

DNA Adenine Methylation Regulates Virulence Gene Expression in *Salmonella enterica* Serovar Typhimurium[∇]

Roberto Balbontín,¹ Gary Rowley,² M. Graciela Pucciarelli,³ Javier López-Garrido,¹
Yvette Wormstone,² Sacha Lucchini,² Francisco García-del Portillo,³
Jay C. D. Hinton,² and Josep Casadesús^{1*}

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41080 Seville, Spain¹; Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom²; and Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología (CSIC), Campus de Cantoblanco, 28049 Madrid, Spain³

Received 14 June 2006/Accepted 6 September 2006

Transcriptomic analyses during growth in Luria-Bertani medium were performed in strain SL1344 of *Salmonella enterica* serovar Typhimurium and in two isogenic derivatives lacking Dam methylase. More genes were repressed than were activated by Dam methylation (139 versus 37). Key genes that were differentially regulated by Dam methylation were verified independently. The largest classes of Dam-repressed genes included genes belonging to the SOS regulon, as previously described in *Escherichia coli*, and genes of the SOS-inducible *Salmonella* prophages ST64B, Gifsy-1, and Fels-2. Dam-dependent virulence-related genes were also identified. Invasion genes in pathogenicity island SPI-1 were activated by Dam methylation, while the fimbrial operon *std* was repressed by Dam methylation. Certain flagellar genes were repressed by Dam methylation, and Dam⁻ mutants of *S. enterica* showed reduced motility. Altered expression patterns in the absence of Dam methylation were also found for the chemotaxis genes *cheR* (repressed by Dam) and STM3216 (activated by Dam) and for the Braun lipoprotein gene, *lppB* (activated by Dam). The requirement for DNA adenine methylation in the regulation of specific virulence genes suggests that certain defects of *Salmonella* Dam⁻ mutants in the mouse model may be caused by altered patterns of gene expression.

In gammaproteobacteria, 5'-GATC-3' sites serve as targets for DNA adenine methyltransferase (or Dam methylase). The genome of *Salmonella enterica* serovar Typhimurium contains over 19,000 GATC sites (41), and a similar number are found in *Escherichia coli* (4). After DNA replication, Dam methylase introduces a methyl group at the N⁶ position of adenosine moieties in the newly synthesized strand, thereby generating methylated DNA (35, 37, 40, 66). Because the Dam methylase protein trails the replication machinery at a small distance, hemimethylated DNA is usually short-lived (40). At certain GATC sites, however, methylation of the newly synthesized strand is hindered by binding of proteins that protect GATC sites from Dam methylase, such as the GATC-binding protein SeqA, the leucine-responsive regulatory protein (Lrp), and the redox-sensitive regulator OxyR (20, 35, 66). Methylation protection can either cause a temporary delay in methylation or generate GATC sites that are stably hemimethylated or unmethylated (35, 66).

When a GATC site is embedded within a protein-binding sequence, its methylation state can affect protein-DNA interactions (46). For instance, the mismatch repair endonuclease MutH is active only on hemimethylated or unmethylated GATC sites, while the replication initiator DnaA binds to the chromosome replication origin only when the appropriate GATC sites are methylated (27). At certain promoters, the

methylation state of specific GATC sites regulates binding of either RNA polymerase or transcription factors (66). Dam methylation can activate or repress transcription, and the regulatory GATC sites can be located in the promoter itself (51), in upstream regulatory regions (8, 24), or in an operator overlapping the coding sequence (20, 63). The classes of published Dam-regulated genes of *E. coli* and *Salmonella* can be assigned to the following categories. (i) Genes with transcription coupled to the cell cycle, such as the transposase gene (*tnp*) of insertion element IS10 (51), the *trpR* gene of *E. coli* (39), and the *traJ* gene of the *Salmonella* virulence plasmid (8). In these examples, Dam methylation acts as a transcriptional repressor, and transcription is activated by hemimethylation. In other cases, Dam methylation can activate transcription in a cell-cycle-coupled fashion. For instance, the p₂ promoter of the *E. coli dnaA* gene is activated by GATC methylation and remains inactive if hemimethylated (5, 33). (ii) Genes regulated by Dam methylation patterns. Paradigms of this class are genes subjected to phase variation, such as the *pap* operon of uropathogenic *E. coli* (24), the *E. coli agn43* gene (20, 63), and the *pef* operon of *Salmonella enterica* serovar Typhimurium (44). In these examples, binding of a regulatory protein to hemimethylated DNA results in methylation hindrance, and the hemimethylated GATC sites become unmethylated after two replication rounds (20, 24, 63).

