promoters in closely related organisms (reviewed in reference 6); therefore, the role of SlyA in *Shigella* virulence-associated phenotypes was investigated. Surprisingly, our data demonstrated that in the absence of *virB*, the exogenous expres-

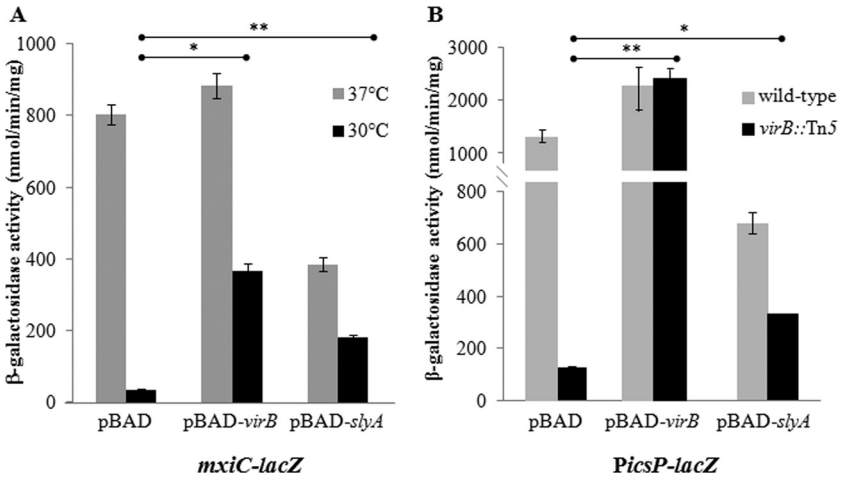

FIG 5 Exogenous expression of *slyA* upregulates VirB-dependent promoters under repressed conditions. (A) Upregulation of *mxiC-lacZ*. β-Galactosidase activity of *mxiC-lacZ* cells harboring pBAD, pBAD-*virB*, or pBAD-*slyA* in derepressed (37°C) or repressed (30°C) conditions. (B) Upregulation of P*icsP-lacZ*. β-Galactosidase activity of cells containing P*icsP-lacZ* and harboring pBAD, pBAD-*virB*, or pBAD-*slyA* in derepressed (wild-type) or repressed (*virB*::Tn*5*) conditions is shown. Data are representative of three independent trials conducted in triplicate. A two-tailed Student's *t* test (assuming equal variance) was used for statistical analysis. *, $P < 0.01$; **, $P < 0.001$.

sion of *slyA* could restore Congo red binding to *S. flexneri* (Fig. 4). We determined that this phenotype was due, at least in part, to SlyA upregulating genes encoding the type three secretion apparatus (i.e., *mxiC-lacZ*) (Fig. 5A), a process known to lead to Congo red binding. In addition, the exogenous expression of *slyA* could upregulate the *icsP* promoter in the absence of *virB* (Fig. 5B) but not the VirB-independent *icsA* promoter (data not shown), suggesting SlyA functions to compensate for the lack of VirB when expressed at high levels. The mechanistically intriguing effect of

