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Transcriptional Regulation of the *assT-dsbL-dsbI* Gene Cluster in *Salmonella enterica* Serovar Typhi IMSS-1 Depends on LeuO, H-NS, and Specific Growth Conditions

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The *assT* gene encodes an arylsulfate sulfotransferase, an enzyme that catalyzes sulfuryl transfer from phenolic sulfate to a phenolic acceptor. In *Salmonella enterica* serovar Typhi IMSS-1, the *assT* gene is located upstream of the *dsbL* and *dsbI* genes, which are involved in a disulfide bond formation required for its activation. The *assT-dsbL-dsbI* gene cluster forms an operon transcribed by a LeuO-dependent promoter, in rich medium A (MA). Interestingly, in the absence of cloned *leuO* and in a Δ*leuO* background, two transcription start sites were detected for *assT* and two for *dsbL-dsbI* in minimal medium. The H-NS nucleoid protein repressed the expression of the *assT-dsbL-dsbI* LeuO-dependent operon, as well as of the *assT* transcriptional units. Thus, the expression of the *assT-dsbL-dsbI* gene cluster depends on the global regulatory proteins LeuO and H-NS, as well as on specific growth conditions.

The *assT* gene encodes an arylsulfate sulfotransferase that is present in several organisms, including mammals and a wide spectrum of microbial genomes (2, 25, 55). Mammalian sulfotransferases are located in the cytosol and in the Golgi apparatus membrane; their activities have been detected in liver, brain, kidney, and intestinal epithelial cells (53, 55). These sulfotransferases use 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as sulfate donor and are involved in a wide variety of biological processes such as cell communication, growth, development, defense, and detoxification (6, 19, 43, 63, 65). Unlike mammals, bacterial sulfotransferases are periplasmic enzymes that require phenolic sulfate esters as donor substrates, and it has been suggested that the intestinal microflora uses this enzyme to detoxify phenolic compounds (31, 32, 34, 35).

In *Salmonella enterica* serovar Typhi, the *assT* gene is located upstream of *dsbL* and *dsbI*, a couple of genes encoding a reduction-oxidation (red-ox) pair of proteins involved in the disulfide bond formation required for AssT activation in the periplasm (25, 40, 44). The nomenclature was adopted from the orthologous genes previously described in uropathogenic *Escherichia coli* (UPEC) and *Salmonella enterica* serovar Typhimurium (25, 40). In contrast to the highly conserved *assT*, the genomic organization of the *assT-dsbL-dsbI* gene cluster is only conserved in *Enterobacter*, *Yersinia*, *Citrobacter*, and *Escherichia* strains, as well as within the *Salmonella* genus (5, 12, 25, 40, 41, 62).

The crystal structures for AssT and DsbL proteins have been solved. AssT forms a homodimer, whose Cys-418 and Cys-424 are involved in disulfide bond formation and its His-436 undergoes the transient sulfurylation necessary for the ping-pong reaction mechanism (44). DsbL is a periplasmic monomer, forming a disulfide bond between Cys-29 and Cys-32, which is oxidized by the transmembrane protein DsbI (25). DsbI contains four predicted transmembrane helixes with two cysteine pairs (Cys-55 and Cys-58; Cys-127 and Cys-153).

In vivo, *assT*, *dsbL*, and *dsbI* expression has been detected in macrophages infected with *Salmonella*; additionally, a transcriptome assay showed *assT* and *dsbL* expression in the urinary tract during UPEC infection (20, 60). Moreover, in *S.* Typhimurium *assT* and *dsbL* have been reported as virulence factors in Nramp1-resistant mice, and a mutation in *assT* results in attenuated virulence in *Edwardsiella tarda* in a fish model (38, 45). However, *assT* mutants in *S.* Typhimurium did not affect the pathogenesis in BALB/c mice or *assT* or *dsbL-dsbI* gene deletions during UPEC colonization in the mouse urinary tract infection model (40, 62).

The *assT-dsbL-dsbI* cluster has been suggested to form an operon in UPEC and in *Enterobacter amnigenus* (25, 37). However, in *S.* Typhimurium, *dsbL* expression can occur independently from *assT* (40). We show that in *S.* Typhi IMSS-1, the transcriptional regulation of the *assT-dsbL-dsbI* cluster depends on global regulatory proteins and specific growth conditions. A LeuO-dependent promoter is necessary for induction of the complete *assT-dsbL-dsbI* operon in rich medium, whereas transcriptional start sites were determined for *assT* and the *dsbL-dsbI* transcriptional units in the absence of cloned *leuO* and in a Δ*leuO* background, in N-minimal medium which is used for induction of *Salmonella* SPI-2 (16). In addition, we also report that the global regulatory protein H-NS represses transcription of the *assT-dsbL-dsbI* gene cluster, both in rich and in minimal medium.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used are listed in Table S1 in the supplemental material. *S.* Typhi IMSS-1 (52) and the *E. coli* strains were grown aerobically at 37°C in LB (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g/liter) for overnight cultures. *S.* Typhi was grown in liquid MA (nutrient broth, 7 g; yeast extract, 1 g; glycerol, 2 g; 3.75 g of K_2HPO_4 and 1.3 g of KH_2PO_4/liter) (33) or in N-minimal medium [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 100 mM Tris-HCl (pH 7.5), 10 μM $MgCl_2$, 0.5% glycerol, 0.1% Casamino acids] (16). Kanamycin (Km) at 15 μg/ml, tetracycline (Tc) at 12 μg/ml, and ampicillin (Ap) at 100 μg/ml were added when required.

DNA and RNA manipulations. Genomic DNA isolation was performed according to published protocols (54). Plasmid DNA was purified with the High-Pure plasmid isolation kit (Boehringer, Mannheim, Germany). Primers for PCR amplifications were provided by the oligonucleotide synthesis facility at our institute (see Table S2 in the supplemental material). Restriction enzymes, ligase, kinases, nucleotides and polymerases were obtained from New England Biolabs (Ipswich, MA) or Gibco-BRL. Sequencing was performed with an automatic Perkin-Elmer/Applied Biosystems 377-18 system. The one-step mutagenesis procedure described by Datsenko and Wanner (15) for bacterial chromosomal genes was used to generate gene deletions and replacements for antibiotic resistance markers.

For reverse transcription-PCR (RT-PCR), total RNA was isolated with Trizol from 1.5 ml of bacterial cells grown in 100 ml of MA medium to an optical density at 595 nm (OD_{595}) of 0.6. For the 5′RACE (5′ rapid amplification of cDNA ends) and primer extension experiments, bacterial cells were grown in 50 ml of N-minimal medium for 12 h. The RNA extraction kit (Qiagen) was used to obtain total RNA samples for 5′RACE experiments, as established in the protocol. For primer extension experiments, 50 ml of N-minimal medium cell culture were treated with RNA*later* (Fermentas) and used for RNA purification with acid-phenol extraction. The RNA concentration was determined by measuring the absorbance at 260 nm, and the integrity of RNA was verified by 1.5% agarose gel electrophoresis.

Construction of transcriptional reporter fusions. Oligonucleotides (see Table S2 in the supplemental material) were designed to amplify by PCR different regions of the *assT*, *dsbL*, and *dsbI* genes. PCR fragments were double digested with BamHI-KpnI or BamHI-MluI and ligated into pKK232-9 (Km), which contains the promoterless *cat* gene. Transcriptional fusions in pKK232-8 (Ap) were generated from pKK232-9 (Km) digested with PstI to eliminate Km gene resistance. Fusions were sequenced in order to verify the correct DNA sequence of the PCR fragments. Each plasmid was then electroporated into different *Salmonella* strains to evaluate its transcriptional activity.

Deletions and substitutions. In order to characterize the *assT* regulatory region, deletions and substitutions by overlap extension were performed according to the method of Sambrook, et al. (54). Two complementary oligonucleotides containing changes in the nucleotide sequence were used (see Table S2 in the supplemental material). The mutated PCR products were digested with the specific restriction enzymes, cloned into pKK232-9, and sequenced to verify the correct changes.

