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# Regulation of the *Salmonella enterica std* Fimbrial Operon by DNA Adenine Methylation, SeqA, and HdfR[▽]

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**DNA adenine methylase (*dam*) mutants of *Salmonella enterica* serovar Typhimurium grown under laboratory conditions express the *std* fimbrial operon, which is tightly repressed in the wild type. Here, we show that uncontrolled production of Std fimbriae in *S. enterica* serovar Typhimurium *dam* mutants contributes to attenuation in mice, as indicated by the observation that an *stdA dam* strain is more competitive than a *dam* strain upon oral infection. Dam methylation appears to regulate *std* transcription, rather than *std* mRNA stability or turnover. A genetic screen for *std* regulators showed that the GATC-binding protein SeqA directly or indirectly represses *std* expression, while the poorly characterized *yifA* gene product serves as an *std* activator. YifA encodes a putative LysR-like protein and has been renamed HdfR, like its *Escherichia coli* homolog. Activation of *std* expression by HdfR is observed only in *dam* and *seqA* backgrounds. These data suggest that HdfR directly or indirectly activates *std* transcription. Since SeqA is unable to bind nonmethylated DNA, it is possible that *std* operon derepression in *dam* and *seqA* mutants may result from unconstrained HdfR-mediated activation of *std* transcription. Derepression of *std* in *dam* and *seqA* mutants of *S. enterica* occurs in only a fraction of the bacterial population, suggesting the occurrence of either bistable expression or phase variation.**

DNA adenine methylase (Dam) catalyzes postreplicative methylation of adenosine moieties located in 5′-GATC-3′ sites, using *S*-adenosyl-methionine as a methyl donor (6, 31, 53). Methylation of daughter DNA strands occurs shortly, but not immediately, after passage of the replication fork. As a consequence, all GATC sites go through a stage called "hemimethylation" in which one DNA strand is methylated and the other is nonmethylated (53). Because Dam trails the replication machinery at a relatively small distance, hemimethylated DNA is usually short-lived (31, 53).

Whenever a GATC site is embedded within a protein-binding sequence, its methylation state can affect DNA-protein interactions (53). For instance, the mismatch repair endonuclease MutH is active only on hemimethylated or nonmethylated GATC sites, while the replication protein DnaA binds more efficiently to the chromosome replication origin when its GATC sites are methylated (31, 53). The methylation state of specific GATC sites can also influence promoter activity. For instance, transient hemimethylation can activate or repress transcription in a cell cycle-coupled fashion (31, 32). Furthermore, in the regulatory regions of certain promoters, binding of proteins can prevent DNA methylase activity, giving rise to stably undermethylated (hemimethylated or nonmethylated) GATC sites. Undermethylation patterns can be maintained beyond cell division, thereby permitting epigenetic inheritance of transcriptional states (6, 32).

*dam* mutants of *Salmonella enterica* are severely attenuated in the mouse model: lack of DNA adenine methylation causes a 10,000 increase in the oral 50% lethal dose, and a 1,000-fold increase in the intraperitoneal 50% lethal dose (17, 19, 21). This extreme attenuation reflects the pleiotropy of *dam* mutations, which cause reduced invasion of epithelial cells (17), reduced cytotoxicity after infection of M cells (17), inefficient colonization of Peyer's patches and mesenteric lymph nodes (17, 21), sensitivity to bile (22, 40, 41), envelope instability accompanied by leakage of proteins (41), reduced motility (3), and probably additional defects still to be discovered. This plethora of virulence-related alterations, combined with the long persistence of *dam* mutants in infected animals, makes Dam-deficient strains highly suitable as live vaccines (13, 14, 22). On the other hand, the essential role played by Dam methylation in the virulence of *Salmonella* and other bacterial pathogens (24) has raised the possibility of using Dam inhibitors as antibacterial drugs (34).

Some of the virulence-related defects so far described in *Salmonella dam* mutants involve alterations in gene expression (3). For instance, the reduced capacity of *dam* mutants to invade epithelial cells (17) seems to be caused by lowered expression of *Salmonella* pathogenicity island I (3), which in turn reflects the existence of reduced levels of the main *Salmonella* pathogenicity island I activator, HilD (J. López-Garrido and J. Casadesus, unpublished data). Altered expression patterns of flagellar and chemotaxis genes may also contribute to deficient invasion (3). Other virulence-related genes with anomalous expression in *dam* mutants are the Braun lipoprotein gene, *lppB* (3), and the *spv* operon of the virulence plasmid (21).

This study deals with an additional virulence-related locus

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under Dam methylation control: the *std* fimbrial operon, initially identified in serovar Typhi (46) and later found in other *Salmonella enterica* serovars, including Typhimurium (2, 7, 27, 38). Std fimbriae belong to the π group (37) and play a role in the adhesion of *Salmonella* to specific intestinal sections, as indicated by the fact that deletion of the *std* operon causes reduced intestinal persistence (51). Synthesis of Std fimbriae is tightly repressed under laboratory conditions, and several lines of evidence suggest that derepression occurs in the intestine of infected animals (9, 51).

The mechanisms that prevent *std* expression outside the animal environment remain unknown, but recent studies have identified the cellular functions involved. Lack of RosE, a protein with homology to the *Escherichia coli* ArgR repressor, permits *std* expression under laboratory conditions (9). Derepression of the *std* operon also occurs in the absence of Dam methylation: *dam* mutants were found among Std-expressing isolates induced by transposon mutagenesis (9). This finding was in agreement with a proteomic study that identified StdA as one of the most abundant proteins in *dam* mutants (1) and with the >100-fold increase in *std* mRNA detected by transcriptomic analysis of *dam* mutants (3). The addition of *std* to the list of Dam-regulated genes strengthens the curious observation that genes under Dam methylation control often encode surface structures such as fimbriae, nonfimbrial adhesins, type III secretion systems, and conjugative pili (32).