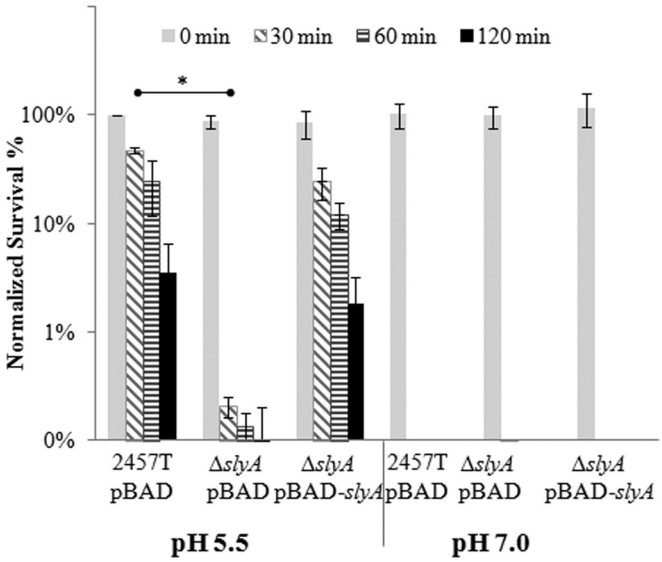

FIG 6 Deletion of *slyA* decreases *S. flexneri* acid resistance. *S. flexneri* 2457T wild-type cells harboring pBAD and Δ*slyA* cells harboring pBAD or pBAD-*slyA* grown under adaptive (pH 5.5) or nonadaptive (pH 7.0) conditions were challenged at pH 2.5. The CFU of challenged cells were determined. The percentage of normalized survival was calculated as CFU after challenge divided by CFU of 2457T pBAD (pH 5.5) before challenge (i.e., 0 min) times 100. Data are the averages from three independent trials. A two-tailed Student's *t* test (assuming equal variance) was used for statistical analysis. *, $P < 0.01$.

SlyA on VirB-dependent promoters could be caused by (i) SlyA binding to VirB binding sites, (ii) SlyA binding to degenerate SlyA binding sites, (iii) SlyA decreasing intracellular H-NS protein levels, or (iv) some combination thereof. While we acknowledge that high levels of SlyA in these particular assays are responsible for the regulatory effects observed, these findings highlight the relative plasticity of the regulatory networks controlling virulence; if *virB* is lost or inactivated, simply increasing SlyA levels through the mutation of its promoter could restore some virulence gene expression and phenotypes. This is especially interesting because *virB* has been shown to be deleted or otherwise inactivated at high frequency when serially passaged at 37°C (60). To our knowledge, our finding that SlyA can compensate for the loss of VirB is the first of its kind to suggest that one derepressor of H-NS-mediated repression compensates for the loss of another. In light of these findings, it is interesting that other derepressors of H-NS-mediated repression have the potential to serve this kind of back-up role when a gene encoding the usual cognate transcriptional regulator is lost or mutated.

Our finding that SlyA is essential for acid resistance in *S. flexneri* is the first to demonstrate that SlyA is required for this crucial virulence phenotype in enterics. The acid resistance controlled by SlyA may have remained uncharacterized to this point, because attributes of *Shigella* virulence most commonly are studied in nongastrointestinal (GI) tract models: the Sereny test, which is a conjunctivitis model in mouse; the rabbit ileal loop model, where shigellae are artificially introduced into a closed-off segment of the ileum, thereby bypassing migration through the upper GI tract; and/or *in vitro* tissue culture assays. None of these assays examine events that lead to the successful passage of shigellae through the stomach, and this may explain why critical factors like SlyA, which promote acid resistance, have not been identified. This is unfortunate, since acid resistance is arguably one of the most critical virulence determinants in *Shigella* spp., because it has been correlated (32, 50, 51) to the extremely low infectious dose of this pathogen (10 to 100 cells) (61). Therefore, our finding that SlyA is essential for acid resistance in *S. flexneri* constitutes a novel and

significant finding in *S. flexneri* pathogenesis and raises the possibility that SlyA plays an important role in determining the infectious dose of this highly infectious enteric organism.

In summary, we have characterized three major aspects of *slyA* in *S. flexneri*. Our study highlights unexpected similarities between the *S. flexneri* and *S.* Typhimurium *slyA* promoters. We describe an unprecedented relationship between two derepressors of H-NS-mediated repression, SlyA and VirB, and discuss possible implications of our findings. Moreover, we emphasize the importance of SlyA in acid resistance. This is a virulence role not previously demonstrated for SlyA but is one that is likely to be essential for the pathogenesis of this pathogen. Clearly, one important future direction of this work will be to identify genes of the *S. flexneri* SlyA regulon and specifically those that are responsible for the acid resistance phenotype, which has been described for the first time in this work.

ACKNOWLEDGMENTS

We thank Ian Blomfield for the polyclonal anti-SlyA antibody and Jim Slauch for the pKG137 plasmid used in this work.

FUNDING INFORMATION

HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Helen J. Wing under grant number R15 AI090573.

This study was supported by National Institutes of Health (NIH) grant R15 AI090573-02.