In many (perhaps all) Dam-regulated promoters, GATC hemimethylation and unmethylation have similar phenotypic effects: for instance, the hemimethylation-activated *tnp*(IS10) and *traJ* promoters are also active in a Dam⁻ background (8, 51), and the hemimethylation-repressed *dnaA2* promoter remains inactive in a Dam⁻ host (5, 33). In turn, the absence of

* Corresponding author. Mailing address: Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, E-41080 Sevilla, Spain. Phone: 34 95 455 7105. Fax: 34 95 455 7104. E-mail: casadesus@us.es.

[∇] Published ahead of print on 22 September 2006.

Dam methylation abolishes phase variation in *agn43* and *pap* and locks both loci in the off state (20, 23). The equivalence of GATC hemimethylation and unmethylation at Dam-regulated promoters facilitates the detection of Dam-regulated loci by comparing gene expression in Dam⁺ and Dam⁻ hosts. One such genetic screen, based on the use of MudJ-generated *lac* fusions, permitted the first description of a Dam-regulated locus in *S. enterica* (60). In the last few years, classical genetic screens have been superseded by high-throughput functional genomic methods (19, 25, 58, 67). In *E. coli*, DNA microarray analysis of the transcriptome, combined with two-dimensional electrophoretic analysis of the proteome, permitted the identification of genes showing impaired expression in Dam⁺ and Dam⁻ hosts (36, 45, 50). The overall number of *E. coli* genes regulated by Dam methylation remains controversial and ranges from 17 known genes (36) to several hundred genes, many of which have unknown functions (45, 50). Furthermore, the vastly different growth conditions chosen in these studies may explain some of the differences.

Dam⁻ mutants of *S. enterica* serovar Typhimurium are severely attenuated in the mouse model (16, 21) and display a plethora of virulence-related defects: reduced secretion of invasion proteins, which impairs invasion of cultured epithelial cells (16); reduced cytotoxicity after infection of M cells (16); inefficient colonization of Peyer's patches and mesenteric lymph nodes (16, 21); sensitivity to bile and to other DNA-damaging agents produced inside the animal (22, 47); and envelope instability accompanied by leakage of proteins that may activate the host immune system (48). This study describes the first transcriptomic study of Dam methylation in *Salmonella enterica* serovar Typhimurium and provides evidence that Dam methylation regulates the invasion genes of pathogenicity island I (SPI-1), the Braun lipoprotein gene, flagellar genes, and the fimbrial operon *stdABC*. Tentative correlations can thus be established between specific alterations of gene expression and certain virulence defects of *Salmonella* Dam⁻ mutants.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *Salmonella enterica* listed in Table 1 belong to serovar Typhimurium. All strains derive from SL1344 (26) unless specified otherwise. The wild type and the mutants used for microarray analysis were derived from the same isolate of SL1344 used for other transcriptomic analyses (14, 38). Strain SV4203 carries a MudJ element inserted in the coding sequence of the *dam* gene (59). Strain JH3294 carries an in-frame deletion in the *dam* coding sequence, constructed by the procedure of Datsenko and Wanner (12). The oligonucleotides used for this construction and the external PCR primers (*dam*-E1 and *dam*-E2) used to verify the predicted deletion are available at <http://alojamientos.us.es/genbac>. Transfer of transposon-tagged alleles between *Salmonella* strains was carried out by P22 HT transduction (54). P22 HT transduction was also used for transfer of the *dam* deletion generated by the procedure of Datsenko and Wanner (12) into a clean SL1344 background. Once transduced, the kanamycin cassette was excised from the *dam* locus by FLP recombinase using plasmid pCP20 (12), rendering an unmarked deletion. The allele *fliC5050::MudJ* was provided by Kelly T. Hughes (Department of Biology, University of Utah, Salt Lake City). The *sipC::lac*(Tn5Tc) transcriptional fusion of strain EE638 was described previously (2). Plasmid pIZ999 is a pMM40 derivative carrying the *dam* gene of *S. enterica* serovar Typhimurium strain SL1344 under the control of a *tac* promoter (A. I. Prieto, unpublished data).