Below, we provide evidence that uncontrolled expression of Std fimbriae may be listed as an additional defect contributing to virulence attenuation in Dam mutants of *S. enterica* serovar Typhimurium. Furthermore, we show that the GATC-binding protein SeqA is a repressor of the *std* operon and that the poorly known protein HdfR, a putative member of the LysR family of transcriptional regulators (29), is an activator of *std* expression whose activity may be antagonized by SeqA.

TABLE 1. Strain list

| Strain designation | Genotype or description | Reference or source |
|---|---|---|
| SV4248 | *traB1*::MudJ *zge-6313*::Tn*10d*Cm (50% linked to *dam*) | Laboratory stock |
| SV4536 | Δ*dam-230* | 40 |
| SV4752 | Δ*seqA1* | 39 |
| SV4783 | *dam-201*::*Tn10dTc* Δ*seqA1* | Laboratory stock |
| SV5031 | Δ*stdA*::Km$^r$ | This study |
| SV5032 | Δ*dam-230* Δ*stdA*::Km$^r$ | This study |
| SV5139 | *stdB*::Cm$^r$ | This study |
| SV5206 | *stdA*::*lacZ* | This study |
| SV5208 | Δ*dam-230 stdA*::*lacZ* | This study |
| SV5637 | Δ*seqA1* Δ*hdfR* | This study |
| SV5638 | Δ*dam-230* Δ*hdfR* | This study |
| SV5643 | *seqA*::Tn*10*dTc *stdA*::*lacZ* | This study |
| SV5645 | Δ*dam-230 seqA*::Tn*10d*Tc *stdA*::*lacZ* | This study |
| SV5651 | Δ*stdAB*::Km$^r$ | This study |
| SV5652 | Δ*dam-230* Δ*stdAB*::Km$^r$ | This study |
| SV5657 | $p_{BAD}$(*stdA stdB*::Cm$^r$) Δ*stdAB*::Km$^r$ | This study |
| SV5658 | $p_{BAD}$(*stdA stdB*::Cm$^r$) Δ*dam-230* Δ*stdAB*::*Km$^r$* | This study |
| SV5716 | Δ*hdfR*::Cm$^r$ | This study |
| SV5717 | Δ*dam-230* Δ*hdfR*::Cm$^r$ *stdA*::*lacZ* | This study |
| SV5718 | Δ*seqA1* Δ*hdfR*::Cm$^r$ *stdA*::*lacZ* | This study |
| SV5719 | Δ*seqA1 stdA*::*lacZ* | This study |

## MATERIALS AND METHODS

**Bacterial strains, media, chemicals, and culture conditions.** The strains of *S. enterica* used in this study (Table 1) belong to serovar Typhimurium and derive from the mouse-virulent strain ATCC 14028. For simplicity, *S. enterica* serovar Typhimurium is often abbreviated as serovar Typhimurium. Luria-Bertani (LB) broth was used as standard liquid medium. Carbon sources were either 0.2% glucose or 0.2% arabinose. Solid LB medium contained agar at a 1.5% final concentration. Green plates were prepared according to Chan et al. (8), except that methyl blue (Sigma-Aldrich, St. Louis, MO) was substituted for aniline blue. Antibiotics were used at concentrations described previously (45). Transductional crosses using phage P22 HT 105/1 *int201* (43; also G. Roberts, unpublished data) were used for strain construction operations involving chromosomal markers. The transduction protocol was previously described elsewhere (18). To obtain phage-free isolates, transductants were purified by streaking on green plates. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5.

**β-Galactosidase assays.** Levels of β-galactosidase activity were assayed as described by Miller (36), using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure.

**Primer extension.** Strain SV4536 was grown in LB medium until late exponential phase; total RNA was then extracted. The oligonucleotide 5′-ACC TGA GCC GAA CGG GCC TG-3′, complementary to an internal region of the *stdA* gene of serovar Typhimurium (GenBank accession number AE008839), was end labeled with [$\gamma^{32}$P]ATP and annealed to 20 μg of RNA. For annealing, $10^6$ cpm of oligonucleotide was used. The end-labeled primer was extended with avian myeloblastosis virus reverse transcriptase (Roche, Basel, Switzerland) under the conditions described by Camacho et al. (5). The extension products were separated in a polyacrylamide gel containing 6% urea. For autoradiography, gels were exposed to X-ray film.

**DNA sequencing.** Sequencing reactions were carried out with a Sequenase, version 2.0, sequencing kit (USB Corporation, Cleveland, OH). The manufacturer's instructions were followed. Additionally, 1 μl of unlabeled 10 μM dATP was added to the reaction mixtures. Sequencing gels were prepared in Tris-borate-EDTA buffer containing 6% acrylamide and 500 g/liter urea. Gels were run in a Sequi-Gen GT System (Bio-Rad, Hercules, CA), dried in a Slab Gel Dryer, model SE1160 (Hoefer Scientific Instruments, Holliston, MA), and developed by exposure to X-ray film.