CAT assay. *S.* Typhi strains were grown in 100 ml of MA medium or 50 ml of N-minimal medium for 12 h; 50 μM IPTG (isopropyl-β-D-thiogalactopyranoside) was added when required. Then, 1.5-ml portions of bacterial cultures were collected by centrifugation and washed with 0.8 ml of TDTT buffer (50 mM Tris-HCl [pH 7.8], 30 μM D,L-dithiothreitol [DTT]). Bacterial cells collected from MA medium and N-minimal medium were resuspended in 0.6 and 0.3 ml of TDTT, respectively, and sonicated on ice for 10-s intervals with 10-s rest periods until the extract was clear. The homogenate was centrifuged, and the supernatant was used for total protein and CAT activity measurement. For CAT assays, 5 μl of each extract were added in duplicate to a 96-well enzyme-linked immunosorbent assay (ELISA) plate, followed by the addition of 0.2 ml of a reaction mixture that contained 1 mM DTNB, 0.1 mM acetyl-CoA and 0.1 mM chloramphenicol in 0.1 M Tris-HCl (pH 7.8). The absorbance at 412 nm was measured every 5 s for 5 min using a scanning autoreader and the microplate workstation Ceres 900. In these studies, 1 μmol of chloramphenicol acetylated/min corresponds to 1 chloramphenicol acetyltransferase (CAT) unit. The protein concentration was determined using BCA protein assay reagent (Pierce). Protein values and the mean rate of product formation by *cat* were used to determine the CAT specific activity as μmol/min/mg protein. The results presented in the figures are the means of three independent experiments.

RT-PCR. Five μg from total RNA was treated with DNase I (Fermentas), and 1.5 μg of RNA (DNA-free) was used for cDNA synthesis with random primers, as established in the Revert-Aid Minus M-MuLV RT protocol (Fermentas). PCR was performed from 1.5 μl of cDNA, and the reaction products were analyzed in a 1% agarose gel. As a positive control, a PCR was amplified for *rsmC*, the gene encoding for the 16S rRNA.

Primer extension analysis. A total of 40 μg of total RNA isolated for bacteria grown in N-minimal medium was denatured at 95°C for 3 min and then slowly cooled to 45°C to anneal with the [γ-^{32}P]ATP-labeled primers. DNA extensions were performed with reverse transcriptase at 42°C for 90 min. The extended products were purified by ethanol precipitation and analyzed by electrophoresis in 8% polyacrylamide–8 M urea gels alongside sequencing ladders. Sequencing ladders were generated from plasmids that contain the entire regulatory region of *assT* or *dsbL*.

5′RACE. Total RNA was treated with DNase I and a RiboMinus transcriptome isolation kit (Invitrogen, Carlsbad, CA) to eliminate DNA and rRNA, respectively. The 5′-monophosphate RNA transcripts were removed by exonuclease treatment with Terminator 5′-phosphate-dependent exonuclease (Epicentre, Madison, WI). The enriched 5′-triphosphate end mRNAs were treated with tobacco acid pyrophosphatase (TAP; Epicentre) to generate 5′-monophosphate ends before ligation to a 5′ RNA adapter (5′-GUUCAGAGUUCUACAGUCCGACGAUC; Illumina, Inc., San Diego, CA) with T4 RNA ligase 1 (ssRNA ligase; New England Biolabs) as described previously (23, 56). A modified 5′RACE methodology (46) was used for the construction of cDNA libraries. A random primer, 5′-CAAGCAGAAGACGGCATACGANNNNNN (adapter 3′), was used for the generation of the cDNA. We performed PCR in order to enrich the yield of the cDNA, using small RNA sequencing primer and small RNA PCR primer 2, which are complementary to the random primer and the 5′ RNA adapter, respectively.

In addition, a second methodology was used to obtain the 5′ ends. Total RNA was used as a template to generate a cDNA library using an oligonucleotide attached to a hexanucleotide random sequence tag at its 3′ end (5′-GCCTTGCCAGCCCGCTCAGNNNNNN). Using terminal deoxynucleotidyltransferase (Fermentas), an adenine polynucleotide tag was incorporated into the 3′ end of the cDNA. A PCR was performed to enrich the cDNA library by using the oligonucleotides B1 (5′-GCCTTGCCAGCCCGCTCAG) and poly(T), which are complementary to the random primer and the 5′ RNA adapter, respectively.

Finally, a PCR was carried out to amplify the upstream region of the intended genes from both methodologies. The PCR products were separated by PAGE in 8% polyacrylamide gels, and DNA bands were isolated and sequenced.

H-NS purification. Purification of H-NS-Myc-6×His proteins was performed with Ni-nitrilotriacetic acid resin (QIAExpress; Qiagen) according to the manufacturer's instructions and the modifications established by De la Cruz (18). Briefly, *E. coli* BL21(DE3) harboring the pMDHNS plasmid was grown in 100 ml of LB supplemented with Ap until an OD_{595} of 0.4 was reached. H-NS induction was performed with 0.1% L-arabinose (Sigma-Aldrich), and the cultures continued to grow for 4 h. The cells were collected and washed with 10 mM Tris-EDTA (pH 8). Cells were resuspended in 8 M urea (pH 8) and lysed by sonication. The cell debris was separated by centrifugation, and the supernatant was stored at −20°C. H-NS was collected with a Ni-nitrilotriacetic acid agarose column (Qiagen), washed with urea buffer at pH 8.0 and 6.5, and eluted with 8 M urea (pH 4.5). Fractions containing purified H-NS-Myc-

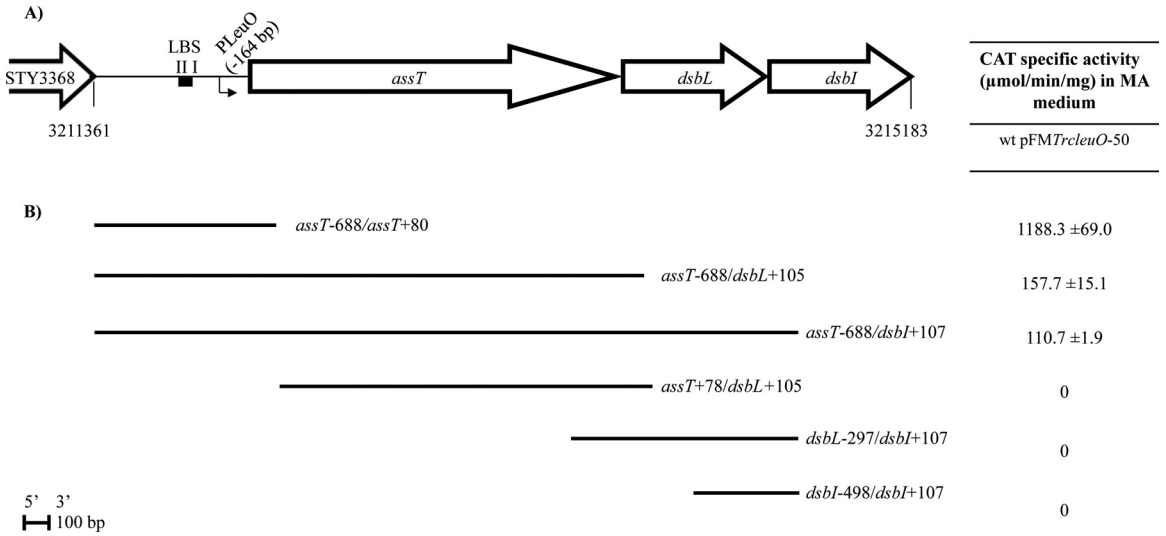

FIG 1 (A) *assT-dsbL-dsbI* gene cluster organization in *S.* Typhi. The LeuO-dependent promoter (PLeuO), as well as the LeuO binding sites I and II (LBS), is shown. The location of the *assT-dsbL-dsbI* cluster is annotated according to the *S. enterica* serovar Typhi CT18 genome (51). (B) DNA fragments fused to the *cat* reporter gene in the 3′ end. The coordinates in each fragment are according to the upstream or downstream distance in nucleotides from the start codon of the corresponding open reading frame. The right column indicates the CAT specific activity obtained from each construction in *S.* Typhi IMSS-1 wild type with pFM*TrcleuO*-50, induced with 50 mM IPTG, in MA medium at 12 h.

