

H-NS Represses *Salmonella enterica* Serovar Typhimurium *dsbA* Expression during Exponential Growth

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Disulfide bond formation catalyzed by disulfide oxidoreductases occurs in the periplasm and plays a major role in the proper folding and integrity of many proteins. In this study, we were interested in elucidating factors that influence the regulation of *dsbA*, a gene coding for the primary disulfide oxidoreductase found in *Salmonella enterica* serovar Typhimurium. Strains with mutations created by transposon mutagenesis were screened for strains with altered expression of *dsbA*. A mutant (NLM2173) was found where maximal expression of a *dsbA::lacZ* transcriptional fusion occurred in the exponential growth phase in contrast to that observed in the wild type where maximal expression occurs in stationary phase. Sequence analysis of NLM2173 demonstrated that the transposon had inserted upstream of the gene encoding H-NS. Western immunoblot analysis using H-NS and StpA antibodies showed decreased amounts of H-NS protein in NLM2173, and this reduction in H-NS correlated with an increase of StpA protein. Northern blot analysis with a *dsbA*-specific probe showed an increase in *dsbA* transcript during exponential phase of growth. Direct binding of H-NS to the *dsbA* promoter region was verified using purified H-NS in electrophoretic mobility shift assays. Thus, a reduction in H-NS protein is correlated with a derepression of *dsbA* in NLM2173, suggesting that H-NS normally plays a role in suppressing the expression of *dsbA* during exponential phase growth.

Salmonella enterica serovar Typhimurium is a major cause of gastroenteritis or food poisoning in humans (34). This gram-negative, facultative, intracellular pathogen has evolved a number of distinct strategies to survive and propagate in a wide variety of cell types in the host. Many of these strategies involve proteins that are exported from the cytoplasm to either the periplasm or outer membrane or secreted out of the cell (15). Some proteins are transported or assembled by means of specialized secretory systems, but many of these proteins pass through the periplasm, where they undergo some degree of folding into their native conformation. Disulfide bonds usually contribute to the stabilization of a folded protein conformation (2, 35). In gram-negative bacteria, disulfide bond formation is mediated by the foldase DsbA, which is part of a disulfide oxidoreductase system that includes other Dsb proteins, such as DsbB, DsbC, and DsbD (2, 24, 35). DsbA, a soluble periplasmic disulfide oxidoreductase, was first discovered in *Escherichia coli* (4) and has also been characterized from a number of gram-negative bacteria, including *S. enterica* serovar Typhimurium (49). Disulfide bonding is an essential step for the proper folding and hence, function, of a number of disulfide bond-containing proteins that are bacterial virulence factors, such as exotoxins, fimbriae, and adhesins (52). Although DsbA is not essential for growth under laboratory conditions, lack of disulfide oxidoreductase activity in serovar Typhimurium renders cells nonmotile and slows growth in defined minimal medium (49). Interestingly, in contrast to the obser-

ervations made in *E. coli* (6), DsbA is growth phase regulated in *S. enterica* serovar Typhimurium, with expression levels increasing during late exponential phase of growth and remaining elevated for at least 72 h in liquid culture (16). This stationary-phase regulation is not dependent upon RpoS (16), a common stationary-phase sigma factor (27, 36) or SlyA, a serovar Typhimurium stationary-phase transcriptional regulator (8).

This study details the investigation of a new facet of DsbA regulation involving the global regulator H-NS. By characterizing mutants that were derepressed for expression of *dsbA* from a plasmid-encoded *dsbA::lacZ* construct in *S. enterica* serovar Typhimurium during exponential phase growth, it was determined that H-NS was involved in the growth phase-dependent regulation of *dsbA*. H-NS is a major protein of the bacterial nucleoid and is involved in the regulation of both housekeeping and virulence genes in *E. coli* (10, 21). H-NS is a small, abundant protein that has affinity for all types of nucleic acids but binds preferentially to curved DNA substrates (37, 47). A number of *hns* mutant alleles have been shown to cause slow growth, reduce motility, and confer mucoid appearance on the mutant strain (5, 19). H-NS has been shown to negatively or positively regulate more than 200 genes in *E. coli* (21). Many of the target genes that are affected by H-NS are also regulated by other global transcription factors, such as LRP, VirF, CfaD, RpoS, and the DNA-binding protein FIS (1, 41). Hence, the effect of H-NS on many target genes is not straightforward. In this study, we demonstrate that H-NS binds to the *dsbA* promoter region and that a reduction in the amount of H-NS protein derepresses *dsbA* expression early in the growth cycle, suggesting that H-NS normally represses *dsbA* until late log or early stationary phase.

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic	Reference or source
Bacterial strains		
CH1794	<i>S. enterica</i> serovar Typhimurium LT2 <i>hns-106::Tn10Δ16Δ17</i>	Hinton et al. (19)
SL1344	<i>S. enterica</i> serovar Typhimurium wild-type strain <i>his rpsL</i>	Wray and Sojka (51a)
NLM331	SL1344 <i>dsbA::kan</i> containing pMEG2	This study
NLM2160	SL1344 containing pMEG2	Goecke et al. (16)
NLM2167	SL1344 <i>dsbA::kan</i>	Turcot et al. (49)
NLM2173 ^a	NLM2160 <i>hns-112::Tn10d</i> (T-POPII) containing pMEG2	This study
NLM2174	NLM2173 suppressor mutant containing pMEG2	This study
NLM2190	NLM331 <i>hns-112::Tn10d</i> (T-POPII) containing pMEG2	This study
SA4105	LT2 <i>osmZ::Tn10</i>	Salmonella Genetic Stock Centre
NLM2275	NLM2160 <i>osmZ::Tn10</i> containing pMEG2	This study
Plasmids		
pMP190	15-kb transcriptional fusion vector containing a promoterless <i>lacZ</i> gene	Spaink et al. (46a)
pMEG2	pMP190 with a 258-bp <i>XhoI/BglIII</i> fragment from immediately upstream of the <i>dsbA</i> translational start site cloned into the multiple cloning site	Goecke et al. (16)
pBluescript	2.9-kb pBR322 vector derivative, Amp ^r	Stratagene

^a The *hns-112::Tn10d*(T-POPII) mutation was originally designated *zde-5A1r::Tn10d*(T-POPII).