**Construction of strains carrying *stdA*, *stdB*, *stdAB*, and *yifA* (*hdfR*) deletions.** All deletions were generated by the method of Datsenko and Wanner (11). Kanamycin resistance cassettes introduced during construction were excised by recombination with plasmid pCP20 (11). Elimination of 351 bp in the putative *stdA* coding sequence (from position +150 to position +506) was achieved with primers 5′-CGC CAG GAG TTG CGG CAT CTG TCA GGG CTA TCA GGC GGG CGT GTA GGC TGG AGC TGC TTC-3′, and 5′-TTT CAC TGG TAC CAT CAC CAA CTC ACC CTG TGA TAT CGC AAT TCC GGG GAT CCG TCG ACC-3′. The resulting deletion eliminates the entire *stdA* gene except for 129 bp at its 5′ end and 81 bp at its 3′ end. PCR amplification using primers from both sides of the *stdA* locus identified kanamycin-sensitive isolates that carried the desired deletion. The sequences of these primers were 5′-GTG GAC GGC TTC TCC CTG TC-3′ and 5′-GCC GCC GAT ACT ACA CCC AC-3′. An internal 2,416-bp deletion in the *stdB* open reading frame (ORF) was created with the primers 5′-CGG AGC CTG CTG GAC AGC GGG AAC CTG TCT AAC GTG GAC CGT GTA GGC TGG AGC TGC TTC-3′ and 5′-GTG CGA GTA AAA TCA CAC GGC TTC TTC TGC TGT TTT TCA CAT GAA TAT CCT CCT TAG TTC-3′. Primers for *stdB* deletion verification by PCR amplification were 5′-CCA TTC TGA TTA CCC TGA CA-3′ and 5′-TAC GGG TCC GGT CAA CAT TG-3′. A 3,310-bp deletion that removed DNA from both *stdA* and *stdB* was obtained with primers 5′-CGG GTC CGG TCA ACA TTG ACG GCC GCC GGG CTG TAC TGG CGT GTA GGC TGG AGC TGC TTC-3 and 5′-GAG TTG TTT TCA GCC TTT GCA AAA TAA TTC TCA TTC ACC CAT TCC GGG GAT CCG TCG ACC-3′. Primers for deletion verification were 5′-CAT ACG AAT CTT TTC TGA AC-3′ and 5′-GGC CAC CGT TTT CTG CGG CG-3′. A 692-bp deletion in the putative *yifA* ORF was generated with primers 5′-AGA AGC ACT TTA CCT GAC GCA ATC CGC GGT GAG CTT TCG TGT GTA GGC TGG AGC TGC TTC-3′ and 5′-TCA TTG TTC ATC CAG CAC ATC CGT TTT TAA CAG ATC GCA GAT GAA TAT CCT CCT TAG TTC-3′ and verified with primers 5′-GGA GAG CAC AGT GGA TAC GG-3′ and 5′-GAT TAT CTG ATC AGG TAA TC-3′.

**Construction of a strain carrying an *stdA*::*lacZ* translational fusion in the *Salmonella* chromosome (SV5206).** The FLT recombinase target site created by *stdA* gene disruption (Δ*stdA*::Km$^r$) in the immediate ancestor of strain SV5031 was used to integrate plasmid pCE40 (15), thereby generating a translational *stdA*::*lacZ* fusion.

**Construction of strains expressing *std* from the arabinose-dependent $p_{BAD}$ promoter (SV5657 and SV5658).** Construction of strains expressing *std* from the arabinose-dependent $p_{BAD}$ promoter followed a procedure similar to that reported by Figueroa-Bossi et al. (16). The chromosomal gene *araB* of strain SV5651 (Δ*stdAB*) was replaced with a DNA fragment that included the start site of the *std* transcript, a complete *stdA* gene, and a portion of the *stdB* gene, using the λ Red technology (11). For this purpose, we generated a PCR product using primers with 40-nucleotide extensions homologous to regions adjacent to *araB*. As a template, we used genomic DNA of strain SV5139 (*stdB*::Cm$^r$). The primers used to amplify *stdA stdB*::Cm$^r$ (with extensions homologous to *araB* boundaries) were 5′-TTA GCA TTT TTG TCC ATA GGA TTA GCG GAT CCT GCC TGA CTA TGC GTA ATA AAA TAA TAC TTG CC-3′ and 5′-GAT GAC GGT TAA TGC ACG GAT CGA GTT CAT CAA AGA AGC TCG CCA GTG CGA GTA AAA TCAC-3′. The resulting PCR product was used to transform derivatives of SV5651 and SV5652 ($dam^+$ Δ*stdAB* and *dam* Δ*stdAB*, respectively) containing the λ Red recombinase-expressing pKD46 plasmid (11). Chloramphenicol-resistant transformants were selected and tested for the presence of the desired gene construct. This was the origin of strains SV5657 and SV5658 ($p_{BAD}$-*stdA stdB*::Cm$^r$ Δ*stdAB* and $p_{BAD}$-*stdA stdB*::Cm$^r$ Δ*stdAB* Δ*dam-230*, respectively).

**Protein extracts and Western blot analysis.** Total protein extracts were prepared from bacterial cultures grown at 37°C in LB medium until stationary phase (final optical density at 600 nm of ~1.2 to 1.4). Bacterial cells contained in 1 ml of culture were collected by centrifugation (20,000 × *g* for 5 min at 4°C), washed in phosphate-buffered saline (PBS), pH 7.4, and suspended in the appropriate volume of Laemmli sample buffer (1.3% sodium dodecyl sulfate, 10% [vol/vol] glycerol, 50 mM Tris-HCl, 1.8% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8). Proteins were resolved by Tris-Tricine-polyacrylamide gel electrophoresis, using 10% gels. Conditions of protein transfer and optimal dilutions of primary (anti-StdA) and secondary antibodies have been described elsewhere (3). Proteins recognized by the antibodies were visualized by chemiluminescence using luciferin-luminol reagents.