6×His were resolved by SDS-PAGE. The selected fractions were dialyzed and stored in the H-NS buffer (50 mM Tris-HCl [pH 8], 10 mM $MgCl_2$, 20% glycerol, 500 mM NaCl, 0.1% Triton X-100). Protein concentrations were determined by the Bradford procedure.

Electrophoretic mobility shift assay (EMSA). The DNA probes used for nonradioactive EMSA were obtained by PCR using the primers described in Table S2 in the supplemental material. A total of 40 ng of each probe was mixed with the protein buffer (10X: 400 mM HEPES, 80 mM $MgCl_2$, 500 mM KCl, 10 mM DTT, 0.5% NP-40, 1 mg of bovine serum albumin/ml) and increasing amounts of H-NS (0 to 600 nM). The DNA fragments and H-NS mixtures were incubated for 20 min at room temperature and analyzed by electrophoresis in 6% native polyacrylamide gels in 0.5× Tris-borate-EDTA buffer. The DNA bands were visualized by ethidium bromide staining. The structural *ler* gene from enteropathogenic *Escherichia coli* (EPEC) was used as a negative control.

RESULTS

The LysR family transcriptional regulator LeuO controls *assT-dsbL-dsbI* expression in *S.* Typhi IMSS-1. Previous reports suggested that the *assT*, *dsbL*, and *dsbI* genes are transcribed as an operon in UPEC and *Enterobacter amnigenus* (25, 37); however, no transcriptional mechanism of expression has been defined for either organism. More recently, in *S.* Typhimurium it has been shown that *dsbL* expression is independent of *assT* transcription (40). Previous studies performed in our laboratory with the *S.* Typhi IMSS-1 wild-type strain showed that the *assT* transcriptional expression depends on the transcriptional regulator LeuO and, by primer extension experiments, a LeuO-dependent promoter (PLeuO), located 164 bp upstream of the putative *assT* translation start site, was reported (26).

To determine whether *assT*, *dsbL*, and *dsbI* form a LeuO-dependent operon, several *cat* transcriptional fusions were designed (Fig. 1), and their expression was evaluated in MA medium, in *S.* Typhi IMSS-1 harboring the pFM*Trc*12 control vector or the pFM*TrcleuO*-50 plasmid (pFM*Trc*12 derivative plasmid encoding *leuO* under an IPTG-inducible p*trc* promoter; see Table S1 in the supplemental material) (18, 21). As previously reported (26), *assT* was not expressed in the wild-type strain with the control vector (data not shown), although it was indeed induced in a fusion that contains 688 bp upstream and 80 bp downstream from the *assT* translation start site (pKK232-9 *assT*−688/*assT*+80), upon LeuO overexpression. In this respect, the coordinates in all fusions are relative to the translational start sites for each gene. The transcriptional fusions *assT*−688/*dsbL*+105 and *assT*−688/*dsbI*+107, containing the *assT* LeuO-dependent promoter, as well as the upstream regions of *dsbL* and *dsbI*, respectively, also showed null transcriptional activity in the wild-type strain with the plasmid vector (data not shown). In contrast, transcriptional expression was detected in the presence of pFM*TrcleuO*-50 induced with 50 μM IPTG (Fig. 1), albeit at lower levels compared to *assT*−688/*assT*+80. Elimination of the *assT* regulatory region, including the LeuO-dependent promoter (*assT*+78/*dsbL*+105, *dsbL*−297/*dsbI*+107, and *dsbI*−498/*dsbI*+107 *cat* fusions), abolished the transcriptional activation generated by LeuO, indicating that the regulatory region of *assT* is essential for *assT-dsbL-dsbI* expression mediated by LeuO, and suggesting a LeuO-dependent operon in *S.* Typhi IMSS-1.

Further support of the *assT*, *dsbL*, and *dsbI* operon organization comes from the RT-PCR amplification of a 2,025-bp fragment that contained most of the *assT* structural gene, the *dsbL*, and part of the *dsbI* genes (*dsbL*−1232 to *dsbI*+107) detected upon LeuO overexpression (Fig. 2). As expected, no transcription of the operon was observed from *S.* Typhi wild type with the pFM*Trc*12 empty vector. These data indicate that *assT*, *dsbL*, and *dsbI* are functionally organized as a LeuO-dependent operon in the rich MA medium.

In order to determine whether the *assT* PLeuO promoter (26) generates the *assT-dsbL-dsbI* polycistronic mRNA, A/T-to-G/C substitutions in the −10 box (TACTAT to CGCCGC) were generated in the transcriptional fusions *assT*−688/*assT*+80, *assT*−688/*dsbL*+105, and *assT*−688/*dsbI*+107. The transcription assay showed null expression when the TATA box was mu-

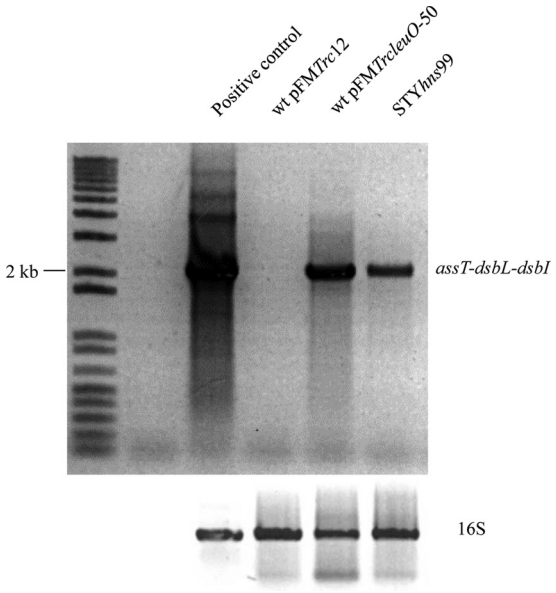

FIG 2 RT-PCR of the *assT-dsbL-dsbI* gene cluster from *S.* Typhi IMSS-1 wild type with either the pFM*Trc*12 empty plasmid vector or the pFM*TrcleuO*-50 induced with 50 mM IPTG and from IMSS-1 STY*hns*99 in MA medium. As a gel loading control, a fragment of the gene encoding the 16S rRNA was amplified. As a full-length positive control, a PCR from genomic DNA was amplified.

tated in all of these fusions in the presence of overexpressed LeuO (data not shown), showing that PLeuO indeed transcribes *assT*, *dsbL*, and *dsbI* as an operon in MA medium.

Effect of deletions and point mutations in the LeuO binding sites of the *assT-dsbL-dsbI* operon. Transcription experiments indicated that *assT-dsbL-dsbI* expression is dependent on LeuO induction in rich medium. Previously, we determined by DNase protection footprinting experiments the presence of two LeuO binding sites (LBS I and LBS II), located at bp −276 to −286 and bp −295 to −328 upstream of the *assT* translation start site (26). In order to identify the nucleotides involved in the *assT* LeuO-dependent activation, deletions and substitution in the LBS were generated (Fig. 3), and their transcriptional expression evaluated in wild-type *S.* Typhi IMSS-1 harboring the pFM*Trc*12 or the pFM*TrcleuO*-50 plasmid.