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. In general, bacteria were grown overnight at 30°C in Luria-Bertani (LB) medium (39) with the appropriate antibiotic selection. When required, antibiotics were used at the following concentrations: chloramphenicol (30 µg ml⁻¹), tetracycline (10 µg ml⁻¹), and ampicillin (100 µg ml⁻¹). When screening for blue or white colonies, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a concentration of 40 µg ml⁻¹.

P22 transduction. P22 transduction was performed by the method of Maloy (32). An aliquot of the lysate containing a pool of random *Tn10d*(T-POPII) insertions in *S. enterica* serovar Typhimurium LT2 was kindly provided by John Roth (38). *Tn10d*(T-POPII) is a *Tn10* derivative that has been modified such that its insertion between any gene or operon and its promoter causes the expression of the gene or downstream gene to become tetracycline dependent. Strain NLM2275 was created by transducing a *hns* null allele (*osmZ*) from strain SA4105 into a SL1344 background.

Motility assays. *Salmonella* strains were grown overnight at 30°C with appropriate antibiotics. The following morning, all the strains were standardized to an A_{600} of 0.04. A flat-ended sterile toothpick was dipped into standardized bacterial culture and stabbed into semisolid 0.3% agar LB plates (19). The plates were incubated for up to 16 h at 30°C, and the swarming behavior of each strain was measured as an indicator of motility.

β-Galactosidase assays. Transcription of plasmid-borne *dsbA-lacZ* fusions were monitored by β-galactosidase assays of cells cultured to mid-exponential phase and stationary phase by the method of Miller (33). Assays were performed in triplicate, and experiments were done at least three times.

RNA protocols. Total RNA was extracted from 3-ml samples of cells at the appropriate growth phase by the Trizol method (Gibco BRL). Diethyl pyrocarbonate-treated water or formamide was used to resuspend the RNA pellets. The concentrations and purity of the RNA samples were determined spectrophotometrically and by visual inspection of formaldehyde-agarose gels (see below).

For Northern blotting, samples of RNA (30 µg) were denatured at 65°C, loaded onto 1.5% formaldehyde-agarose gels, electrophoresed within buffer containing 20 mM MOPS [3(*N*-morpholino) propanesulfonic acid], 5 mM sodium acetate, and 1 mM EDTA at 80 V for 2 to 3 h, and transferred to Hybond-N nylon membranes (Amersham). The membranes were washed, denatured, neutralized, air dried, and cross-linked following established protocols (40). The templates used for the DNA probe were a PCR fragment amplified from the sequence of the *dsbA* gene using NLM88 (5'-CTGGCGAACCCAGGTACT G-3') and NLM78 (5'-CGCATCAACGAACACTTTACGG-3') or an amplicon specific for *hns* using primers NM93 (5'-ATAAGCTCTTTTGTGCGGTG-3') and NM94 (5'-TATTTTTTTTCGCGCCTAAATG-3'). The DNA fragment was labeled with digoxigenin, and prehybridization and hybridization were performed as recommended by the manufacturer (Amersham). Chemiluminescence detection as described by the Genius guide was used for probe detection.

For reverse transcriptase PCR (RT-PCR), RNA isolated from *S. enterica* serovar Typhimurium strains at various phases of growth was subjected to DNase treatment (RQ1 RNase-free DNase; Promega) and subsequent purification using RNeasy columns (RNeasy Mini kit; Qiagen). Reverse transcription reactions (Retroscrip kit; Ambion) using 100 pmol of primer NM112 *hns* primer (5'-GC AGTTTACGAGTGCCTTCTCC-3') were performed on approximately 3 µg of purified RNA (Trizol method). Reverse transcription negative-control reactions were performed simultaneously where water was added instead of the RT enzyme. PCR amplification was performed using forward NM111 (5'-TAGCG ACAGACGGTGAGTATCC-3') and reverse NM112 (5'-GCAGTTTACGAGT GCGTTCTTCC-3') *hns*-specific primers. PCRs (50 µl) were performed using 2.5 U of *Taq* DNA polymerase (Gibco BRL), 5 µl of reverse transcription reaction mixture sample as the template, 50 pmol of each PCR primer pair, 1× PCR buffer, 0.2 mM (each) deoxynucleoside triphosphate, and 1.5 mM MgCl₂. Template cDNA was denatured for 2 min at 94°C before *Taq* DNA polymerase was added. Twenty cycles of PCR were performed, with 1 cycle consisting of denaturation (45 s at 4°C), annealing (30 s at 60°C), and extension (1 min at 72°C). The final extension step was 7 min at 72°C. To aid in qualitative analysis, we normalized the RT-PCR product to an established endogenous internal control (*tsf* encoding the elongation factor EF-Tsf) (20). After the PCR, 5 µl of PCR product was visualized by agarose gel electrophoresis.