Construction of transcriptional *lac* fusions in SPI-1 genes. The *hilA*, *hilC*, *invF*, *sipB*, *sicA*, and *prgH* genes of strain SL1344 were disrupted by gene targeting using the procedure of Datsenko and Wanner (12). The oligonucleotide pairs used for these constructions and the external ("E") PCR primers employed to verify the predicted deletions are available from the authors. FRT sites gener-

TABLE 1. Bacterial strains

Strain	Genotype	Reference or source ^a
SL1344	<i>rpsL hisG</i>	26
EE638	<i>sipC::Tn5(Tc)lacZ</i>	2
JH3294	Δ <i>dam-231</i>	
SV3000	LT2 <i>dam-201::Tn10dTc</i>	60
SV4203	<i>dam-225::MudJ</i>	59
SV4204	<i>dam-201::Tn10dTc</i>	Laboratory stock
SV4536	ATCC 14028 Δ <i>dam-230</i>	Laboratory stock
SV5197	<i>fliC5050::MudJ</i>	
SV5198	<i>fliC5050::MudJ dam-201::Tn10dTc</i>	
SV5202	<i>sipC::Tn5(Tc)lacZ dam-225::MudJ</i>	
SV5246	ϕ <i>hilA</i> '- <i>lacZY</i>	
SV5247	ϕ <i>hilA</i> '- <i>lacZY</i> Δ <i>dam-231</i>	
SV5248	ϕ <i>hilC</i> '- <i>lacZY</i>	
SV5249	ϕ <i>hilC</i> '- <i>lacZY</i> Δ <i>dam-231</i>	
SV5252	ϕ <i>sicA</i> '- <i>lacZY</i>	
SV5253	ϕ <i>sicA</i> '- <i>lacZY</i> Δ <i>dam-231</i>	
SV5254	ϕ <i>sipB</i> '- <i>lacZY</i>	
SV5255	ϕ <i>sipB</i> '- <i>lacZY</i> Δ <i>dam-231</i>	
SV5258	ϕ <i>invF</i> '- <i>lacZY</i>	
SV5259	ϕ <i>invF</i> '- <i>lacZY</i> Δ <i>dam-231</i>	
SV5262	ϕ <i>prgH</i> '- <i>lacZY</i>	
SV5263	ϕ <i>prgH</i> '- <i>lacZY</i> Δ <i>dam-231</i>	

^a Omitted for strains constructed for this study.

ated by excision of kanamycin resistance cassettes were used to integrate plasmid pCE37 (13), generating transcriptional *lac* fusions.

Culture media and conditions. Unless otherwise indicated, the experiments described in this study were carried out with cultures grown in Luria-Bertani (LB) medium. Solid LB medium contained agar at a 1.5% final concentration. Antibiotics were used at the final concentrations described elsewhere (61). For complementation analysis using the *dam*-carrying plasmid pIZ999, IPTG (isopropyl- β -D-thiogalactopyranoside) was used at a final concentration of 1 mM. Motility assays were carried out in LB medium prepared without yeast extract (17). Solid motility medium contained agar at a 0.25% final concentration (17).