In the absence of cloned *leuO*, null transcriptional activity was observed in all constructions analyzed (data not shown). Interestingly, compared to *assT*−688/*assT*+80, CAT activity levels in the presence of LeuO decreased more than 75% when the LBS II or both the LBS I-II were eliminated (Fig. 3). In contrast, a null effect was observed with the elimination of LBS I, showing that the LBS II nucleotide sequence is crucial for *assT* transcriptional activation mediated by LeuO.

Because LeuO still activates *assT* when both LBS I and II have been deleted (*assT*−688/*assT*+80ΔLBS I-II construction), although at <25% of the wild-type levels, we analyzed, by footprinting of a DNA fragment lacking both LBS I and II sites, whether there are other LeuO-binding sites in this region. However, no DNase protection was observed (data not shown). As a control, a wild-type DNA fragment from *assT* was used for footprinting assays and, as reported previously (26), the two LeuO binding sites were detected. This result suggests that there are no other high-affinity LeuO binding sites in this region.

In order to determine the nucleotides in LBS II involved in *assT* activation by LeuO, substitutions were generated on the *assT*−688/*assT*+80 gene fusion (Fig. 3B). Three regions with high A+T content were replaced by G+C, considering that LeuO binds preferentially to sites with high A+T (9, 10, 11, 26). The substitutions *assT*−688/*assT*+80 LBS-B and *assT*−688/*assT*+80 LBS-C, decreased LeuO-mediated *assT* activation by 53 and 35%, respectively (Fig. 3), whereas no changes in transcriptional activity were detected from *assT*−688/*assT*+80 LBS-D, containing a 3-bp substitution in LBS I. These results indicate, in agreement with data

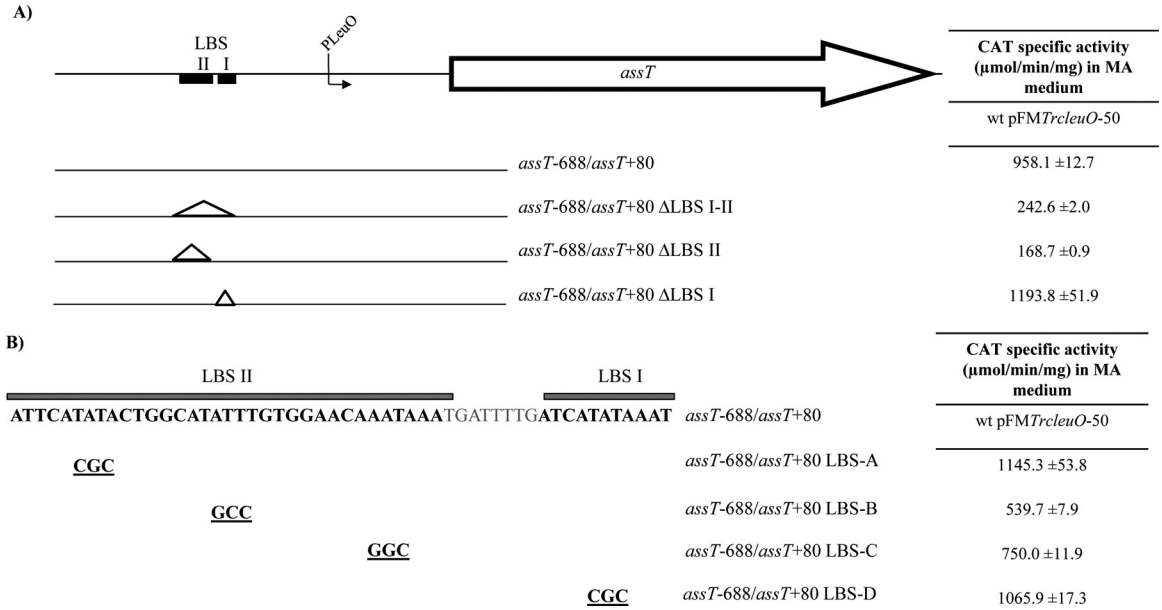

FIG 3 (A and B) Deletions (A) and substitutions (B) in the LeuO binding sites (LBS I and II) generated in the *assT*−688/*assT*+80 fusion. The right columns indicate CAT specific activity from each fusion in *S.* Typhi IMSS-1 containing the pFM*TrcleuO*-50 in MA medium induced with 50 mM IPTG at 12 h.

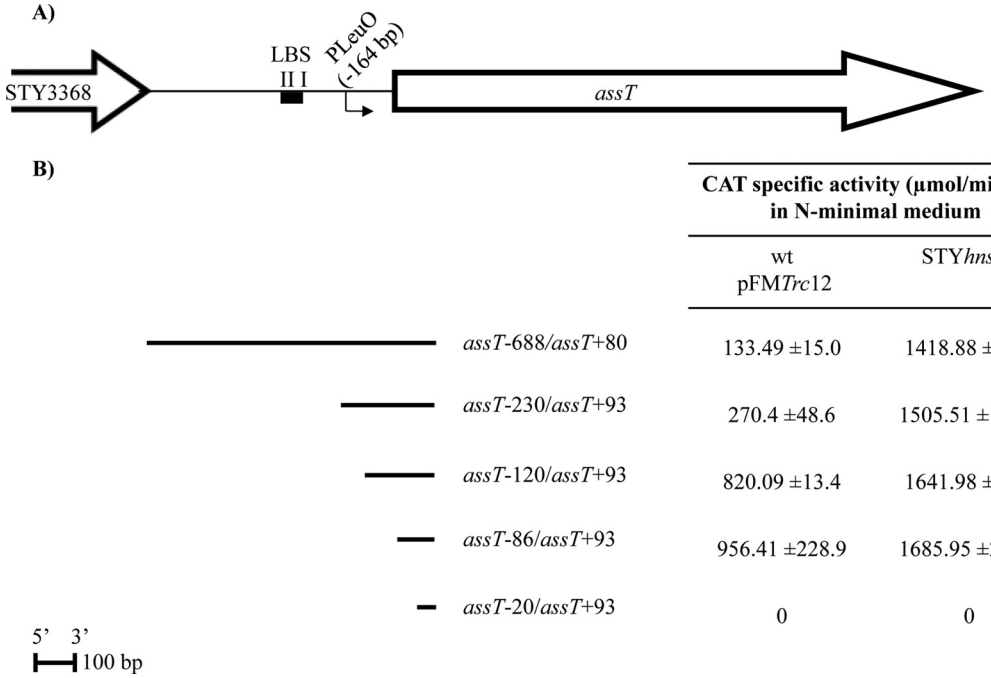

FIG 4 (A) *assT* gene organization in *S.* Typhi. The LeuO-dependent promoter (PLeuO), as well as the LeuO binding sites I and II (LBS), is shown. (B) DNA fragments fused to the *cat* reporter gene in the 3′ end. The right columns indicate the CAT specific activity obtained from each construction in *S.* Typhi IMSS-1 wild type with pFM*Trc*12 and in IMSS-1 STY*hns*99 in N-minimal medium at 12 h.

obtained from LBS deletions, that the nucleotides of LBS II are more relevant for LeuO transcriptional activation.

***assT* transcriptional activation in N-minimal medium and repression by H-NS.** *In vivo*, *assT*, *dsbL*, and *dsbI* expression has been detected in macrophages infected with *Salmonella* (20). In order to determine *assT* activation in this environmental condition, the transcriptional activity of *cat* gene fusions, containing different lengths of the 5′ *assT* upstream region, were analyzed in N-minimal medium, a laboratory growth condition widely used for induction of SPI-2 pathogenicity island expression (16).