Gel electrophoresis and Western blotting. Proteins were separated by the method of Laemmli (26) using a sodium dodecyl sulfate-12% polyacrylamide gel. Cell cultures were centrifuged at a specific optical density, and the cell pellet was resuspended in loading buffer and boiled before loading. The amount of protein in each whole-cell lysate was determined, and equal amounts of protein (2 × 10⁷ cells) were loaded in each lane. The expression of StpA and H-NS proteins in *S. enterica* serovar Typhimurium was determined using anti-StpA polyclonal antibody (S100) that does not cross-react with H-NS and anti-H-NS monoclonal antibody (H113) that does not cross-react with StpA (45).

Electrophoretic mobility shift assay. Purified H-NS protein from *S. enterica* serovar Typhimurium was kindly provided by John Ladbury (Department of Biochemistry and Molecular Biology, University College London). The band shift reaction contained various concentrations of H-NS protein in the picomolar range and 10 ng of radiolabeled probe DNA in binding buffer (10 mM Tris-HCl [pH 7.5], 15 mM KCl, 0.1 mM EDTA, 2 mM spermidine, 15% glycerol) and was performed as described previously (23). The template used for the DNA probe was a PCR fragment amplified from the promoter region of the *dsbA* gene using primer pair NLM22 (5'-ACAAGATCTATTAATACATTGGCGTT-3') and NLM24 (5'-CCCTCGAGAAGCTTATCAAGAAGTT-3') and primer pair NM111 (5'-TAGCGACAGACGGTGAGTATCC-3') and NM112 (5'-GCAGT TTACGAGTGCCTTCTCC-3') for the promoter region of *hns*. The reaction mixture was incubated at room temperature for 30 min, and the samples were loaded onto a 5% polyacrylamide gel in Tris-acetate-EDTA and electrophoresed at 35 mA for 2 h. After electrophoresis, the gel was dried, and radiolabeled DNA was detected by autoradiography.

RESULTS

Transposon mutagenesis strategy for the isolation of *dsbA* regulatory mutants. A pool of random Tn10d(T-POPII) (38) insertions from *S. enterica* serovar Typhimurium LT2 was obtained as a P22 phage lysate and used to transduce *S. enterica* serovar Typhimurium SL1344 carrying a *dsbA::lacZ* transcriptional fusion on a low-copy-number plasmid, pMEG2 (strain NLM2160). This *dsbA::lacZ* fusion is normally regulated by growth phase, with maximal induction of expression occurring upon entry into the stationary phase of growth (16). The pool of mutants resulting from T-POPII insertion mutations in strain NLM2160 were screened for those altered in *dsbA* regulation by monitoring β -galactosidase activity of isolated colonies using X-Gal. White colonies were chosen, as these colonies were considered potential regulatory mutants. Of several white colonies isolated, the phenotypes of three of these colonies was caused by a single transposon insertion, as confirmed by 100% linkage of the *dsbA::lacZ* phenotype with the tetracycline resistance marker upon transduction into a fresh strain of SL1344 (NLM2160). Two of these insertion mutants contained the T-POPII insertion in the same location, and this mutant was designated NLM2173, while the third remains to be characterized. The mutant phenotype of NLM2173 was somewhat unstable at temperatures higher than 30°C where secondary mutations led to renewed expression of β -galactosidase. These suppressor mutants were visible as blue sectors in the colonies on agar plates containing X-Gal. This suppressor phenotype, as exemplified by strain NLM2174, was able to grow in the presence of tetracycline, confirming the maintenance of the transposon. The suppressor phenotype was not, however, cotransduced with the tetracycline resistance marker, confirming that the secondary mutations were not closely linked to the transposon insertion site. The ratio of the appearance of suppressor mutants to the original mutant was observed to be much lower at 30°C (0.55%) than at 37°C (24%).

On solid LB agar plates, the mutant NLM2173 produced colonies that were generally smaller in size than strain SL1344 or the suppressor mutant, NLM2174. When grown in LB broth at 30°C, the growth rate of strain NLM2173 was lower than that of the parental strain (Fig. 1A). In addition to the difference in the growth rate of strain NLM2173, a decrease in motility was also observed. The parental strain NLM2160 was fully motile (diameter of the motility zone, 5.0 ± 0.4 cm), whereas strain NLM2173 had reduced motility (diameter of the motility zone, 2.8 ± 0.2 cm).

Expression of *dsbA::lacZ* transcriptional fusion in *S. enterica* serovar Typhimurium strains. In order to measure the effects of the transposon insertion on *dsbA* promoter activity, the β -galactosidase activity from strain NLM2173 containing the *dsbA::lacZ* transcriptional fusion was measured throughout the growth cycle (Fig. 1B). As expected, maximal expression of the *dsbA::lacZ* fusion occurred at the onset of stationary phase in wild-type strain NLM2160. In contrast, strain NLM2173, in either the absence or presence of tetracycline, showed a shift in the activation of the *dsbA* promoter to earlier in the growth cycle, from stationary phase to log phase. If the Tn10d(T-POPII) transposon were to disrupt a promoter, then the expression of any downstream gene could become tetracycline

dependent (38). This was not the case, however, for NLM2173. In addition, stationary-phase levels of *dsbA* promoter activity in NLM2173 are lower than in the wild type. The *dsbA::lacZ* expression was also assayed in the suppressor strain NLM2174, where no shift in the induction of *dsbA::lacZ* expression was observed and the stationary-phase β -galactosidase levels were the same as those of the wild type (Fig. 1B). When the Tn10d(T-POPII) interrupted locus was transduced into a *dsbA* null strain, the overall level of *dsbA* promoter induction was even higher than in the wild type but still occurred prior to the onset of stationary-phase growth (Fig. 1C). Previous studies have shown that the *dsbA::lacZ* activity from pMEG2 is higher in a *dsbA* null background than in a wild-type background (16). These observations led to the hypothesis that a feedback loop exists for the expression of DsbA in *S. enterica* serovar Typhimurium, whereby the absence of DsbA activity signals the cell to produce more DsbA (16), and this mechanism of autoregulation appears to be independent of the effect generated by the T-POPII insertion.

