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 unmethylated 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 “hemi-methylation” 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 associated 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 methylation 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 S. enterica, 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.

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 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.

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 50 μl/giter agar. 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′-CCG CAG GAG TTG CAG GTG CTA GGA CTA GCC GGG CGT GTA GGC TGG AGC AGC TTC TCG TTC-3′ and 5′-TTT GCT TAC TAC CAT CAA CTC ACC TGA TAT CGG GAT CCT TTC-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′-GGG GAC GGC TTC TCC TTC TCC TTG TCC CTG TTC ACC-3′ and 5′-GCC GCC GAT ACC AAT CCT GGG GAT GAG TCT AGC CCC CTA TCC ACC-3′. An internal 2,416-bp deletion in the stdA open reading frame (ORF) was created with the primers 5′-GGG GAC GGC TTC TCC TTC TCC TTG TCC CTG TTC ACC-3′ and 5′-GCC GCC GAT ACC AAT CCT GGG GAT GAG TCT AGC CCC CTA TCC ACC-3′. This 3,310-bp deletion that removed DNA from both stdA and stdB was obtained with primers 5′-GGG GAC GGC TTC TCC TTC TCC TTG TCC CTG TTC ACC-3′ and 5′-GCC GCC GAT ACC AAT CCT GGG GAT GAG TCT AGC CCC CTA TCC ACC-3′.

β-Galactosidase assays. Levels of β-galactosidase activity were assayed as described by Miller (36), using the CHCl₃-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 [γ-³²P]ATP and annealed to 20 μg of RNA. For annealing, 10⁴ 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.
was used to integrate plasmid pCE40 (15), thereby generating a translational stdA::lacZ fusion.

Construction of strains expressing std from the arabinose-dependent pBAD promoter (SV5657 and SV5658). Construction of strains expressing std from the arabinose-dependent pBAD promoter followed a procedure similar to that reported by Figueroa-Bossi et al. (16). The chromosomal gene araB of strain SV5651 (ΔstdA::Cmr) 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 arabinose. As a template, we used genomic DNA of strain SV5139 (stdB::Cmr). The primers used to amplify stdA::stdB::Cmr (with extensions homologous to araB boundaries) were 5′-TTA GCA TTT TGG TCC ATA GGA TTA GCG GAT CCT GCC TGA CTA GTC GTA ATA AAA TAA TAC TTG CC-3′ and 5′-GAT GAC GGT TAA TAC CGA ACG CTA CCG CTA CGA GTA AAA TCAC-3′. The resulting PCR product was used to perform transformations of derivatives of SV5651 and SV5652 (dam ΔstdA and dam ΔstdB, 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 (pBAD::std stdA::Cmr ΔstdB and pBAD::std stdB::Cmr ΔstdA Δlam-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 stacking SDS-PAGE gel buffer (1% sodium dodecyl sulfate, 30% [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 luminol/ luminol reagents.

Quantitative reverse transcriptase PCR (real-time PCR) and calculation of relative expression levels. Salmonella RNA was extracted from stationary phase cultures using a 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 (Turbogene Probe, 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 in 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 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://frodo.wi.mit.edu/primer3), 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′-GCC CTG CGA TTA GTA TGA-3′ and 5′-GGG CCG GTG CTT GCG CTA GAC-3′; and for stdB, 5′-CGC CCG CCC TCT CTT CAG-3′ and 5′-GAG GGT GAC GTC TGC ATT ACT G-3′.

Cloning and molecular characterization of Tn10Tc insertions. Amplification of DNA sequences close to Tn10Tc insertions was achieved by inverse PCR. Genomic DNA from each Tn10Tc-carrying isolate was digested with Smal and PstI. The resulting fragment was autolysed and used as a template in a two-step PCR amplifications with the primer 5′-ATT TGA TCA TAT GAC AAG ATG TG-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 table top 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 table top 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 × 10^8 cells/ml. For each sample, the fluorescence of 10,000 particles (bacterial cells) was measured by flow cytometry (FACS Calibur; Becton Dickson, 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 10^9 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 “canceled-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 rseE 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
In silico analysis of the DNA sequence upstream of the +1 site identified DNA sequences with features similar to those of canonical, σ70-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.