RNA isolation, microarray procedures, and data analysis. To prepare cells for RNA extraction, 25 ml of fresh LB medium was inoculated at 1:100 from an overnight bacterial culture and grown in a 250-ml flask incubated with shaking at 250 rpm in a New Brunswick Innova 3100 water bath at 37°C. Three biological replicates were performed for each strain, and RNA was extracted at an optical density at 600 nm of 0.3 (mid-exponential phase). RNA was extracted using Promega's SV 96 total RNA purification kit. RNA quality was assessed on an Agilent 2100 Bioanalyser (38). Transcriptomic analyses were performed on a SALSA microarray that contained the 5,000 open reading frames (ORFs) identified from the partial sequence of *S. enterica* serovar Typhimurium SL1344. Unpublished genome sequence data from strains SL1344, DT104, and PT4 were obtained from the Wellcome Trust Sanger Institute (<ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella>). Regions unique to each strain were identified by comparison with the published LT2 genome (PubMed identifier [PMID] 11677609) using MUMmer (PMID, 14759262). Putative coding sequences within these regions, as identified using Glimmer (PMID, 10556321), were used in the design of the SALSA microarray. Hybridization, microarray scanning, and data analysis were performed as described previously (32), using a false-discovery rate of 0.1.

β -Galactosidase assays. Levels of β -galactosidase activity were assayed as described by Miller (42), using the CHCl₃-sodium dodecyl sulfate permeabilization procedure.

Subcellular fractionation and Western analysis. Bacteria grown statically overnight at 37°C in LB medium were spun down by centrifugation at 15,000 \times g for 15 min at 4°C. The supernatant was filtered through Millipore 0.2- μ m-pore-size filters, and extracellular proteins were precipitated by a 10% trichloroacetic acid/acetone washing procedure (31). To prepare membrane extracts, pelleted bacteria were resuspended in cold phosphate-buffered saline (PBS) buffer, pH 7.4, and disrupted by sonication. Unbroken cells were further removed by low-speed centrifugation (5,000 \times g; 5 min; 4°C). The supernatant was centrifuged at high speed (200,000 \times g; 15 min; 4°C). The pellet containing envelope material was suspended in PBS buffer, pH 7.4, and an appropriate volume of Laemmli buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 1%

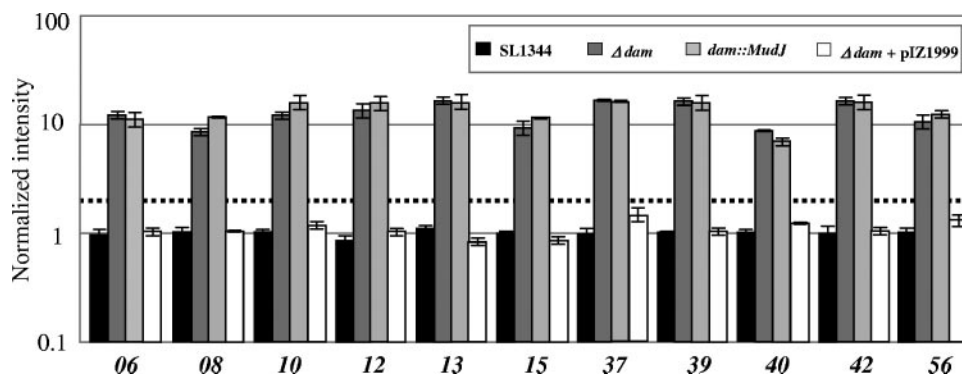


FIG. 1. Relative expression levels of selected prophage ST64B gene transcripts in a Dam^+ strain (SL1344), in two Dam^- mutants (JH3294 and SV4203), and in a Dam^- mutant (JH3294) complemented with a wild-type dam^+ allele (carried on pIZ999). The prophage gene numbers (bottom) are taken from its genome sequence, accession number NC004313. The error bars represent the standard errors of the mean. The dashed line represents the twofold cutoff applied. Normalized intensity refers to normalization to a wild-type SL1344 relative expression value of 1.0.