Localization and identification of the site of transposon insertion. Genomic DNA from strain NLM2173 was digested with *SacI*, *SalI*, and *HindIII*, shotgun cloned into pBluescript, and selected by screening for clones that conferred tetracycline resistance. Using outward facing primers specific to the Tn10d(T-POPII) transposon, the regions flanking the transposon were sequenced and localized to a 3,629-bp contig (B-STM1107) from the *S. enterica* serovar Typhimurium sequencing project. The transposon insertion occurred 580 bp upstream of the *hns* coding region and 116 bp upstream of the *tdk* gene on the opposite strand encoding a thymidine kinase. Two other open reading frames could be recognized on this genomic DNA fragment; downstream of *hns*, a putative *galU* gene was detected, and upstream of *hns*, a putative *adhE* gene was also found (Fig. 2). Mutations at the *hns* locus are highly pleiotropic (5, 19, 53). In general, *hns* mutant strains grow more slowly than wild-type strains, show reduced motility, and are mucoid in nature (5). These phenotypic characteristics of an *hns* mutant were also shared by the mutant strain NLM2173, consistent with *hns* being the locus that affected *dsbA* regulation.

It was not immediately apparent how the T-POPII insertion 580 bp upstream of the *hns* coding region, produced an *hns* phenotype. However, Hinton et al. (19) and Hulton et al. (22) have demonstrated that *S. enterica* serovar Typhimurium strain CH1794, which contains a Tn10 insertion 377 bp upstream of the translational initiation codon of *hns*, produced reduced levels of H-NS protein than other *hns* mutants and the wild type. Unlike mutations within the *hns* structural gene, this *hns-106::Tn10d* insertion caused only a low level of derepression of the *proU* locus and had no detected effect on DNA supercoiling (19). This *hns* mutant strain was also included in this study for comparison.

***hns* mutations differentially affect the levels of *dsbA* mRNA at mid-logarithmic growth.** To examine the effects of *hns* mutations on *dsbA* transcription, Northern blot analysis was performed on total RNA extracted from several strains using a probe complementary to the transcribed *dsbA* gene. Goecke et al. (16) previously showed that two transcripts were consistently detected for *dsbA* and that the amount of these two transcripts varied with growth conditions. In the present study,

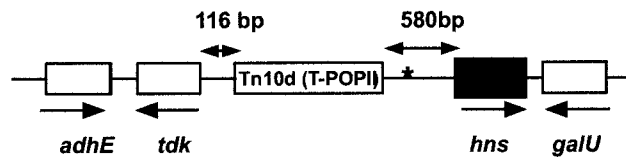
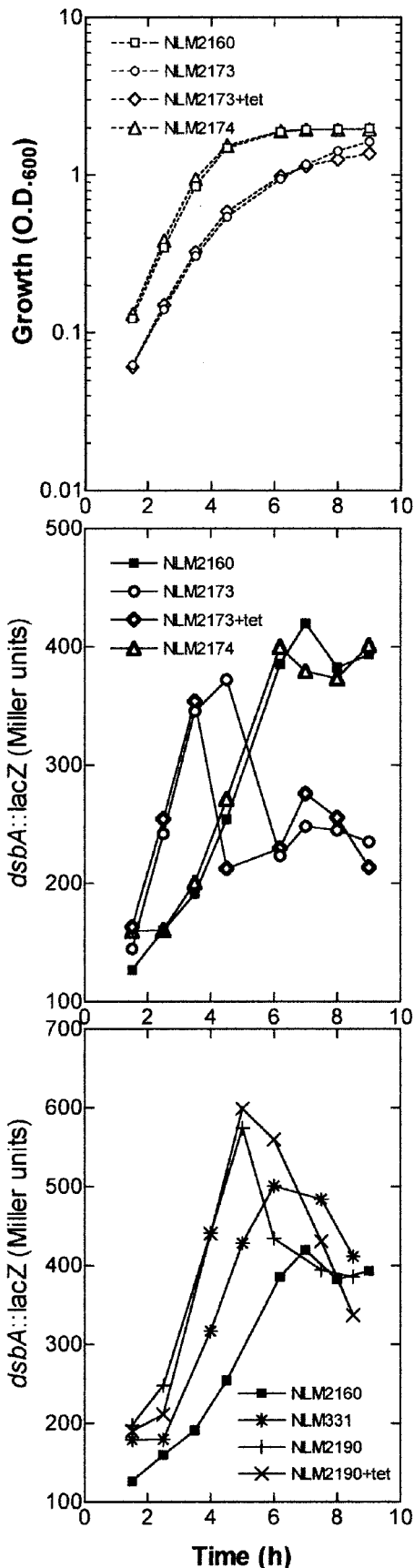


FIG. 2. Schematic diagram illustrating the position of the transposon insertion in *S. enterica* serovar Typhimurium SL1344. Sequencing analysis of strain NLM2173 demonstrated that the 2.5-kb Tn10d(T-POPII) transposon had inserted 580 bp upstream of the *hns* gene (encoding H-NS) and 116 bp upstream of the *tdk* gene (encoding a thymidine kinase). The positions of *adhE* (encoding an alcohol dehydrogenase) and *galU* (encoding glucose-1-phosphate uridylyltransferase) are also shown. The position of the Tn10 insertion 377 bp upstream of the translational initiation codon of *hns* in strain CH1794 is shown by an asterisk.

this transcription pattern was observed in the wild-type strain NLM2160 and the suppressor mutant NLM2174. However, there was a substantial increase in the amount of the shorter *dsbA*-specific transcripts compared to the larger transcript in strains NLM2173 and CH1794 relative to the wild type or the suppressor strain (Fig. 3). This increase in the amount of *dsbA* transcript during log phase growth correlated with the increase in expression of the *dsbA*::*lacZ* fusion, suggesting that *dsbA* promoter activity is elevated in strain NLM2173.