**Quantitative reverse transcriptase PCR (real-time PCR) and calculation of relative expression levels.** *Salmonella* RNA was extracted from stationary phase cultures using an SV Total RNA Isolation System (Promega Corporation, Madison, WI). The quantity and quality of the extracted RNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). To diminish genomic DNA contamination, the preparation was treated with DNase I (Turbo DNA free; Applied Biosystems/Ambion, Austin, TX). An aliquot of 0.5 μg of DNase I-treated RNA was used for cDNA synthesis using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCRs were performed in an Applied Biosystems 7500 Fast Real-Time PCR System. Each reaction was carried out in a total volume of 15 μl on a 96-well optical reaction plate (Applied Biosystems) containing 7.5 μl of Power Sybr Green PCR Master Mix (Applied Biosystems), 6.9 μl of cDNA (1/10 dilution), and two gene-specific primers at a final concentration of 0.2 μM each. Real-time cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. No-template controls and controls lacking reverse transcriptase were included for each primer set and template. Melting curve analysis verified that each reaction contained a single PCR product. Reported gene expression levels were normalized to transcripts of *ompA*, a housekeeping gene that served as an internal control. Gene-specific primers, designed with PRIMER3 software (http://primer3.sourceforge.net), were as follows: for *ompA*, 5′-TGT AAG CGT CAG AA CCG ATA CG-3′ and 5′-GAG CAA CCT GGA TCC GAA AG-3′; for *stdA*, 5′-CGG CTG CCG GTA TGA TGT-3′ and 5′-GGG CCT GCT GTG GGT GTA-3′; and for *stdB*, 5′-CTG CCG CCC TCT CTT CAG-3′ and 5′-GAC GGT GAC CTG TGC ATT ACT G-3′.

**Cloning and molecular characterization of Tn*10d*Tc inserts.** Amplification of DNA sequences close to Tn*10d*Tc insertions was achieved by inverse PCR. Genomic DNA from each Tn*10d*Tc-carrying isolate was digested with SmaI and PstI. The resulting fragment was autoligated and used as a template in two serial PCR amplifications with the primer 5′-ATT TGA TCA TAT GAC AAG ATG TGT-3′ (49). The final PCR product was purified and cloned onto pGEM-T (Promega Corporation, Madison, WI). Plasmid inserts were sequenced at the facilities of Sistemas Genómicos SL, Parque Tecnológico de Valencia, Paterna, Valencia, Spain, using the M13L and M13R universal primers.

**Flow cytometry.** Approximately 5 × $10^8$ cells were incubated with an equal volume of 4% paraformaldehyde (EM Science, Fort Washington, PA) at room temperature for 20 min. Cells were washed twice with 0.5 ml of 0.02% gelatin in PBS (PBS-gel). To block nonspecific binding, cells were harvested and resuspended in 0.5 ml of filter-sterilized 2% normal goat serum (Sigma) and incubated at room temperature for 30 min on a tabletop rotator. Polyclonal rabbit anti-StdA serum was added to the cells at a final dilution of 1:250 for detection of StdA, and cells were incubated at room temperature for 60 min on a tabletop rotator. After the cells were washed three times in PBS-gel, bacteria were resuspended in 0.5 ml of a solution of 0.04 mM propidium iodide in 2% normal goat serum with secondary antibody (fluorescein isothiocyanate [FITC]-conjugated goat anti-rabbit immunoglobulin G [IgG]) (Jackson ImmunoLabs, West Grove, PA), added at a dilution of 1:250. The mixture was rotated at room temperature for 1 h in the dark. Samples were washed three times with PBS-gel, and bacteria were resuspended in PBS to a final concentration of 5 × $10^6$ cells/ml. For each sample, the fluorescence of 10,000 particles (bacterial cells) was measured by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA).

**CI virulence assays.** Eight-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were used for virulence tests. Groups of three to four animals were inoculated with a 1:1 ratio of two strains. Bacteria were grown overnight at 37°C in LB medium without shaking. Oral inoculation was performed by feeding the mice with 25 μl of saline (0.9% NaCl) containing 0.1% lactose and $10^8$ bacterial CFU. Bacteria were recovered from the mouse spleen 6 days after inoculation, and CFU were enumerated on appropriate medium. A competitive index (CI) for each mutant was calculated as the ratio between the wild type and the mutant strain in the output divided by their ratio in the input (4). To compare the virulence of a double mutant with that of a single mutant, a "cancelled-out" competitive index (COI) was calculated. A COI is the ratio between the double mutant and the single mutant in the output divided by their ratio in the input (4). Assays were carried out in triplicate. A Student's *t* test was used to analyze CIs and COIs. The null hypothesis was that CIs were not significantly different from 1. COIs were analyzed with two null hypotheses: (i) mean COI is not significantly different from 1; (ii) mean COI is not significantly different from the CI of the corresponding single mutant. *P* values of 0.01 or less were considered significant.

## RESULTS

**Overexpression of Std fimbriae contributes to virulence attenuation in *S. enterica* serovar Typhimurium *dam* mutants.** *dam* mutants of *S. enterica* serovar Typhimurium display a plethora of virulence-related defects whose basis is only partially understood (17, 40, 41). The recent observation that overexpression of Std fimbriae in a *rosE* mutant caused attenuation of serovar Typhimurium virulence (9) led us to examine whether Std overproduction, a conspicuous phenotype of *Salmonella dam* mutants (1, 3), might likewise contribute to their attenuation. To examine this hypothesis, BALB/c mice were inoculated with pairs of strains at a 1:1 ratio. Infections were performed by the oral route, and a CI was calculated. An *stdA* mutation caused a small but significant reduction of virulence by the oral route (Fig. 1). In contrast, an *stdA* mutation increased the COI in a *dam* background (Fig. 1). The latter observation indicates that ectopic expression of Std fimbriae does contribute to attenuation in *Salmonella dam* mutants and thus may be added to the list of virulence-related defects caused by lack of Dam methylation. On the other hand, the contribution of *std* overexpression to attenuation in *dam* mutants is in agreement with the view that ectopic expression of Std fimbriae is more detrimental than their absence during the intestinal stage of *Salmonella* infection (9).