REFERENCES

1. **George AM, Levy SB.** 1983. Gene in the major cotransduction gap of the *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. J Bacteriol **155:**541–548.
2. **Srikumar R, Kon T, Gotoh N, Poole K.** 1998. Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive *Escherichia coli* strain. Antimicrob Agents Chemother **42:**65–71.
3. **Thomson NR, Cox A, Bycroft BW, Stewart GS, Williams P, Salmond GP.** 1997. The rap and hor proteins of *Erwinia*, *Serratia* and *Yersinia*: a novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. Mol Microbiol **26:**531–544. http://dx.doi.org/10.1046/j.1365-2958.1997.5981976.x.
4. **Galan B, Kolb A, Sanz JM, Garcia JL, Prieto MA.** 2003. Molecular determinants of the hpa regulatory system of *Escherichia coli*: the HpaR repressor. Nucleic Acids Res **31:**6598–6609. http://dx.doi.org/10.1093/nar/gkg851.
5. **Roper DI, Fawcett T, Cooper RA.** 1993. The *Escherichia coli* C homoprotocatechuate degradative operon: *hpc* gene order, direction of transcription and control of expression. Mol Gen Genet **237:**241–250.
6. **Ellison DW, Miller VL.** 2006. Regulation of virulence by members of the MarR/SlyA family. Curr Opin Microbiol **9:**153–159. http://dx.doi.org/10.1016/j.mib.2006.02.003.
7. **Libby SJ, Goebel W, Ludwig A, Buchmeier N, Bowe F, Fang FC, Guiney DG, Songer JG, Heffron F.** 1994. A cytolysin encoded by *Salmonella* is required for survival within macrophages. Proc Natl Acad Sci U S A **91:**489–493. http://dx.doi.org/10.1073/pnas.91.2.489.
8. **Ludwig A, Tengel C, Bauer S, Bubert A, Benz R, Mollenkopf HJ, Goebel W.** 1995. SlyA, a regulatory protein from *Salmonella typhimurium*, induces a haemolytic and pore-forming protein in *Escherichia coli*. Mol Gen Genet **249:**474–486. http://dx.doi.org/10.1007/BF00290573.
9. **Fass E, Groisman EA.** 2009. Control of *Salmonella* pathogenicity island-2 gene expression. Curr Opin Microbiol **12:**199–204. http://dx.doi.org/10.1016/j.mib.2009.01.004.
10. **Norte VA, Stapleton MR, Green J.** 2003. PhoP-responsive expression of the *Salmonella enterica* serovar *typhimurium slyA* gene. J Bacteriol **185:**3508–3514. http://dx.doi.org/10.1128/JB.185.12.3508-3514.2003.
11. **Shi Y, Latifi T, Cromie MJ, Groisman EA.** 2004. Transcriptional control of the antimicrobial peptide resistance *ugtL* gene by the *Salmonella* PhoP and SlyA regulatory proteins. J Biol Chem **279:**38618–38625. http://dx.doi.org/10.1074/jbc.M406149200.
12. **Buchmeier N, Bossie S, Chen CY, Fang FC, Guiney DG, Libby SJ.** 1997. SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. Infect Immun **65:**3725–3730.
13. **Oscarsson J, Mizunoe Y, Uhlin BE, Haydon DJ.** 1996. Induction of haemolytic activity in *Escherichia coli* by the *slyA* gene product. Mol Microbiol **20:**191–199. http://dx.doi.org/10.1111/j.1365-2958.1996.tb02500.x.
14. **Corbett D, Bennett HJ, Askar H, Green J, Roberts IS.** 2007. SlyA and H-NS regulate transcription of the *Escherichia coli* K5 capsule gene cluster, and expression of *slyA* in *Escherichia coli* is temperature-dependent, positively autoregulated, and independent of H-NS. J Biol Chem **282:**33326–33335. http://dx.doi.org/10.1074/jbc.M703465200.
15. **Xue P, Corbett D, Goldrick M, Naylor C, Roberts IS.** 2009. Regulation of expression of the region 3 promoter of the *Escherichia coli* K5 capsule gene cluster involves H-NS, SlyA, and a large 5′ untranslated region. J Bacteriol **191:**1838–1846. http://dx.doi.org/10.1128/JB.01388-08.