Comparison of expression from the *dsbA* promoter in *hns* mutant strains. The effect on *dsbA* transcription of the Tn10 insertion 377 bp upstream of the translational initiation codon of *hns* (strain CH1794) was compared to that of the T-POPII insertion mutant NLM2173 under log phase growth conditions (Fig. 4). Both NLM2173 and CH1794 showed increased *dsbA* promoter activity at mid- and late log phase, suggesting that although the transposons are inserted 200 bases apart from each other, their effect on *hns* is similar. An *hns* null strain (NLM2275) was also tested and had even higher levels of β -galactosidase activity than NLM2173. NLM2174 was also included in this comparison, and although the *dsbA* promoter activity is closer to the wild type in this suppressor strain, it is not identical to that of the wild type.

H-NS binds to the *dsbA* promoter region. In order to determine whether H-NS could interact directly with the *dsbA* promoter region, band shift assays were performed. As H-NS had previously been shown to bind to its own promoter with high affinity (11, 51), the *hns* promoter region was used as a control for binding specificity. The results show that the *dsbA* promoter fragment begins to shift at an H-NS concentration equivalent to that required to demonstrate binding to the *hns*

FIG. 1. Comparison of growth and the levels of transcription initiated from the *dsbA* promoter in the construct pMEG2 in the wild type (NLM2160), Tn10d(T-POPII) mutant (NLM2173), and suppressor strain (NLM2174). (A) Growth of NLM2173, NLM2174, and NLM2160 at 30°C. O.D.₆₀₀, optical density at 600 nm. (B) Expression of the *dsbA*::*lacZ* fusion in strains NLM2160, NLM2174, and NLM2173 (in the presence and absence of tetracycline [tet]). (C) Expression of the *dsbA*::*lacZ* fusion in a *dsbA* null *S. enterica* serovar Typhimurium SL1344 strain (NLM331) containing the transposon-interrupted locus (NLM2190) (in the presence and absence of tetracycline). The results are representative of three independent trials.

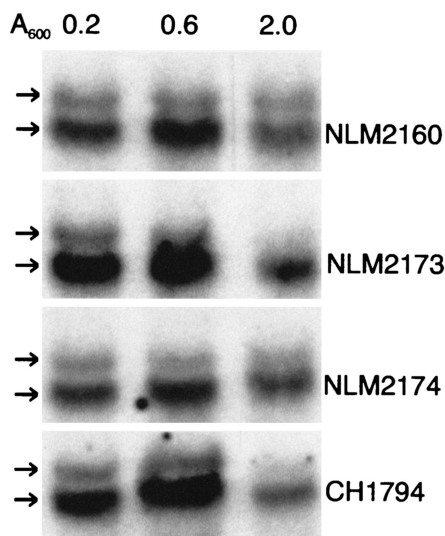


FIG. 3. Northern blot analysis of the transcription of *dsbA* in several strains at different time points. Using a *dsbA* DNA probe, two transcripts of approximately 700 and 800 nucleotides (arrows) were detected in all the strains. The amount of the shorter *dsbA* transcript in strains NLM2173 and CH1794 at A_{600} s of 0.2 and 0.6 is higher than in strains NLM2160 (wild type) and NLM2174. Equal amounts of total RNA were loaded in each lane. Results are representative of three independent trials.

promoter (Fig. 5). This binding is specific, as demonstrated by the fact that a mobility shift does not occur in the 112-bp digested *hns* fragment but does occur in the 267-bp fragment previously shown to contain the H-NS binding domain (Fig. 5).

H-NS expression is altered in strains NLM2173 and CH1794. As the results indicated that H-NS was affecting the

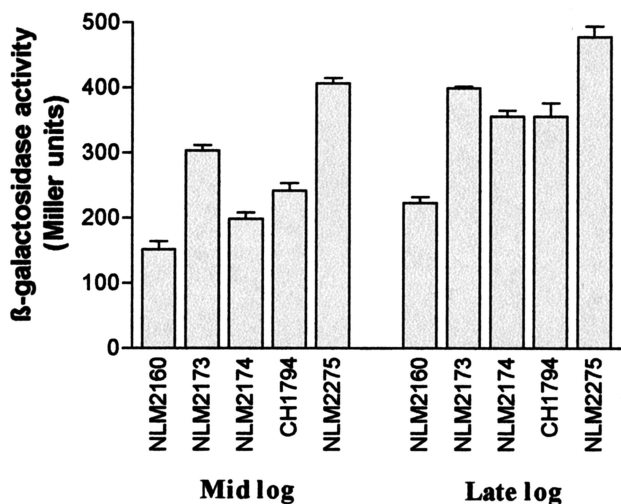


FIG. 4. Expression of the *dsbA::lacZ* fusion was measured in strains NLM2160, NLM2173, NLM2174, CH1794, and NLM2275 in mid- and late log growth phase. Cultures of the various strains were grown at 30°C, and samples were taken at A_{600} s of 0.3 and 0.6. β -Galactosidase activity was plotted for each strain. Data are means \pm standard errors of the means (error bars) of three independent experiments, each with duplicate samples.

dsbA promoter activity, Western immunoblotting using a H-NS-specific monoclonal antibody was undertaken to monitor steady-state H-NS levels. As previous work had demonstrated that both H-NS and the homologous protein, StpA, are implicated in a global regulatory system and that the *stpA* gene is derepressed in *hns* mutants of *E. coli* (12, 44, 4), StpA protein levels were also monitored using an StpA polyclonal antibody. If a reduction in H-NS protein was occurring in NLM2173 and CH1794, increased expression of StpA would be expected. The amount of H-NS protein produced was lower in strain NLM2173 than in wild-type strain NLM2160, and the reverse pattern was observed when the same samples were probed with the StpA-specific antibody (Fig. 6). Thus, H-NS protein production was decreased in the transposon mutant, and this reduction in H-NS protein was associated with an increase in expression of StpA. A similar decrease in H-NS and increase in StpA levels were also observed in CH1794 (data not shown).