**Identification of the *std* operon start site.** Primer extension was used to map the 5′ terminus of the *std* transcript (Fig. 2). Because the *std* operon is not expressed in wild-type serovar Typhimurium under laboratory conditions, a *dam* mutant (strain SV4536) was used. A sequencing reaction was run in parallel and used as a size marker. DNA sequencing was primed by the same oligonucleotide employed for primer extension. The *std* transcript was found to start 21 bp upstream of

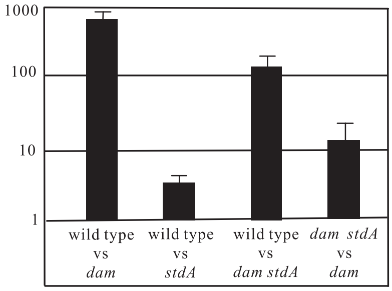


FIG. 1. CI and COI analysis of *dam*, *std*, and *dam std* strains after oral infection of BALB/c mice. The mixed infections performed were with the following strains: wild type (ATCC 14028) and *dam* (SV4536) (CI), wild type (ATCC 14028) and *stdA* (SV5031) (CI), wild type (ATCC 14028) and *dam stdA* (SV5032) (COI), and (d) *dam stdA* (SV5032) and *dam* (SV4536) (COI). The CIs and COI's represented are the means from three infections. Error bars represent the standard deviations. vs, versus.

the putative start codon of the *stdA* gene. In silico analysis of the DNA sequence upstream of the +1 site identified DNA sequences with features similar to those of canonical, $\sigma^{70}$-dependent promoters (25): (i) a putative −10 module including the motif 5′-TGTATAAT-3′, which has 6/8 matches with the consensus sequence; (ii) a putative spacer 19 nucleotides long; (iii) a 5′-TTATTTAAG-3′ sequence defining a putative −35 module, with 7/11 matches with the consensus sequence.

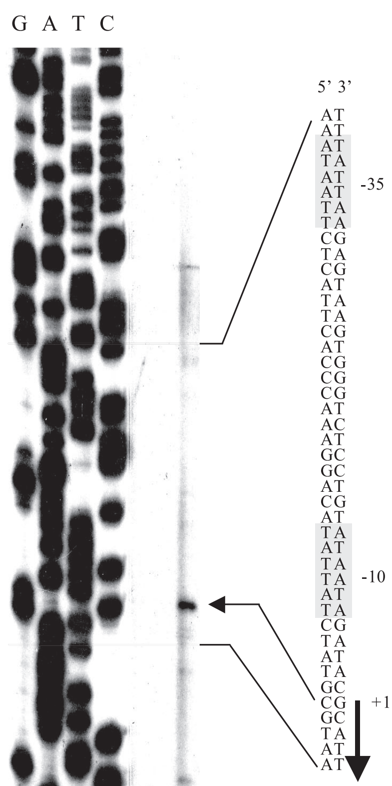


FIG. 2. Extension of an *std* mRNA primer with avian myeloblastosis virus reverse transcriptase. The extension product is indicated by an arrow. The DNA sequence of the *std* promoter region, the putative −10 and −35 modules, and the transcription start site are shown.

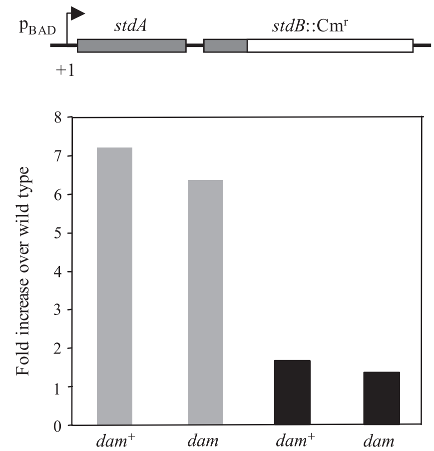


FIG. 3. (Top) Diagram of the construction that permits *std* expression under the control of $p_{BAD}$. (Bottom) Relative amounts of *stdA* mRNA transcribed from the heterologous $p_{BAD}$ promoter, normalized to *ompA* mRNA. Each bar represents the average from three independent experiments. Strains were grown in LB-arabinose (gray histograms) and LB-glucose (black histograms) media. Strains were SV5657 (*dam*$^+$) and SV5658 (*dam*).

Some of the DNA sequences found in the *std* promoter region are also compatible with the existence of an RpoS-dependent promoter (50); however, this possibility was judged unlikely since a previous study showed that *std* is expressed in *dam* derivatives of LT2 (3), a serovar Typhimurium strain known to be lacking RpoS (52). Upstream from the *std* promoter, a potential regulatory region containing three GATC motifs in a 25-bp interval was found, as previously described (3, 9).

The identification of a promoter upstream of the *stdA* gene does not rule out the possibility that internal promoters may also exist. However, transcriptomic analysis has provided evidence that the five genes that are part of the *std* cluster (*stdA*, *stdB*, *stdC*, STM3026, and STM3025) undergo coordinate expression (3). Hence, we propose that the cluster formed by *stdA*, *stdB*, *stdC*, STM3026, and STM3025 constitutes a polycistronic operon, which is transcribed from the promoter identified in this study. The decreasing gradient of mRNA levels detected in downstream *std* genes (3) is typical of polycistronic operons with natural polarity (30).