16. **McVicker G, Sun L, Sohanpal BK, Gashi K, Williamson RA, Plumbridge J, Blomfield IC.** 2011. SlyA protein activates *fimB* gene expression and type 1 fimbriation in *Escherichia coli* K-12. J Biol Chem **286:**32026–32035. http://dx.doi.org/10.1074/jbc.M111.266619.
17. **Turner EC, Dorman CJ.** 2007. H-NS antagonism in *Shigella flexneri* by VirB, a virulence gene transcription regulator that is closely related to plasmid partition factors. J Bacteriol **189:**3403–3413. http://dx.doi.org/10.1128/JB.01813-06.
18. **Castellanos MI, Harrison DJ, Smith JM, Labahn SK, Levy KM, Wing HJ.** 2009. VirB alleviates H-NS repression of the *icsP* promoter in *Shigella flexneri* from sites more than one kilobase upstream of the transcription start site. J Bacteriol **191:**4047–4050. http://dx.doi.org/10.1128/JB.00313-09.
19. **Basta DW, Pew KL, Immak JA, Park HS, Picker MA, Wigley AF, Hensley CT, Pearson JS, Hartland EL, Wing HJ.** 2013. Characterization of the *ospZ* promoter in *Shigella flexneri* and its regulation by VirB and H-NS. J Bacteriol **195:**2562–2572. http://dx.doi.org/10.1128/JB.00212-13.
20. **Stoebel DM, Free A, Dorman CJ.** 2008. Anti-silencing: overcoming H-NS-mediated repression of transcription in Gram-negative enteric bacteria. Microbiology **154:**2533–2545. http://dx.doi.org/10.1099/mic.0.2008/020693-0.
21. **Wyborn NR, Stapleton MR, Norte VA, Roberts RE, Grafton J, Green J.** 2004. Regulation of *Escherichia coli* hemolysin E expression by H-NS and *Salmonella* SlyA. J Bacteriol **186:**1620–1628. http://dx.doi.org/10.1128/JB.186.6.1620-1628.2004.
22. **Navarre WW, Halsey TA, Walthers D, Frye J, McClelland M, Potter JL, Kenney LJ, Gunn JS, Fang FC, Libby SJ.** 2005. Co-regulation of *Salmonella enterica* genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ. Mol Microbiol **56:**492–508. http://dx.doi.org/10.1111/j.1365-2958.2005.04553.x.
23. **Lithgow JK, Haider F, Roberts IS, Green J.** 2007. Alternate SlyA and H-NS nucleoprotein complexes control *hlyE* expression in *Escherichia coli* K-12. Mol Microbiol **66:**685–698. http://dx.doi.org/10.1111/j.1365-2958.2007.05950.x.
24. **Kong W, Weatherspoon N, Shi Y.** 2008. Molecular mechanism for establishment of signal-dependent regulation in the PhoP/PhoQ system. J Biol Chem **283:**16612–16621. http://dx.doi.org/10.1074/jbc.M800547200.
25. **Song H, Kong W, Weatherspoon N, Qin G, Tyler W, Turk J, Curtiss R, III, Shi Y.** 2008. Modulation of the regulatory activity of bacterial two-component systems by SlyA. J Biol Chem **283:**28158–28168. http://dx.doi.org/10.1074/jbc.M801058200.
26. **Stapleton MR, Norte VA, Read RC, Green J.** 2002. Interaction of the *Salmonella typhimurium* transcription and virulence factor SlyA with target DNA and identification of members of the SlyA regulon. J Biol Chem **277:**17630–17637. http://dx.doi.org/10.1074/jbc.M110178200.
27. **Tran HJ, Heroven AK, Winkler L, Spreter T, Beatrix B, Dersch P.** 2005. Analysis of RovA, a transcriptional regulator of *Yersinia pseudotuberculosis* virulence that acts through antirepression and direct transcriptional activation. J Biol Chem **280:**42423–42432. http://dx.doi.org/10.1074/jbc.M504464200.
28. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006.0008.