***hns* transcription and growth phase.** Although the connection between decreased H-NS protein and increased *dsbA* transcription was established, it was not clear how transposons inserted either 377 or 580 bases upstream of the *hns* coding region could cause a decrease in H-NS protein levels. Transcriptional analysis of *hns* was undertaken to assess the effects of these transposons on *hns* transcript abundance. RT-PCR was used to assess the amount of *hns* transcript produced at different time points during the growth cycle (Fig. 7). For each strain, an *hns*-specific transcript was detected at all growth phases tested. In the wild-type strain (NLM2160), different amounts of the RT-PCR product were produced in the different growth phases, with more abundant transcript being detected earlier in the growth cycle, as expected. However, the relative intensities of the RT-PCR product in strains NLM2173 and CH1794 were higher in mid-log phase than those of the wild-type strain, NLM2160, and the suppressor strain, NLM2174. These RT-PCR results were surprising, as they did not correlate with the data showing a reduction in the steady-state H-NS protein levels, but these results were confirmed when Northern blotting or multiplex RT-PCR (17) were used to assess the *hns* transcript (data not shown).

DISCUSSION

The growth phase-regulated expression of *dsbA* in *S. enterica* serovar Typhimurium has been shown to be independent of the stationary-phase sigma factor, RpoS, and the transcriptional activator, SlyA (16). This study was thus initiated to determine the factors that may be involved in influencing the levels of *dsbA* expression in the cell. Screening a T-POPII mutant library using a *dsbA::lacZ* transcriptional fusion led to the isolation of strain NLM2173 that exhibited alterations in its ability to regulate *dsbA*. Cloning and sequencing of the transposon-containing DNA fragments from strain NLM2173 revealed that the *zde-5A1r::Tn10d*(T-POPII) locus is around 38.4 min on the chromosome of *S. enterica* serovar Typhimurium, 116 bp upstream of the thymidine kinase gene (*tdk*) and 580 bp upstream of the *hns* gene. Several phenotypic characteristics of the mutant, such as increased mucoidy, decreased growth rate, and decreased motility, suggested that modification of expression of the *hns* locus was being affected. The *zde-5A1r::Tn10d* mutation was later designated *hns-112::Tn10d*(T-

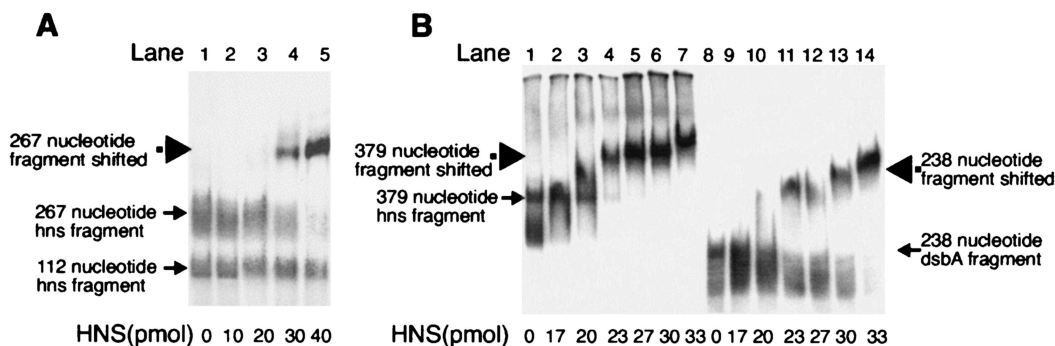


FIG. 5. Electrophoretic mobility shift assays showing an interaction between purified H-NS and the promoter regions of *hns* and *dsbA*. (A) All lanes contain a ³²P-labeled 379-bp amplicon covering the promoter region of *hns* that has been digested to give 112- and 267-bp fragments. The 112-bp fragment does not shift, while the 267-bp fragment, which contains the predicted H-NS binding region, does shift, in agreement with previously published results (11). (B) Lanes 1 to 7 lanes contain a ³²P-labeled 379-bp amplicon covering the promoter region of *hns*. Lanes 8 to 14 contain a ³²P-labeled 238-bp *dsbA* promoter amplicon and show that the *dsbA* fragment binds H-NS at concentrations as low as 20 pmol. Purified H-NS was added to each reaction mixture, as indicated at the bottom of the gel. These results are representative of several independent experiments.