2-mercaptoethanol, and 0.002% bromophenol blue) was then added. For the analysis of the bacterium-associated SipC content, the bacterial pellet was directly suspended in an appropriate volume of PBS buffer, pH 7.4, and Laemmli buffer was then added. All samples were heated (100°C; 5 min) and cleared by centrifugation (15,000 \times g; 5 min; room temperature) before being loaded into gels. Proteins were resolved by SDS-Tris-Tricine electrophoresis using 10% acrylamide gels (53) and transferred onto polyvinylidene-difluoride membranes using a semidry electrophoresis transfer apparatus (Bio-Rad). Specific proteins were detected with the following primary antibodies: polyclonal rabbit anti-StdA (28) and polyclonal rabbit anti-SipC (48). Goat anti-rabbit immunoglobulin G conjugated to peroxidase was used as a secondary antibody to detect specific proteins by the ECL assay (Amersham Biosciences Europe, Cerdanyola, Spain).

Motility assays. Cultures were prepared as described elsewhere (10). At the stage of mid-exponential growth, a sterile toothpick was soaked in the culture and used to inoculate a motility agar plate. Bacterial growth halos were compared after incubation at 37°C.

RESULTS

Transcriptomic analyses of Dam methylation-dependent gene expression. Wild-type (SL1344) isogenic Dam^- mutants (JH3294 and SV4203) and a JH3294 derivative carrying a dam^+ complementing plasmid were grown to mid-exponential phase in LB medium.

Two distinct Dam^- mutants were studied: one had a deletion of the complete dam coding sequence; the other had a *MudJ* insertion inside the dam gene. Changes in gene expression patterns were detected by comparing each Dam^- mutant with the wild type, and differences putatively caused by absence of Dam methylation were confirmed by complementation. Because overproduction of Dam methylase reproduces certain phenotypes of Dam^- mutants (40, 60), complementation was achieved with a low-copy-number plasmid carrying the *S. enterica* serovar Typhimurium dam gene (pIZ999). Only genes which showed altered transcription in both Dam^- strains after statistical filtering were taken into account. Overall, the mRNA levels of 139 genes were >2-fold higher in both Dam^- mutants, and the mRNA levels of 37 genes were >2-fold lower in both Dam^- mutants (data available at <http://alojamientos.us.es/genbac>). Higher activity in a Dam^- background indicated that Dam methylation represses gene expression in the wild type. In turn, lower activity in a Dam^- background indicated that Dam methylation activates gene expression in the wild type. The relevant transcriptomic data can be summarized as follows.

(i) SOS regulon genes. A number of genes belonging to the SOS response regulon (e.g., *umuD*, *yebG*, *yebE*, *yebF*, and *ysdB*) showed higher activity in a Dam^- background, in accordance with data previously described in an *E. coli* transcriptomic study (36). Because SOS induction in the absence of Dam methylation is a well-known phenomenon in both *E. coli* and *Salmonella* (18, 40, 60), increased SOS gene expression in Dam^- mutants provided useful confirmatory data for our transcriptomic analyses.

(ii) Prophage genes. Genes belonging to the ST64B, Gifsy-1, and Fels-1 prophages showed increased expression in a Dam^- host. Increased gene expression in the absence of Dam methylation was especially remarkable in ST64B: most of the prophage genes were affected (see the data for 11 arbitrarily chosen ST64B ORFs in Fig. 1).

(iii) Conjugal-transfer genes. The transfer operon (*tra*) of the virulence plasmid (pSLT) showed increased expression in a Dam^- host, confirming previous reports (7, 60, 61).

(iv) Virulence-related genes. A number of virulence-related genes showed differential Dam-dependent expression (Table 2). Genes that were potentially related to previously known phenotypes of Dam^- mutants (e.g., reduced invasion of epithelial cells and increased release of proteins [16, 48]) were chosen for further study. Additional virulence-related genes regulated by Dam methylation, not included in Table 2, were STM3216, which encodes a putative chemotaxis protein (64), and the STM3026 and STM4261 ORFs, which are required for colonization of bovine ileal loops (43). STM3216 and STM4261 are activated by Dam methylation, while STM3026 is a Dam-repressed locus (data not shown).