Surprisingly, *assT* expression was observed in wild-type pFM*Trc*12 from the *assT*−688/*assT*+80 transcriptional fusion (Fig. 4). Interestingly, the elimination of nucleotides −230 to −120 (*assT*−120/*assT*+93), showed the highest transcriptional levels of *assT* gene expression in *S.* Typhi IMSS-1 wild type, containing the empty vector pFM*Trc*12, and similar CAT activity levels were obtained for fusion *assT*−86/*assT*+93, whereas no activity could be detected from the *assT*−20/*assT*+93 *cat* fusion. The data indicate that *assT* is induced in N-minimal medium in the absence of LeuO, since the same activity was observed in all of the constructions in a Δ*leuO* isogenic background (data not shown). Moreover, the results indicate the presence of a promoter closer to the ATG (between bp −86 and −20 regarding the *assT* translation start site), as well as a negative regulatory elements located between bp −230 and −120 upstream of the *assT* translational start site.

Since previous reports have shown that H-NS negatively regulates *assT* transcription in *S.* Typhi IMSS-1 (26, 42, 49), the *assT* transcriptional fusions were analyzed in an *hns* background, in N-minimal medium (Fig. 4). Interestingly, derepression of *assT* was observed and similar transcriptional levels were obtained for all fusions (with the exception of the *assT*−20/*assT*+93 *cat* fusion in which no activity could be detected), indicating that H-NS is an *assT* major negative regulator in this culture condition. The results also showed that H-NS represses expression from the transcription start site located proximal to the ATG (Fig. 4).

Determination of the *assT* transcriptional start site in N-minimal medium. Transcriptional fusions suggested that an *assT* promoter, located between nucleotides −20 and −86 regard to the translation start site, directed *assT* expression in N-minimal medium. Primer extension assays and 5′RACE were used to identify such promoter.

For primer extension assays, RNA was isolated from the *S.* Typhi IMSS-1 wild type harboring the pFM*Trc*12 empty vector or *S.* Typhi STY*hns*99, both containing the *assT*−120/*assT*+93 fusion. These strains were selected due to the high transcriptional levels detected in the CAT expression assays in N-minimal medium (Fig. 4). Primer extension analyses showed two 5′ ends located at bp −31 (P1) and −36 (P2) from the *assT* translation start site (Fig. 5), with a higher level of transcription in an *hns* mutant, according with the data obtained from transcriptional assays (Fig. 4).

The 5′RACE experiments were performed from total RNA extracted from cells grown in N-minimal medium, using wild-type pFM*Trc*12, and using two strategies: polynucleotide addition by terminal transferase and ligation of an oligonucleotide adapter. Interestingly, only a 5′ end located at bp −30 upstream of the *assT* ATG was identified, which differs by 1 bp from P1, identified by primer extension (see Fig. S1 in the supplemental material). Moreover, the same 5′ end was identified in STY*hns*99 (not shown).

To validate the transcription start sites identified by primer extension, substitutions in the −10 box (A/T to G/C) from each promoter, P1 and P2, or a double substitution P1-P2, were generated in fusions *assT*−688/*assT*+80 and *assT*−120/*assT*+93, and

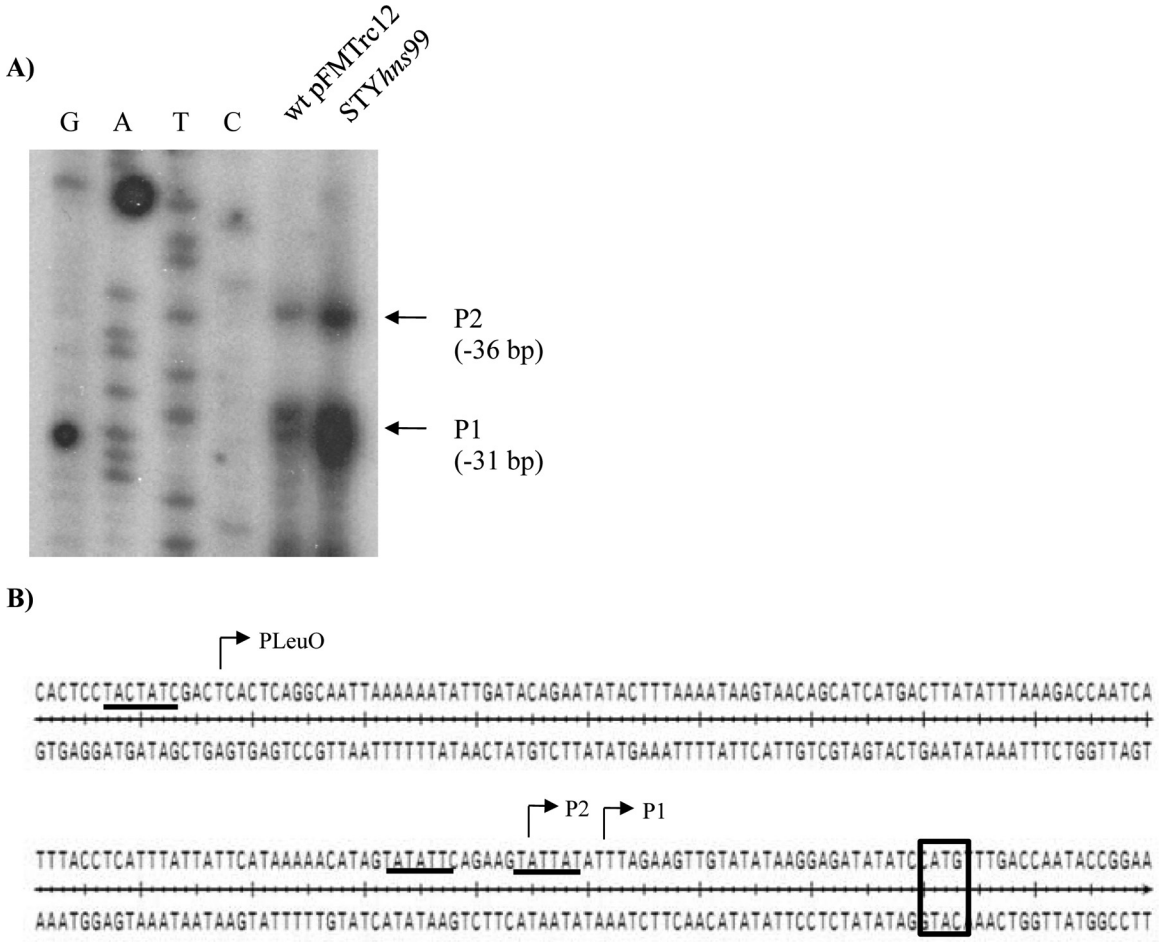

FIG 5 *assT* promoter region in N-minimal medium. (A) Primer extension assay performed in N-minimal medium at 12 h from *S.* Typhi IMSS-1 wild type with pFM*Trc*12 and from IMSS-1 STY*hns*99, with both containing the *assT*−120/*assT*+93 gene fusion. (B) Upstream regulatory sequence of *assT*. The location of the PLeuO (−164) promoter observed in MA medium and the two transcription start sites, P1 (−31) and P2 (−36), observed in N-minimal medium are shown, as well the corresponding −10 TATA boxes, which are underlined. The initiation codon for *assT* is indicated in a rectangle.

transcriptional activity assays were performed in N-minimal medium (Fig. 6). The substitutions in the −10 region of P1 or P2 in the *assT*−688/*assT*+80 fusions generated lower transcriptional levels in either wild-type pFM*Trc*12 or STY*hns*99 mutant, whereas no CAT activity was detected from a double promoter substitution in a wild-type background, indicating that P1 and P2 are the *assT* promoters expressed in N-minimal medium. In contrast, low levels of *assT* expression were detected in the absence of P1 and P2, in a STY*hns*99 strain, suggesting that the upstream PLeuO promoter activity is also repressed by the H-NS global regulator in N-minimal medium.

Transcriptional assays from *assT*−120/*assT*+93 derivatives showed no changes in expression in independent promoter substitutions in wild-type pFM*Trc*12, and STY*hns*99 (Fig. 6); in contrast, double promoter substitutions generated no transcriptional activity in N-minimal medium. These results validate the data obtained by primer extension, showing two *assT* transcription start sites, located at bp −31 and −36 upstream the *assT* translation start site, preferentially expressed in N-minimal medium and negatively regulated by H-NS.