POPII). NLM2173 was able to grow on plates containing thymidine kinase (25), confirming that the *tdk* locus was not affected by *hns-112::Tn10d* (data not shown). The motility of strain NLM2173 is about 56% that of the wild-type strain, suggesting that the transposon insertion does not completely eliminate the expression of H-NS but changes the level of H-NS expressed in the cell. Motility is reduced to different levels by several *hns* alleles, and it was previously shown in strain CH1794 that the insertion 377 bp upstream of the translation initiation codon of *hns* also resulted in a partial loss of motility (55% of the wild type), while a strain with an *hns* null allele lacks flagella (19). In *E. coli*, the presence of an *hns* mutation decreased the transcription of *flhD* and *fljA* genes required for the synthesis of flagella (7, 46), a rather rare example of H-NS acting as a positive regulator. DsbA is also involved in flagellar biosynthesis. Work done by Bardwell et al. (3) showed that DsbA is essential for flagellar assembly and function in *E. coli*, and Turcot et al. (49) demonstrated that an *S. enterica* serovar Typhimurium *dsbA* null strain was also immotile. Thus, the decrease in motility observed in the transposon mutant strains in the present study is unlikely to be related to reduced expression of the wild-type *dsbA* gene, since the expression of *dsbA* is derepressed in NLM2173.

The phenomenon of spontaneous second-site mutations arising in *hns* mutants has been observed previously (5, 18, 30). In addition, Barth et al. (5) found that *hns* suppressor strains had lost the increased mucoidy characteristic for *hns* mutants and grew faster, exhibiting shorter doubling times, than the parental strains, as was seen with the present study. Barth et al. (5) found that some of their suppressor mutants carried alterations at the *rpoS* locus, raising the possibility that the suppressor mutation in strain NLM2174 was in the *rpoS* gene. Qualitative assays of catalase activity showed an increase in NLM2173 relative to the wild type, but catalase activity in NLM2174 was similar to NLM2173 (data not shown). This increase in catalase activity in the *hns* mutant probably resulted from increased *rpoS* transcription (5) and, because activity was unchanged in NLM2174, suggests that the suppressor mutation in NLM2174 is not located in the *rpoS* gene.

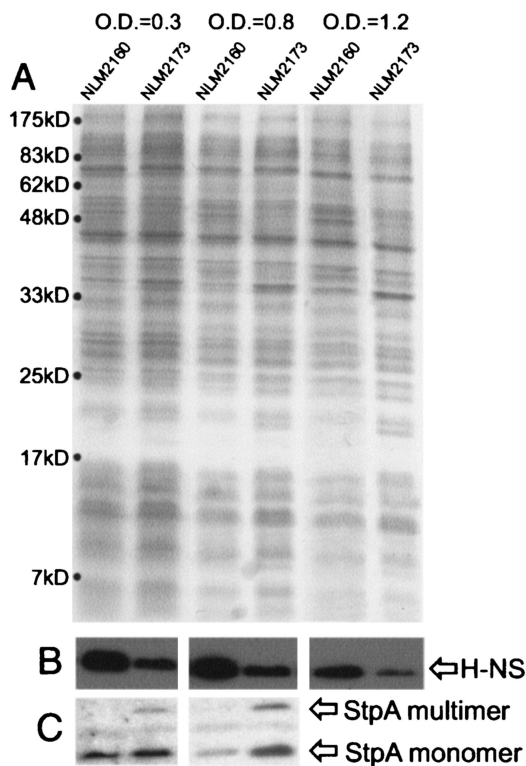


FIG. 6. Western immunoblot analysis of H-NS and StpA levels in strains NLM2160 and NLM2173. Cells were grown at 30°C in LB and harvested sequentially at optical densities (O.D.) at 600 nm of 0.3, 0.6, and 1.2. Whole-cell lysates of samples, normalized to total cell number, of each strain were separated on a sodium dodecyl sulfate–15% polyacrylamide gel and probed with anti-H-NS (H113) antibody and anti-StpA (S100) antibody. Panel A is the Coomassie blue-stained gel. There is no visible difference in the banding pattern at the 17-kDa marker where both H-NS and StpA are located. Panel B shows the immunoblot from the same samples as in panel A using anti-H-NS antibodies. Under these growth conditions, H-NS protein levels are higher in the wild type than in strain NLM2173 during log phase growth, and the levels in both strains decrease later in the growth cycle. Panel C shows the immunoblot from the same samples as in panel A using anti-StpA antibodies. There is slightly more StpA protein in NLM2173 than in the wild type, and there is also an StpA-specific band running at the size of a StpA dimer.

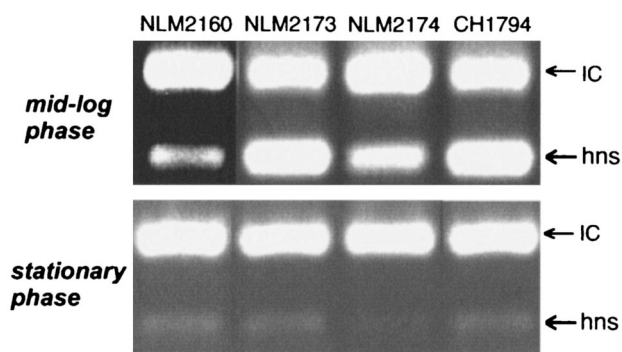


FIG. 7. RT-PCR was performed on RNA isolated from strains NLM2160, NLM2173, NLM2174, and CH1794 to detect *hns*-specific transcript at optical densities at 600 nm of 0.3 (mid-log phase) and 2 (stationary phase). The specific *hns* transcript is designated by an arrow and is approximately 378 bp in size. The 563-bp band corresponds to the amplified *tsf* gene that was used as the internal control (IC) in this experiment. These data are representative of three independent trials.