**Evidence that regulation of *std* expression by Dam methylation is transcriptional.** To determine whether Dam methylation-mediated control of *std* expression was transcriptional or posttranscriptional, we constructed *dam*$^+$ and *dam* strains (SV5657 and SV5658, respectively) in which the first gene of the *std* operon, *stdA*, was expressed from the *araBAD* promoter. In these constructs, the transcription start site of *stdA* was conserved (described in Materials and Methods). The effect of Dam methylation on *std* expression from the heterologous $p_{BAD}$ promoter was examined by quantitative real-time PCR. Strains SV5657 and SV5658 were grown overnight in LB medium containing either 0.2% arabinose or 0.2% glucose. Total RNA was then extracted and retrotranscribed. The resulting cDNA preparations were analyzed by quantitative reverse transcriptase PCR. As expected, expression from the $p_{BAD}$ promoter was dependent on the presence or absence of arabinose (Fig. 3). However, the total amount of retrotrans-

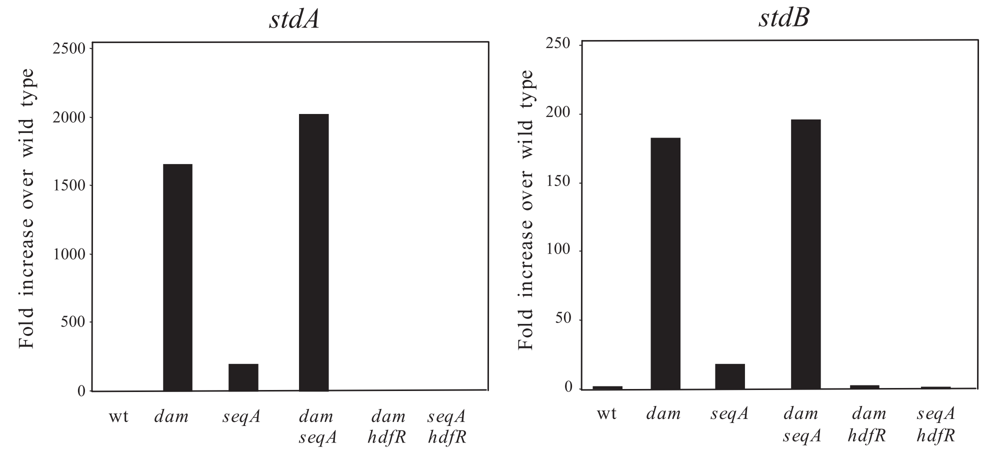


FIG. 4. Relative amounts of *stdA* mRNA and *stdB* mRNA in various genetic backgrounds, normalized to *ompA* mRNA. The strains used were as follows: wild type (ATCC 14028), *dam* (SV4536), *seqA* (SV4752), *dam seqA* (SV4783), *dam hdfR* (SV5638), and *seqA hdfR* (SV5637). Each bar represents the average from three independent experiments. wt, wild type.

cribed DNA was similar in $dam^+$ and *dam* strains, indicating that transcription from the $p_{BAD}$ promoter is insensitive to the presence or absence of Dam methylation (Fig. 3). Because the construct conserves the +1 site of the *std* operon, the 5′ end of the *std* mRNA can be expected to remain unaltered when transcription occurs from the $p_{BAD}$ promoter. Thus, these experiments provide evidence that Dam-dependent control of *std* expression requires its native promoter. This observation is consistent with the hypothesis that Dam-dependent control of *std* expression is transcriptional.

**Genetic screens for transcriptional regulators of *std* operon expression.** Tn*10d*Tc insertions that altered the expression of the *std* operon were initially sought in a $dam^+$ strain carrying a *stdA*::*lacZ* translational fusion (SV5206). Because the operon is not expressed in wild-type serovar Typhimurium, this fusion is $Lac^-$. SV5206 was transduced with 10 pools of Tn*10d*Tc insertions, each containing around 3,000 independent inserts. $Tc^r$ transductants were selected on LB plates containing tetracycline and 5-bromo-4-chloro-3-indolyl-β"(X-Gal), and $Lac^+$ (blue) colonies were visually identified. Tn*10d*Tc insertions in the *dam* gene (as well as upstream insertions polar on *dam*) were identified by cotransduction analysis. For this purpose, phage-free derivatives of the initial isolates were transduced with a lysate of SV4248, a strain that carries a Tn*10d*Cm element 50% linked to *dam*. Whenever ~50% of the $Cm^r$ transductants were $Tc^s$, the isolate was judged to carry a Tn*10d*Tc insertion in or near *dam*. Such mutants were the major class among the candidates analyzed (24/30). Six additional candidates whose insertions did not map in the *aroB-damX-dam* region were subjected to further study. Reverse PCR cloning and sequencing of one Tn*10d*Tc boundary indicated that all six insertions were in *seqA*. Use of the previously characterized Δ*seqA1* allele (39) confirmed that lack of SeqA derepressed *std* expression (Fig. 4, 5, and 6).

A second screen involved use of a $SeqA^-$ strain carrying an *stdA*::*lacZ* translational fusion (SV5719). Because the *std* operon is expressed in a $SeqA^-$ background, this fusion is $Lac^+$. Strain 5719 was transduced with 10 pools of Tn*10d*Tc insertions, each containing some 3,000 independent fusions. $Tc^r$ transductants were selected on LB plates containing tetracycline, kanamycin, and X-Gal, and $Lac^-$ (white) colonies were visually identified. Two candidates of this kind were analyzed as above. Both contained a Tn*10d*Tc insertion in *yifA*. This locus is part of the uncharacterized *yifA-yifE-yifB* region, which lies between *trpT* and *ilvL* at centisome 85 in the *Salmonella* genome map (35). In *E. coli*, the *yifA* homolog encodes a putative LysR-like transcriptional regulator called HdfR (29). Because 82% identity is found between the *E. coli* HdfR and *Salmonella* YifA ORFs (data not shown), the serovar Typhimurium *yifA* gene was renamed *hdfR*.

To rule out potential artifacts associated with the Tn*10d*Tc insertion (e.g., caused by the existence of outward promoters), we constructed an *hdfR* deletion (described in Materials and Methods). Insertion and deletion mutations caused identical effects on *std* expression (data not shown), indicating that the *hdfR*::Tn*10d*Tc insertion alleles described above were null.