Regulation of *dsbL* and *dsbI* expression in N-minimal medium. As previously mentioned, *S.* Typhimurium expressed *assT*, as well as *dsbL* and *dsbI*, during macrophage infection (20); however, the mechanisms of the transcriptional regulation of *dsbL* and *dsbI* have not been elucidated. As shown above, transcriptional assays and RT-PCR showed LeuO-dependent activation of the *assT-dsbL-dsbI* operon in rich MA medium, whose expression was enhanced under a LeuO-dependent promoter (Fig. 1 and 2). Moreover, RT-PCR performed in an *hns* background in MA medium generates a 2-kb PCR product, which corresponds in length to the polycistronic fragment (Fig. 2), showing that the *assT-dsbL-dsbI* operon is regulated negatively by the H-NS global regulator. However, because *assT* expression could be detected in the absence of LeuO from P1 and P2 in N-minimal medium, indicating a different regulatory mechanism, it was thus of interest to analyze the transcriptional organization of the *assT-dsbL-dsbI* gene cluster in N-minimal medium, for which the appropriate *cat* transcriptional fusions were designed (Fig. 1 and Table 1).

The transcriptional assays performed in N-minimal medium showed *dsbL* expression in the wild-type strain containing pFM*Trc*12 from the *assT*+78/*dsbL*+105 fusion, which does not contain the *assT* promoters or sequences upstream of the *assT* translation start site (the construction begins at 1,738 bp upstream of the *dsbL* translation start site). The results suggest an alternative

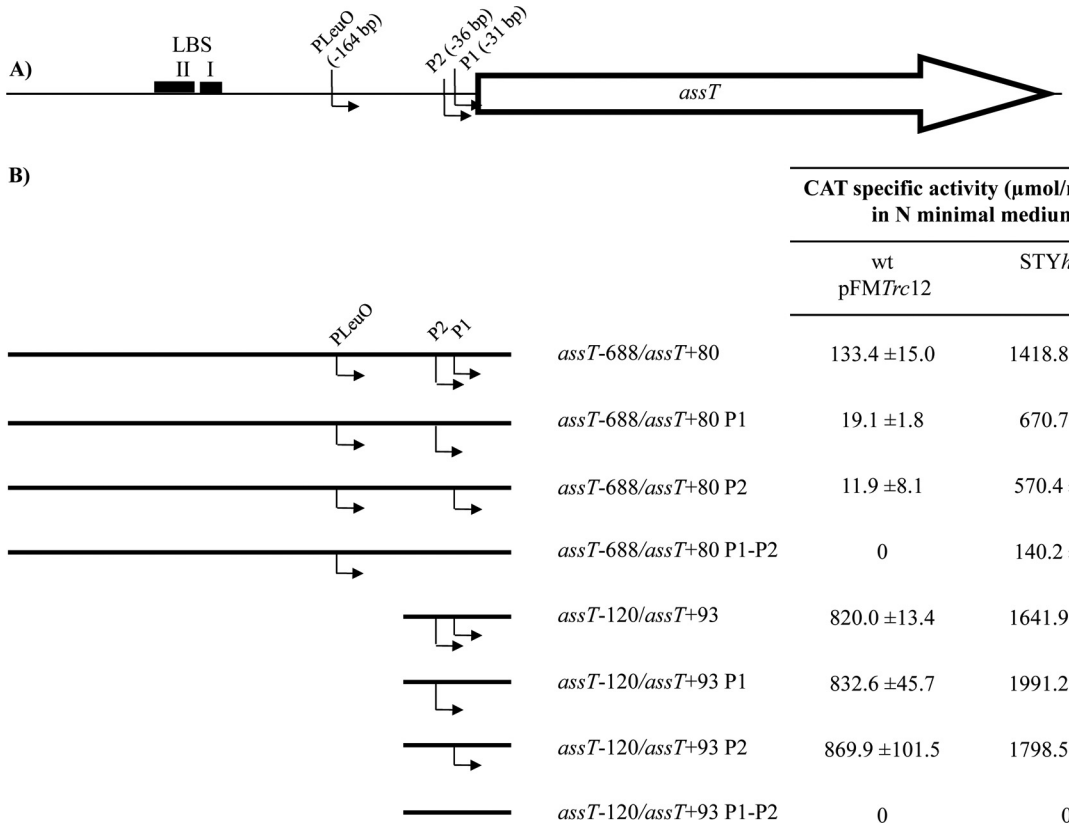

FIG 6 (A) *assT* gene organization in *S.* Typhi. The three *assT* promoters (P1, P2, and PLeuO), as well as the LeuO binding sites I and II (LBS), are shown. (B) DNA fragments fused to the *cat* reporter gene in the 3′ end with substitution in the −10 box of the P1 and/or P2 promoters in the regulatory region of *assT*, in the constructions containing the region *assT*−688/*assT*+80 or *assT*−120/*assT*+93. For P1, the nucleotides TATTAT were changed for CGCCGC, whereas for P2, the nucleotides TATATT were changed for CGCGCC. The right columns indicate the CAT specific activity obtained from each construction in *S.* Typhi IMSS-1 wild type with pFM*Trc*12 and in IMSS-1 STY*hns*99 in N-minimal medium at 12 h.

promoter for *dsbL*, independent of *assT* expression that is activated in N-minimal medium. It is noteworthy that expression of *dsbL* (*assT*+78/*dsbL*+105) was not influenced by the *hns* mutation (Table 1).

Transcriptional activity could be detected in N-minimal medium in the wild-type pFM*Trc*12 from *cat* fusion *dsbL*−297/*dsbI*+107, containing the 5′ regulatory region and the whole structural *dsbL* gene and a portion of the structural *dsbI* gene (Table 1). Higher transcriptional expression was observed in the STY*hns*99 strain. Interestingly, no transcriptional activity was detected with the elimination of the *dsbL* regulatory region, in *dsbI*−498/*dsbI*+107 in the two backgrounds analyzed. These data indicate that *dsbL* and *dsbI* are transcribed as an operon in N-minimal medium.

TABLE 1 CAT specific activity in N-minimal medium at 12 h from *S.* Typhi IMSS-1 wild type with the pFM*Trc*12 empty vector or STY*hns*99

	Mean CAT sp act (μmol/min/mg) ± SD in N-minimal medium	
PCR fused to *cat*[a]	Wild-type pFM*Trc*12	STY*hns*99
assT+78/*dsbL*+105	392.8 ± 48.2	343.0 ± 44.4
dsbL−297/*dsbI*+107	30.3 ± 11.6	320.0 ± 57.4
dsbI−498/*dsbI*+107	0	0

[a] The PCR fragments fused to the *cat* reporter gene, containing the upstream region of *dsbL* and/or *dsbI*, are indicated.

To determine the *dsbL* transcriptional start sites expressed in N-minimal medium, primer extension assays were performed from *S.* Typhi wild-type strain harboring the pFM*Trc*12 plasmid or in *S.* Typhi STY*hns*99, both containing the *assT*+78/*dsbL*+105 fusion (Fig. 7). Two putative transcriptional initiation sites were determined: P1-L and P2-L, located at bp −189 and −245 upstream of the *dsbL* translation start codon, respectively. Neither promoter exhibited activation in the *hns* mutant, in accord with the expression values from Table 1. Interestingly, P1-L contains a recognition sequence for σ^{32}, which is involved in heat shock stress (13); however, the promoter was activated in wild-type pFM*Trc*12 and in STY*hns*99 backgrounds in the absence of a heat shock induction. On the other hand, σ^{32} induction has been detected inside the macrophage during *Salmonella* Typhimurium infection (20), a condition that should be explored in future studies.