Figure 1B clearly shows that expression of the *dsbA::lacZ* transcriptional fusion in strain NLM2173 occurs earlier in the growth cycle, with a twofold derepression of *dsbA* expression in mid- and late log phase (Fig. 4). This twofold derepression of *dsbA* was also seen for the expression of the *dsbA::lacZ* transcriptional fusion in another *hns* mutant strain (CH1794) that also contains a Tn10 insertion upstream of the *hns* coding region, while analysis of the *hns* null mutant NLM2275 showed even higher levels of *dsbA* promoter activity (Fig. 4). Taken together, these results imply that the level of H-NS in the cell influences the transcription of *dsbA* in *S. enterica* serovar Typhimurium. H-NS is known to act as a transcriptional repressor by binding to DNA in the promoter region (51) and shows a preference for binding to intrinsically curved DNA (9). Sequence analysis of the region upstream of the *dsbA* translation start site revealed the presence of a region predicted to bend (data not shown). Band shift assays with purified H-NS demonstrated high-affinity binding to the *dsbA* promoter region, suggesting that normally *dsbA* expression is directly repressed by H-NS (Fig. 5). By surveying the literature, Atlung and Ingmer (1) determined that H-NS has a larger effect on target gene expression in scenarios where expression is not also mediated by positive transcription factors and noted that any repression by H-NS is virtually eliminated when positive transcription factors are artificially induced. We see an increase in *dsbA* promoter activity in NLM2173 during exponential phase of only two- to threefold, the magnitude of which could be influenced by a positive regulator; it also could be due to the fact that H-NS is not completely abolished.

H-NS is autoregulated (11, 12, 50), positively regulated at the transcriptional level (41), and posttranscriptionally regulated by DsrA RNA (28). Free and Dorman (12) found that the *hns* transcript is virtually absent in stationary-phase cells but is present at high levels within 1 h of subculturing a stationary-phase culture. Additionally, Dorman et al. (10) showed that the ratio of H-NS synthesis to DNA synthesis was constant, which could explain why *hns* expression is reduced in stationary phase when DNA synthesis slows. In the present study, steady-state levels of H-NS in the wild-type strain were

seen to be slightly higher in exponential phase growth than in stationary phase. The levels of H-NS were decreased in NLM2173 than in the wild type, correlating with the observed derepression of the *dsbA* promoter in exponential phase.

The steady-state levels of StpA were also examined. StpA is a paralogue of H-NS that shows 52% identity at the amino acid level and has a DNA-binding affinity that is comparable to that of H-NS (45). H-NS and StpA can act cooperatively to repress many H-NS-regulated genes (13, 14). Furthermore, it has been demonstrated that the expression of *stpA* is derepressed in an *hns* mutant strain (14, 44). This increase in StpA expression in an *hns* mutant strain appears to compensate for the lack of H-NS and allows repression of many H-NS-regulated genes (45), although not all H-NS-repressed loci can be regulated by StpA (10, 54). In this study, StpA levels were higher in strain NLM2173 than in the wild type (NLM2160), showing that the *hns-112::Tn10d* mutation caused a sufficient decrease in H-NS levels to exert a biologically relevant effect. However, the increase in StpA protein levels in NLM2173 did not allow StpA to substitute for H-NS in the repression of the *dsbA* promoter.

It was not clear how transposon insertions significantly upstream of the *hns* coding region caused a decrease in H-NS levels, especially since the transposons were inserted further from the promoter region than any previously described regulatory regions. Transcriptional autorepression occurs as a result of H-NS binding to extended regions of DNA 150 nucleotides upstream of its coding region (11, 48). In the present study, it was hypothesized that the T-POPII and Tn10 transposons somehow affect the *hns* promoter region, lowering the transcription of the *hns* locus. Using RT-PCR (Fig. 7), it was shown that the level of *hns* transcript is abundant early in the growth cycle and lower when the cells reach stationary phase in the wild-type strain. However, there was a marked increase in the amount of *hns* transcript in strains NLM2173 and CH1794 at mid-log phase compared to that of the wild type, suggesting that the transposon insertions enhanced *hns* transcription. Both Northern blotting and multiplex primer extension approaches to measuring the *hns* transcript abundance also showed increased transcription in NLM2173 and CH1794 than in the wild type (data not shown). The data clearly establish enhanced *hns* transcription, suggesting that the transposons have affected a previously uncharacterized regulatory element upstream of *hns*. The data also suggest that autoregulation of *hns* transcription has been disrupted, as the results are similar to that seen in an *hns* deletion strain where basal *hns* transcription levels are more than twofold higher than the level in the wild type (29).

With *hns* transcription increased, the observed decrease in H-NS protein levels still requires an explanation. It is hypothesized that DsrA, a small RNA, is involved in the repression of H-NS in these mutants. DsrA is an untranslated, regulatory RNA that is involved in the expression of RpoS (31, 43) and H-NS (29, 31, 42). It is thought that DsrA and *hns* mRNA interact and that this interaction enhances the turnover of *hns* mRNA, resulting in the production of less H-NS protein (28). In experiments performed by Lease et al. (29), DsrA expression decreased H-NS protein levels in a wild-type background and had no effect on the level of *hns* transcript. Lease et al. (29) also showed that StpA is still produced when DsrA is overexpressed, and it appears that the DsrA-mediated reduction in

H-NS actually leads to an increase in StpA levels (29). A direct connection between increased *hns* transcription and increased DsrA activity remains to be established in future experiments.

In this study, we have demonstrated that a reduction of H-NS protein correlates with a derepression of *dsbA* expression in log phase. Since the regulation of DsbA is growth phase dependent, the involvement of H-NS, a protein abundant in log phase, fits with the expression profile of this disulfide oxidoreductase. There must also be as yet unidentified positively regulating factors involved in *dsbA* transcription to account for the increase in stationary-phase expression. DsbA appears to facilitate protein folding in stationary phase rather than exponential growth phase where it is expected that protein secretion would require foldases in order to be rapid and efficient. Given the involvement of H-NS in the expression of genes related to cell survival under stressful growth conditions, DsbA expression may reflect the need for foldase activity in the context of environmental factors causing stress to the bacterial cell.

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