29. **Ellermeier CD, Janakiraman A, Slauch JM.** 2002. Construction of targeted single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene **290:**153–161. http://dx.doi.org/10.1016/S0378-1119(02)00551-6.
30. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
31. **Maurelli AT, Sansonetti PJ.** 1988. Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. Proc Natl Acad Sci U S A **85:**2820–2824. http://dx.doi.org/10.1073/pnas.85.8.2820.
32. **Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H.** 2005. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. DNA Res **12:**291–299.
33. **Goldman SR, Tu Y, Goldberg MB.** 2008. Differential regulation by magnesium of the two MsbB paralogs of *Shigella flexneri*. J Bacteriol **190:**3526–3537. http://dx.doi.org/10.1128/JB.00151-08.
34. **Miller J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. **Nelson DL, Kennedy EP.** 1971. Magnesium transport in *Escherichia coli*. Inhibition by cobaltous ion. J Biol Chem **246:**3042–3049.
36. **Wing HJ, Yan AW, Goldman SR, Goldberg MB.** 2004. Regulation of IcsP, the outer membrane protease of the *Shigella* actin tail assembly protein IcsA, by virulence plasmid regulators VirF and VirB. J Bacteriol **186:**699–705. http://dx.doi.org/10.1128/JB.186.3.699-705.2004.
37. **Waterman SR, Small PL.** 1996. Identification of sigma S-dependent genes associated with the stationary-phase acid-resistance phenotype of *Shigella flexneri*. Mol Microbiol **21:**925–940. http://dx.doi.org/10.1046/j.1365-2958.1996.00058.x.
38. **Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW.** 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J Bacteriol **177:**4097–4104.
39. **Hensley CT, Kamneva OK, Levy KM, Labahn SK, Africa LA, Wing HJ.** 2011. Two promoters and two translation start sites control the expression of the Shigella flexneri outer membrane protease IcsP. Arch Microbiol **193:**263–274. http://dx.doi.org/10.1007/s00203-010-0669-2.
40. **Moss JE, Fisher PE, Vick B, Groisman EA, Zychlinsky A.** 2000. The regulatory protein PhoP controls susceptibility to the host inflammatory response in *Shigella flexneri*. Cell Microbiol **2:**443–452. http://dx.doi.org/10.1046/j.1462-5822.2000.00065.x.
41. **Kato A, Tanabe H, Utsumi R.** 1999. Molecular characterization of the PhoP-PhoQ two-component system in *Escherichia coli* K-12: identification of extracellular $Mg2^{+}$-responsive promoters. J Bacteriol **181:**5516–5520.
42. **Groisman EA.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol **183:**1835–1842. http://dx.doi.org/10.1128/JB.183.6.1835-1842.2001.
43. **Yamamoto K, Ogasawara H, Fujita N, Utsumi R, Ishihama A.** 2002. Novel mode of transcription regulation of divergently overlapping promoters by PhoP, the regulator of two-component system sensing external magnesium availability. Mol Microbiol **45:**423–438. http://dx.doi.org/10.1046/j.1365-2958.2002.03017.x.
44. **Hromockyj AE, Tucker SC, Maurelli AT.** 1992. Temperature regulation of *Shigella* virulence: identification of the repressor gene *virR*, an analogue of *hns*, and partial complementation by tyrosyl transfer RNA (tRNA1(Tyr)). Mol Microbiol **6:**2113–2124. http://dx.doi.org/10.1111/j.1365-2958.1992.tb01385.x.
45. **Maurelli AT, Blackmon B, Curtiss R, III.** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect Immun **43:**195–201.
46. **Porter ME, Dorman CJ.** 1994. A role for H-NS in the thermo-osmotic regulation of virulence gene expression in *Shigella flexneri*. J Bacteriol **176:**4187–4191.
47. **Porter ME, Dorman CJ.** 1997. Differential regulation of the plasmid-encoded genes in the *Shigella flexneri* virulence regulon. Mol Gen Genet **256:**93–103. http://dx.doi.org/10.1007/s004380050550.