H-NS binding sites in the *assT-dsbL-dsbI* gene cluster. In order to define whether H-NS regulates directly *assT* and *dsbL*, EMSAs were performed with the 5′ upstream region of each gene and the purified H-NS protein (Fig. 8). A sequence from the structural *ler* gene from EPEC was used as a negative control (22). H-NS bound to the complete 5′ region of *assT* (F1) at a concen-

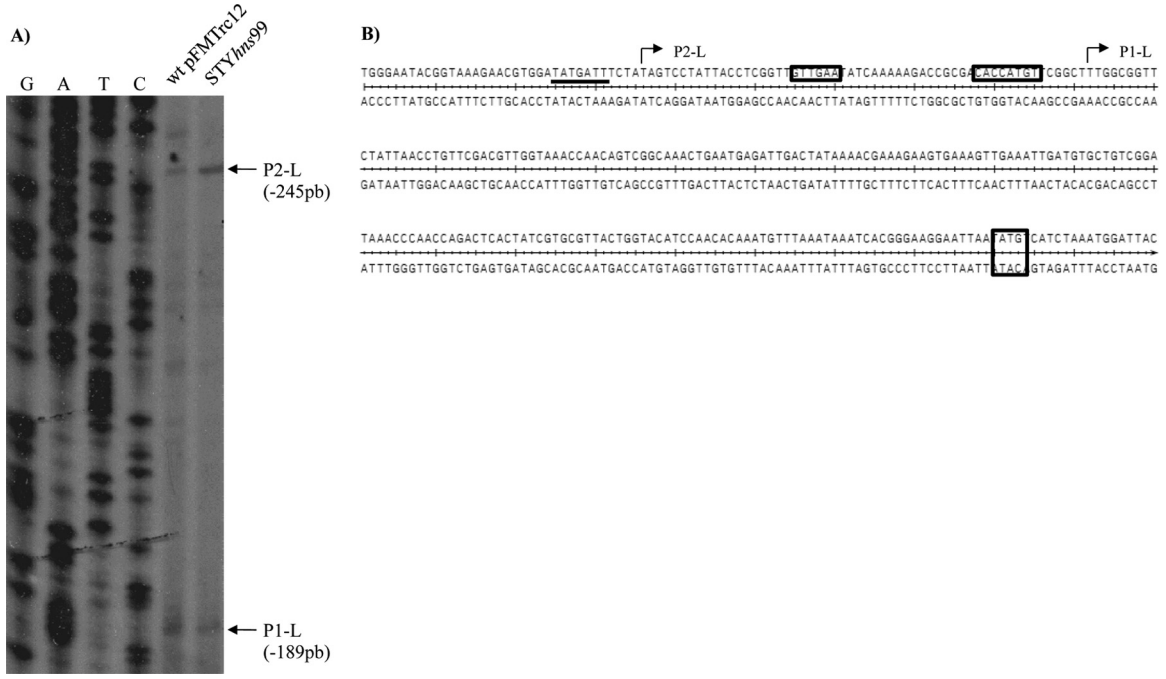

FIG 7 *dsbL* promoter region in N-minimal medium. (A) Primer extension assay results for *dsbL*, performed in N-minimal medium at 12 h from *S.* Typhi IMSS-1 wild type with pFM*Trc*12 and from IMSS-1 STY*hns*99, both containing the *assT*+78/*dsbL*+105 gene fusion. (B) Upstream regulatory sequence of *dsbL*. The locations of the two transcription start sites (P1-L and P2-L) are shown. The putative −10 and −35 σ^{32} promoter sequences for P1-L are shown in rectangles, whereas the −10 TATA box for the P2-L σ^{70} promoter is underlined. The initiation codon for the *dsbL* gene is indicated in a rectangle.

tration of ≥200 nM H-NS (Fig. 8B). To narrow down the H-NS binding site, F1 was divided into two DNA fragments, F2 and F3. An H-NS-F2 complex was observed at 500 nM protein, while no interaction with H-NS was observed for F3.

Additional DNA fragments were designed with the elimination of 5′ upstream sequences contained in F1. Thus, F4, F5, and F6 were generated: F4 contains the region between *assT*−271 and *assT*+57, F5 contains the region between *assT*−230 and *assT*+57, and F6 contains the region between *assT*−120 and *assT*+57 (Fig. 8C). H-NS bound to F4 and F5 at the same concentration (500 nM); interestingly, the elimination of bp −120 to −230 lowered the DNA-protein interaction (F6), a finding in agreement with the derepression observed in the transcriptional assays (Fig. 4). Hence, a full shift was only obtained with the full 5′ regulatory fragment (F1), indicating that the whole region is necessary for the strongest H-NS interaction.

A DNA fragment of the *dsbL* 5′ region was amplified (F7), containing 1,232 bp upstream and 5 bp downstream of the *dsbL* ATG (or +584 of *assT* on the left end). H-NS bound to F7, resulting in a full interaction at 400 nM (Fig. 8D). Interestingly, this fragment is the most similar to the one in fusion *assT*+78/*dsbL*+105 whose expression was not influenced by the *hns* mutation in the transcriptional assays and the primer extension (Table 1 and Fig. 7). Nevertheless, when F7 was divided into two smaller fragments (F8 and F9), containing the 5′ and 3′ regions of F7, respectively, no H-NS affinity for both F8 (−297 to +105) and F9 (−1232 to −258) was detected, suggesting nonspecific binding of H-NS to F7 in accordance with the null effect of the *hns* mutation on *dsbL* expression (Table 1 and Fig. 7) and suggesting that H-NS may act indirectly on the *dsbL*−297/*dsbI*+107 fusion (Table 1), which contains the whole *dsbL* gene and the initial portion of *dsbI*.

The H-NS interaction also was evaluated for *dsbI* (*dsbI*−530/*dsbI*+107) and no DNA-protein interaction could be observed, which is in agreement with previous results where neither promoters nor regulatory regions were detected close to the translation start site of *dsbI* (Fig. 8E). It is clear that more studies will be necessary to further understand the regulatory mechanisms for this operon.

DISCUSSION

The *assT-dsbL-dsbI* cluster is conserved in a small number of *Enterobacteriaceae* (5, 25, 41, 62). The *assT* gene encodes an arylsulfate sulfotransferase, and *dsbL-dsbI* encodes an oxidoreductase system whose function is to generate disulfide bonds in the AssT protein in the periplasm.

The *Salmonella* and pathogenic *E. coli* genomes encode a major oxidoreductase system composed of DsbA and DsbB, paralogues of DsbL and DsbI, respectively (3, 4, 14). DsbA is a periplasmic monomer that generates disulfide bonds in periplasmic proteins and contains, as well as DsbL, a CXXC motif characteristic of the thioredoxin family of proteins (24, 25). DsbB is an inner membrane protein with four transmembrane helices and two periplasmic loops, each one containing a cysteine pair, whose function is to keep DsbA in its active form by reoxidation (4, 28, 30). DsbA plays a central role in periplasmic protein folding, and diverse protein substrates for DsbA have been described, including proteins involved in transport of amino acids and peptides, RNA and protein degradation, a flagellar and type III secretion system apparatus, and some proteins such as toxins and chaperons (27, 29, 47, 50, 66). In addition to the similarity in protein structure between DsbA and DsbL, differences in the active site and the hydrogen bond network might limit potential substrate for the