**Effects of *dam*, *seqA*, and *hdfR* mutations on *std* operon expression.** The amounts of *std* mRNA in strains carrying *dam*, *seqA*, and *hdfR* mutations were analyzed by quantitative reverse transcription-PCR. Levels of mRNA were monitored for the first gene of the operon, *stdA*, and for the second gene

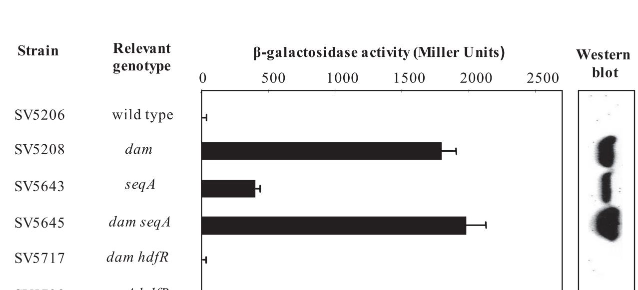


FIG. 5. Expression of the *stdA* gene in *S. enterica* serovar Typhimurium strains carrying *std* regulatory mutations. The left panel shows β-galactosidase activities of a *stdA*::*lacZ* translational fusion constructed on the *Salmonella* chromosome. The right panel shows the levels of StdA protein in protein extracts from the same collection of strains, detected by Western blotting with anti-StdA serum.

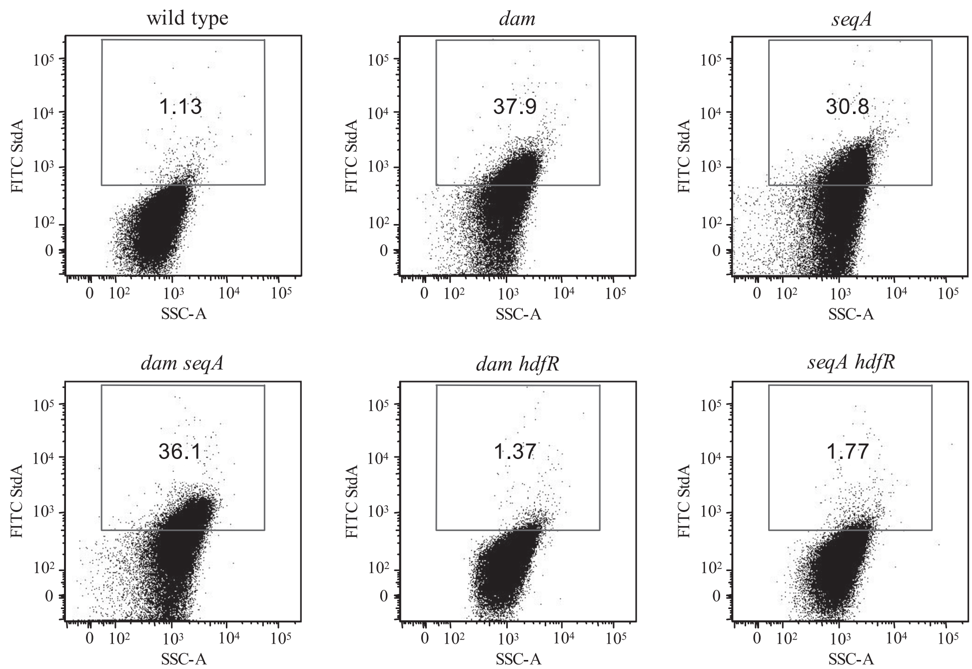


FIG. 6. Flow cytometry analysis of *std* expression. Rabbit anti-StdA antiserum and FITC-conjugated goat anti-rabbit IgG were used for the detection of StdA antigen (*y* axes). Propidium iodide was used for the detection of DNA (*x* axes).The gate for the detection of StdA expression was set such that cells of the wild type (ATCC 14028) were considered positive for expressing StdA antigen when their FITC fluorescence intensity exceeded that of all but a small fraction (<2%) of the control population of the *stdA* mutant (not shown). The strains used were as follows: wild type (ATCC 14028), *dam* (SV4536), *seqA* (SV4752), *dam seqA* (SV4783), *dam hdfR* (SV5638), and *seqA hdfR* (SV5637). SSC-A, side scatter area.

(*stdB*) as well. Data shown in Fig. 4 can be summarized as follows: (i) neither *stdA* nor *stdB* was expressed in the wild type, as previously reported (3, 27); (ii) extremely high levels of *stdA* mRNA (and, to a lesser extent, of *stdB* mRNA) were detected in a *dam* strain, again in agreement with previous observations (3); (iii) *stdA* and *stdB* mRNAs were also detected in a *seqA* background, but their amounts were smaller than in *dam* mutants; (iv) similar amounts of *stdA* and *stdB* mRNAs were found in *seqA dam* and *dam* mutants, indicating that a *dam* mutation is epistatic over a *seqA* mutation; (v) an *hdfR* mutation suppressed *stdAB* expression in both *seqA* and *dam* strains. Altogether, these observations suggest that Dam methylation and SeqA repress *std* operon expression and that HdfR activates *std* expression if either SeqA is absent or the genome lacks $N^6$-methyl-adenosine. Lack of HdfR does not alter *std* expression in a $seqA^+$ $dam^+$ background (data not shown). The epistatic effect of a *dam* mutation over a *seqA* mutation is consistent with the well-known incapacity of SeqA to bind nonmethylated DNA (53).

The effects of *dam*, *seqA*, and *hdfR* mutations on *std* operon expression were also examined by β-galactosidase assays using a translational *stdA*::*lacZ* fusion and by Western blotting with polyclonal anti-StdA antibody. The results, shown in Fig. 5, were fully consistent with those described above: Dam methylation and the GATC-binding protein SeqA are *std* repressors, and HdfR is an *std* activator in the absence of either Dam methylation or SeqA.