48. **Sankaran K, Ramachandran V, Subrahmanyam YV, Rajarathnam S, Elango S, Roy RK.** 1989. Congo red-mediated regulation of levels of *Shigella flexneri* 2a membrane proteins. Infect Immun **57:**2364–2371.
49. **Maurelli AT, Blackmon B, Curtiss R, III.** 1984. Loss of pigmentation in *Shigella flexneri* 2a is correlated with loss of virulence and virulence-associated plasmid. Infect Immun **43:**397–401.
50. **Bahrani FK, Sansonetti PJ, Parsot C.** 1997. Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation. Infect Immun **65:**4005–4010.
51. **Adler B, Sasakawa C, Tobe T, Makino S, Komatsu K, Yoshikawa M.** 1989. A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. Mol Microbiol **3:**627–635. http://dx.doi.org/10.1111/j.1365-2958.1989.tb00210.x.
52. **Beloin C, Dorman CJ.** 2003. An extended role for the nucleoid structuring protein H-NS in the virulence gene regulatory cascade of *Shigella flexneri*. Mol Microbiol **47:**825–838. http://dx.doi.org/10.1046/j.1365-2958.2003.03347.x.
53. **Spory A, Bosserhoff A, von Rhein C, Goebel W, Ludwig A.** 2002. Differential regulation of multiple proteins of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium by the transcriptional regulator SlyA. J Bacteriol **184:**3549–3559. http://dx.doi.org/10.1128/JB.184.13.3549-3559.2002.
54. **Foster JW, Hall HK.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J Bacteriol **172:**771–778.
55. **Gorden J, Small PL.** 1993. Acid resistance in enteric bacteria. Infect Immun **61:**364–367.
56. **Hong W, Wu YE, Fu X, Chang Z.** 2012. Chaperone-dependent mechanisms for acid resistance in enteric bacteria. Trends Microbiol **20:**328–335. http://dx.doi.org/10.1016/j.tim.2012.03.001.
57. **Kaoukab-Raji A, Biskri L, Bernardini ML, Allaoui A.** 2012. Characterization of SfPgdA, a *Shigella flexneri* peptidoglycan deacetylase required for bacterial persistence within polymorphonuclear neutrophils. Microbes Infect **14:**619–627. http://dx.doi.org/10.1016/j.micinf.2012.01.009.
58. **Zwir I, Shin D, Kato A, Nishino K, Latifi T, Solomon F, Hare JM, Huang H, Groisman EA.** 2005. Dissecting the PhoP regulatory network of *Escherichia coli* and *Salmonella enterica*. Proc Natl Acad Sci U S A **102:**2862–2867. http://dx.doi.org/10.1073/pnas.0408238102.
59. **Monsieurs P, De Keersmaecker S, Navarre WW, Bader MW, De Smet F, McClelland M, Fang FC, De Moor B, Vanderleyden J, Marchal K.** 2005. Comparison of the PhoPQ regulon in *Escherichia coli* and *Salmonella typhimurium*. J Mol Evol **60:**462–474. http://dx.doi.org/10.1007/s00239-004-0212-7.
60. **Schuch R, Maurelli AT.** 1997. Virulence plasmid instability in *Shigella flexneri* 2a is induced by virulence gene expression. Infect Immun **65:**3686–3692.
61. **DuPont HL, Levine MM, Hornick RB, Formal SB.** 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J Infect Dis **159:**1126–1128. http://dx.doi.org/10.1093/infdis/159.6.1126.
62. **Pogliano JA, Beckwith J.** 1994. SecD and SecF facilitate protein export in *Escherichia coli*. EMBO J **13:**554–561.
63. **Formal SB, Dammin GJ, Labrec EH, Schneider H.** 1958. Experimental *Shigella* infections: characteristics of a fatal infection produced in guinea pigs. J Bacteriol **75:**604–610.
64. **Guzman LM, Belin D, Carson MJ, Beckwith J.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol **177:**4121–4130.
65. **Schuch R, Sandlin RC, Maurelli AT.** 1999. A system for identifying postinvasion functions of invasion genes: requirements for the Mxi-Spa type III secretion pathway of *Shigella flexneri* in intercellular dissemination. Mol Microbiol **34:**675–689. http://dx.doi.org/10.1046/j.1365-2958.1999.01627.x.
66. **Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.