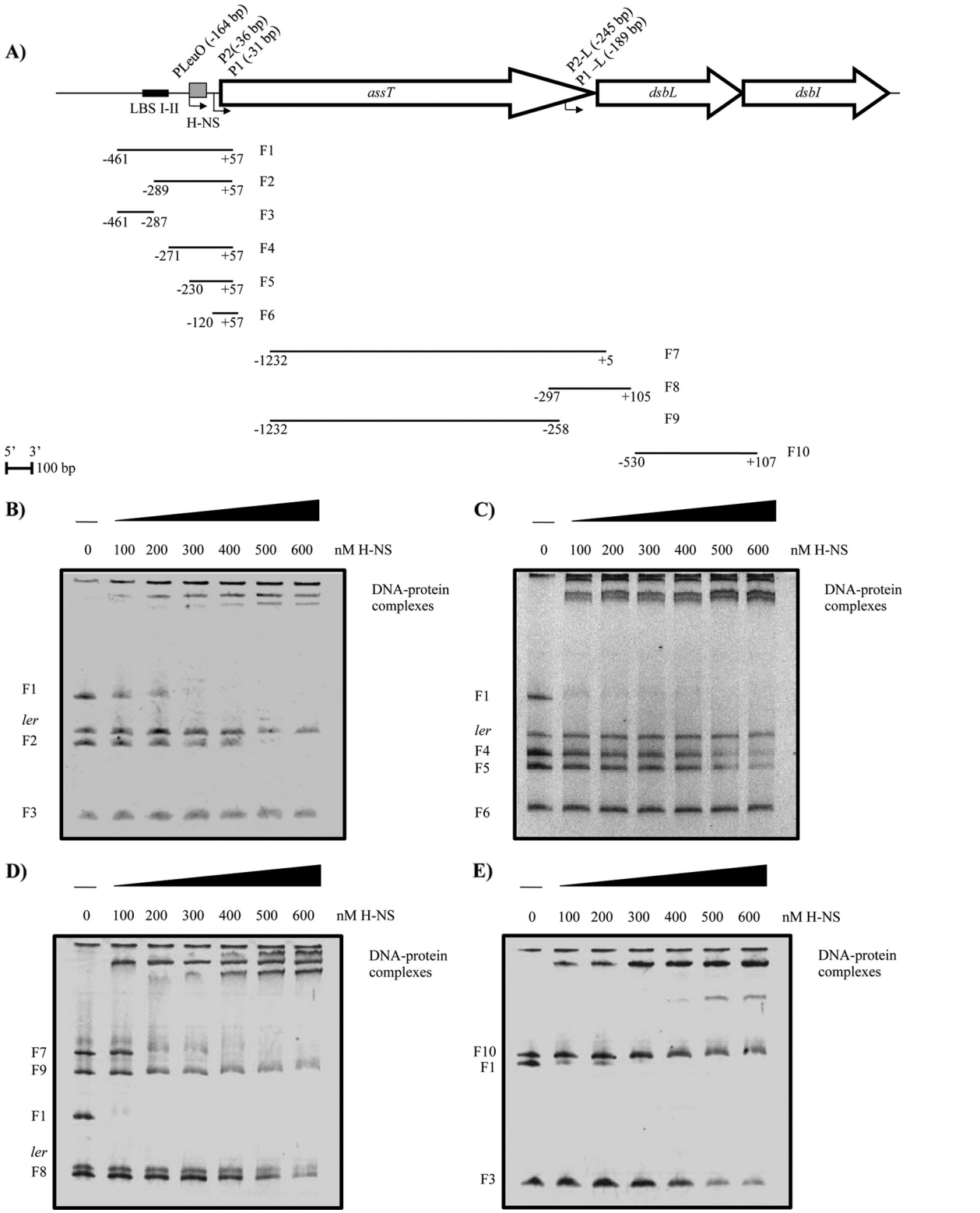

FIG 8 EMSAs of H-NS with various DNA fragments from the *assT-dsbL-dsbI* cluster. (A) DNA fragments utilized. The coordinates of the fragments according to the ATG of each gene are shown. EMSAs were performed with increasing amounts of H-NS (0 to 600 nM) for *assT* (B and C), *dsbL* (D), and *dsbI* (E). The structural *ler* gene from EPEC was used as a negative control for some experiments.

DsbL-DsbI oxidoreductase system (25). However, overexpression of the DsbL-DsbI system restored the absence of the major red-ox system in some DsbA targets, such as PhoA, FlgI, and PapD (40, 62).

Contrasting with the well-characterized structure and function of AssT, DsbL, and DsbI, the transcriptional regulation of the corresponding gene cluster has not been thoroughly studied. In *S. enterica* serovar Typhi IMSS-1, we showed that the LeuO-dependent promoter (PLeuO), located at bp −164 upstream of the *assT* translation start site (26), is activated only in the presence of LeuO, resulting in the transcription of *assT*, *dsbL*, and *dsbI* as an operon (Fig. 1 and 2). Moreover, negative regulation was observed by the global transcriptional factor H-NS (Fig. 2). Interestingly, LeuO and H-NS coregulation have been observed for several genes (8, 26, 36, 39, 57, 61, 64).

LeuO has been described recently as a global transcriptional regulator (26, 58, 59); however, a consensus DNA-binding sequence has not been established for this protein. The analysis of the LBS showed a deficient LeuO activation when LBS II, which is the farthest from PLeuO, is eliminated from the *assT* regulatory region (Fig. 3). Moreover, substitutions in the LBS II affect *assT* activation mediated by LeuO. Similar results were obtained for *ompS1*, where changes in the LBS located farthest from the transcription start site affect LeuO derepression and binding (18).

Multiple promoters have been identified for several genes; more than 10% of the genes contain two promoters, whereas in some cases more than four promoters per gene have been found (46). In this respect, aside from PLeuO, the regulatory region of *assT* contains two additional promoters (P1 and P2), located 36 and 31 bp upstream the *assT* translation start site, whose activation occurs in the absence of cloned *leuO* and in a Δ*leuO* background and whose repression is mediated by H-NS upon growth in N-minimal medium (Fig. 5 and 6). The binding site for H-NS was ascribed to the −230 to −120 *assT* region, slightly upstream of P1 and P2 and encompassing PLeuO. This site was based on the activity results in Fig. 4, where removal of such region results in lesser fold induction in the *hns* background. However, the facts that subdivisions of the whole *assT* 5′ regulatory region into fragments F2, F4, and F5 results in lower binding and F3, encompassing the furthest 5′ upstream region, does not bind H-NS (Fig. 8) suggest that the whole segment is needed for the tightest binding, and yet the initial nucleation site appears to be at −230 to −120.

Moreover, in the upstream region of *dsbL* two promoters were also identified (P1-L and P2-L) in N-minimal medium (Fig. 7), which were active in the absence of cloned *leuO* and in a Δ*leuO* background. Interestingly, no promoter was observed in the upstream region of *dsbI*, although transcriptional activity was detected in a *dsbI* gene fusion that contains both P1-L and P2-L, indicating that *dsbL-dsbI* form an operon that is expressed in this growth condition (Table 1). However, it cannot be excluded that the *assT-dsbL-dsbI* gene cluster is also transcribed as an operon in N-minimal medium. P1-L contains a putative σ^{32} promoter sequence, whereas P2-L is very likely a σ^{70} promoter (7, 13). In this respect, it is interesting that no heat shock stress was utilized for *dsbL* expression; however, growth in N-minimal medium could be an artificial stress signal for the *dsbL* activation under σ^{32}, since *rpoH* activation has been detected when *Salmonella* is inside macrophages (20).

N-minimal medium, which contains low concentrations of magnesium and phosphate, has been utilized to activate virulence gene expression in *Salmonella* pathogenicity island 2 (SPI-2), which is preferentially expressed inside the macrophage when *Salmonella* replicates in the vacuole (1, 16, 17, 20). Despite the activation of *assT*, *dsbL*, and *dsbI* in N-minimal medium, a mutation in the gene encoding the major SPI-2 global regulator, i.e., PhoP (48), had no considerable effect on transcription of *assT* or *dsbL-dsbI* (data not shown). Hence, it will be of interest to elucidate the regulators that allow such activation.

In conclusion, the transcriptional regulation of the *assT-dsbL-dsbI* gene cluster in *Salmonella enterica* serovar Typhi is dependent on growth culture conditions, where the global regulators LeuO and H-NS are involved in positive and negative regulation, respectively. The complex regulation observed suggests that these genes need to be expressed with high precision in space and time during infection.

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