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

**Genetic screens for transcriptional regulators of \( \text{std} \) operon expression.** Tn10dTc insertions that altered the expression of the \( \text{std} \) operon were initially sought in a \( \text{dam}^{+} \) strain carrying a \( \text{stdA} : \text{lacZ} \) 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 Tn10dTc insertions, each containing around 3,000 independent insertions. Tc⁺ transductants were selected on LB plates containing tetracycline, kanamycin, and X-Gal, and Lac⁺ (white) colonies were visually identified. Tn10dTc insertions that altered the expression of the \( \text{std} \) gene (as well as upstream insertions polar on \( \text{dam} \)) 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 Tn10dCm element 50% linked to \( \text{dam} \). Whenever ~50% of the Cm⁺ transductants were Tc⁺, the isolate was judged to carry a Tn10dTc insertion in or near \( \text{dam} \). Such mutants were the major class among the candidates analyzed (24/30). Six additional candidates whose insertions did not map in the \( \text{aroB-dam-dam} \) region were subjected to further study. Reverse PCR cloning and sequencing of one Tn10dTc boundary indicated that all six insertions were in \( \text{seqA} \). Use of the previously characterized \( \Delta \text{seqA1} \) allele (39) confirmed that lack of SeqA derepressed \( \text{std} \) expression (Fig. 4, 5, and 6).

A second screen involved use of a SeqA⁻ strain carrying an \( \text{stdA} : \text{lacZ} \) translational fusion (SV5719). Because the \( \text{std} \) operon is expressed in a SeqA⁻ background, this fusion is Lac⁺. Strain 5719 was transduced with 10 pools of Tn10dTc insertions, each containing some 3,000 independent fusions. Tc⁺ 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 Tn10dTc insertion in \( \text{yifA} \). This locus is part of the uncharacterized \( \text{yifA-yifE-yifB} \) region, which lies between \( \text{trpT} \) and \( \text{ihbL} \) at centisome 85 in the Salmonella genome map (35). In \( E. \text{coli} \), the \( \text{yifA} \) homolog encodes a putative LysR-like transcriptional regulator called HdfR (29). Because 82% identity is found between the \( E. \text{coli} \) HdfR and Salmonella YifA ORFs (data not shown), the serovar Typhimurium \( \text{yifA} \) gene was renamed \( \text{hdfR} \).

To rule out potential artifacts associated with the Tn10dTc insertion (e.g., caused by the existence of outward promoters), we constructed an \( \text{hdfR} \) deletion (described in Materials and Methods). Insertion and deletion mutations caused identical effects on \( \text{std} \) expression (data not shown), indicating that the \( \text{hdfR} \)::Tn10dTc insertion alleles described above were null.

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

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

**FIG. 5.** Expression of the \( \text{stdA} \) gene in \( S. \text{enterica} \) serovar Typhimurium strains carrying \( \text{std} \) regulatory mutations. The left panel shows \( \beta \)-galactosidase activities of a \( \text{stdA} : \text{lacZ} \) 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.
derivatives SV4536 (H9004), SV4752 (H9004), SV4783 (seqA), expression of StdA in individual serovar Typhimurium cells, methylation or SeqA.

N lacks stdsors, and HdfR is an ylation and the GATC-binding protein SeqA are were fully consistent with those described above: Dam methylation and the GATC-binding protein SeqA are 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 (Δdam), SV4752 (ΔseqA), SV4783 (Δdam ΔseqA), SV5638 (Δdam ΔhdfR), and SV5637 (ΔseqA ΔhdfR) 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 Tn10Tc 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 mutant 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 Pr promoter (44) and the agrn43 gene of E. coli (10). In the wild type, SeqA binding to methylated and hemimethylated GATC positions 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 fthDC 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 agrn43 (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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REFERENCES