**Flow cytometry analysis of StdA production.** To monitor expression of StdA in individual serovar Typhimurium cells, cultures of strains ATCC 14028 (wild type) and its isogenic derivatives SV4536 (Δ*dam*), SV4752 (Δ*seqA*), SV4783 (Δ*dam* Δ*seqA*), SV5638 (Δ*dam* Δ*hdfR*), and SV5637 (Δ*seqA* Δ*hdfR*) were subjected to flow cytometry analysis, using rabbit anti-StdA antiserum and FITC-conjugated goat anti-rabbit IgG for the detection of StdA antigen and propidium iodide for the detection of DNA. Only 1.3% of wild-type cells produced StdA (Fig. 6). In contrast, expression of StdA was detected in 37.9% of cells in the *dam* mutant, 30.8% in the *seqA* mutant, and 36.0% in the double mutant *dam seqA*. Knockout of *hdfR* abolished StdA expression: only 1.37% of cells expressed StdA in the *dam hdfR* double mutant, and 1.77% of cells expressed StdA in the *seqA hdfR* double mutant. These observations confirm the regulatory patterns described above. Furthermore, individual cell analysis shows that the *std* derepression observed in *dam* and *seqA* mutants does not involve a massive, uniform response of the bacterial population but the formation of an Std-expressing subpopulation.

## DISCUSSION

The genome of *S. enterica* serovar Typhimurium contains 13 fimbrial loci that constitute a potential arsenal of antigens and attachment factors for the interaction with host tissues (26, 28). One such locus is the *std* operon (35). Like the majority of *S. enterica* serovar Typhimurium fimbrial operons, *std* is not expressed under laboratory conditions (27). In contrast, Std fimbriae are synthesized during infection and play a role in virulence, as indicated by the following observations: (i) StdA, the major protein of Std fimbriae, is detected upon infection of bovine ileal loops (27); (ii) mice infected with serovar Typhimurium seroconvert to StdA (26); and (iii) *std* deletion reduces intestinal persistence of serovar Typhimurium infection (51).

However, synthesis of Std fimbriae inside the animal host must remain under tight control, as indicated by the observation that uncontrolled *std* expression in *rosE* mutants reduces their ability to colonize both the cecum and the spleen of mice (9). In this study, we provide additional evidence that massive synthesis of Std fimbriae is detrimental for *Salmonella* virulence: the extreme attenuation of Dam mutants upon oral infection (17, 21) is partially suppressed by deletion of *std*. Hence, optimal *Salmonella* infection may require either restrained levels or *std* expression or formation of an Std-expressing bacterial subpopulation (see below).

Our genetic screens for the identification of mutations that derepressed *std* operon expression in vitro identified Tn*10d*Tc insertions in or upstream of *dam*, as previously described (3), and in *seqA*. Expression of *std* was found to be identical in *dam* and *dam seqA* mutants, indicating that SeqA is unable to repress *std* in the absence of Dam methylation. Because *seqA* mutations are less pleiotropic than *dam* mutations (and *seqA* mutants are healthier than *dam* mutants), the screen for mutations that suppressed *std* expression was performed in a *seqA* strain instead of a *dam* mutant as initially planned. This second screen identified HdfR (previously, YifA) as a function needed for *std* expression in *dam* and *seqA* mutants. Hence, HdfR appears to be an *std* activator whose action is antagonized by both Dam methylation and SeqA.

The mechanisms underlying SeqA- and HdfR-mediated regulation of the *std* operon remain to be investigated. However, because SeqA is a DNA-binding protein (33), it seems reasonable to suspect that it might be a transcriptional repressor of *std*. This view is supported by the involvement of SeqA in transcriptional regulation of other genes, such as the lambda $P_R$ promoter (44) and the *agn43* gene of *E. coli* (10). In the wild type, SeqA binding to methylated and hemimethylated GATCs could endlessly maintain repression under laboratory conditions, thereby explaining why *std* is not expressed in batch cultures of serovar Typhimurium. In *dam* mutants, however, the well-known inability of SeqA to bind nonmethylated DNA (53) would permit *std* derepression. This hypothesis is supported by the observation that *dam* mutations are epistatic over *seqA* mutations regarding *std* operon derepression. With respect to HdfR, its *E. coli* counterpart has been characterized as an LysR-like transcriptional regulator that represses *flhDC* transcription (29). However, many LysR-like proteins are transcriptional activators (42). It is thus conceivable that HdfR might be a transcriptional activator of *std* transcription.

Although previous studies had detected enormous amounts of *std* mRNA and StdA protein in extracts from serovar Typhimurium *dam* strains (1, 3), examination of *std* expression in individual cells provided the noteworthy observation that synthesis of Std fimbriae occurs in only a fraction (≥30%) of *dam* and *seqA Salmonella* cells. The possibility that *std* undergoes either bistable expression (12) or phase variation (47) in *dam* and *seqA* mutants of serovar Typhimurium can thus be considered. Additional, intriguing questions concern the mechanisms that derepress *std* transcription inside the animal host. The observation that excess Std synthesis by *rosE* and *dam* mutants is detrimental in vivo (9; also above) argues in favor of self-limited Std expression in the animal environment, perhaps involving bistable or phase-variable *std* expression, as observed under laboratory conditions in *dam* and *seqA* mutants. An attractive (albeit speculative) model is that competition between a transcriptional activator (HdfR) and transcriptional repressors (SeqA and perhaps RosE) might create lineages of $std^+$ and *std* cells, in a manner reminiscent of the self-propagating states described for phase-variable loci like *pap* (23) and *agn43* (20, 48). However, *std* regulation presents unique features. One is that the operon is fully repressed under laboratory conditions, and subpopulation formation is observed only upon derepression by *dam* and *seqA* mutations. Another specific trait of *std* regulation is the involvement of poorly understood cell functions such as those of RosE and HdfR, whose study might unveil novel mechanisms of fimbrial control.

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