Regulation of *std* fimbrial genes. Among the Dam-repressed genes, *stdA* and *stdB* showed the highest levels of Dam-dependent regulation (Table 2 and Fig. 2). Significant derepression in a Dam^- background was also found for *stdC* and to a lesser extent for the downstream genes STM3026 and STM3025 (Table 2 and Fig. 2). Dam-mediated repression of the *std* operon was confirmed by Western hybridization: large amounts of StdA protein were found in both the membrane and the supernatant of Dam^- strains grown in LB, while little or no StdA protein was found in the wild type (Fig. 3). Repression of *std* by Dam methylation is not only observed in strain SL1344; iden-

TABLE 2. Dam methylation-dependent virulence genes identified by transcriptomic analyses of *S. enterica* serovar Typhimurium^a

Gene	Protein function	<i>dam</i> mutant/wild-type expression ratio	
		Δdam^b	<i>dam::MudJ</i> ^c
<i>fliC</i>	Flagellin structural protein	3.72	3.07
<i>fliD</i>	Flagellar capping protein	2.04	3.02
<i>lppB</i>	Braun lipoprotein	0.36	0.26
<i>prgH</i>	SPI-1 TTSS ^d structural protein	0.43	0.70
<i>prgI</i>	SPI-1 TTSS structural protein	0.38	0.60
<i>prgJ</i>	Cytoplasmic cell invasion protein	0.31	0.57
<i>sipB</i>	SPI-1 translocated effector protein	0.35	0.40
<i>sipC</i>	SPI-1 translocated effector protein	0.25	0.46
<i>sipD</i>	SPI-1 translocated effector protein	0.28	0.49
<i>sicA (sipE)</i>	SPI-1 translocated effector protein	0.33	0.52
<i>pipC</i>	SPI-1 protein, unknown function	0.33	0.37
<i>cheR</i>	Response regulator for chemotaxis	2.04	2.55
<i>stdA</i>	Fimbrial protein	147.60	139.10
<i>stdB</i>	Outer membrane usher protein	17.94	17.20
<i>stdC</i>	Fimbrial chaperone	10.37	7.21
STM3025	Putative cytoplasmic protein	2.55	2.01
STM3026	Putative outer membrane protein	4.77	4.86

^a Dam methylation is an activator for genes with a relative expression value below 1.0 and a repressor for genes with values above 1.0.

^b Strain JH3294.

^c Strain SV4203.

^d TTSS, type three secretion system.

tical expression patterns are found in ATCC 14028 and LT2 (Fig. 3). Std fimbriae are required for long-term intestinal carriage of *S. enterica* serovar Typhimurium; however, they are dispensable for acute infection in the mouse model (65). Sim-

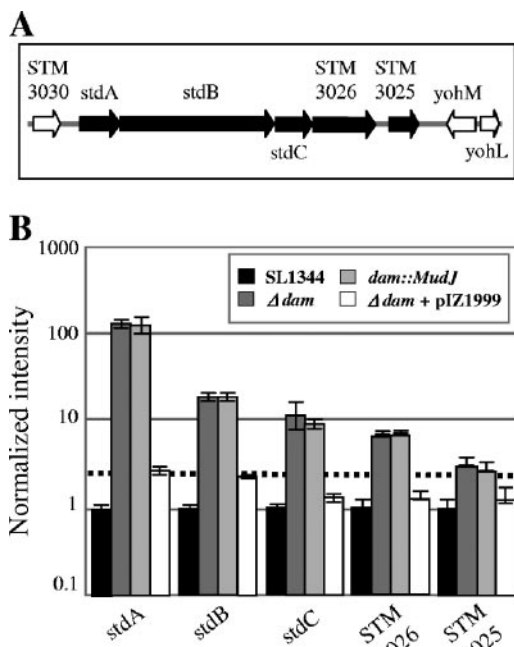


FIG. 2. (A) Diagram of the *std* operon showing its five ORFs, all arranged in the same orientation. The flanking genes STM3030 and *yohM* are also shown. (B) Relative expression levels of *std* genes in a *Dam*⁺ strain (SL1344), two *Dam*⁻ mutants (JH3294 and SV4203), and a *Dam*⁻ mutant complemented with a wild-type *dam*⁺ allele (JH3294/pIZ999). The error bars represent the standard errors of the mean. The dashed line represents the twofold cutoff applied.

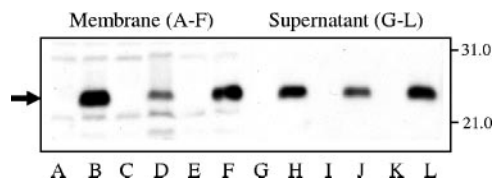


FIG. 3. Levels of StdA fimbrial protein (indicated by an arrow) in membrane and supernatant protein extracts prepared from various wild-type strains of *S. enterica* serovar Typhimurium and from isogenic *Dam*⁻ mutants. In all samples, the amount of extract loaded corresponded to the equivalent of 2×10^7 colony-forming units. Lanes: A and G, SL1344; B and H, SV4203 (*Dam*⁻); C and I, ATCC 14028; D and J, SV4536 (*Dam*⁻); E and K, LT2; F and L, SV3000 (*Dam*⁻). Lanes A to F are from membrane protein preparations, and lanes G to L are from supernatant protein preparations. The StdA protein was identified by Western blotting with a polyclonal anti-StdA antibody.

ilar types of functional redundancy have been reported for other fimbrial operons, and a current view is that alternative adhesins can replace the loss of individual fimbrial types (62). The *stdABC* operon is repressed in LB medium and becomes up-regulated in bovine ileal loops (28). Our observations suggest that Dam methylation may be a key factor for repression of the *stdABC* operon outside the intestinal milieu, and they are supported by a previous study showing that StdA is one of the most abundant protein species in a *Dam*⁻ strain (1). In silico examination of the *std* DNA sequence reveals the existence of a cluster of GATC motifs upstream of the putative *stdA* promoter: three GATC motifs in a 25-bp interval, a density 10-fold higher than expected from a random distribution (data not shown). This GATC clustering is reminiscent of that of other *Dam*-regulated genes, in which the methylation state of critical GATC sites affects the binding of regulatory proteins (6, 20, 24, 63). Accordingly, we speculate that Dam methylation might repress *stdABC* transcription either by preventing the binding of a transcriptional activator or by favoring the binding of a transcriptional repressor.

Regulation of pathogenicity island 1 genes. Several genes of SPI-1 were shown to be *Dam* activated (Table 2). Use of a *sipC::lac* transcriptional fusion (2) confirmed that expression of the *sipC* gene is activated by *Dam* methylation (Fig. 4A). Transcription of *sipC* was low in exponential cultures and increased as the culture entered the stationary phase, as previously described (56). Activation of *sipC* expression by *Dam* methylation was also more pronounced in stationary-phase cultures: *sipC* expression appeared to be insensitive to growth phase-dependent activation in a *Dam*⁻ host (Fig. 4A). This pattern of expression is typical of genes repressed by *Dam* hemimethylation, which does not occur in the absence of DNA replication and so is absent in stationary-phase cultures. Hence, our data suggest that that *sipC* expression may be repressed by *Dam* hemimethylation during active growth.

The effect of *Dam* methylation upon the production of SipC protein was confirmed by Western blotting (Fig. 4B). More SipC was found in the bacterium-associated fraction of *Dam*⁺ than in *Dam*⁻ hosts, providing further evidence that *Dam* methylation regulates SipC synthesis.

Transcriptomic evidence that other SPI-1 genes were also activated by *Dam* methylation (Table 2) prompted the analysis of additional SPI-1 genes using transcriptional *lac* fusions. For

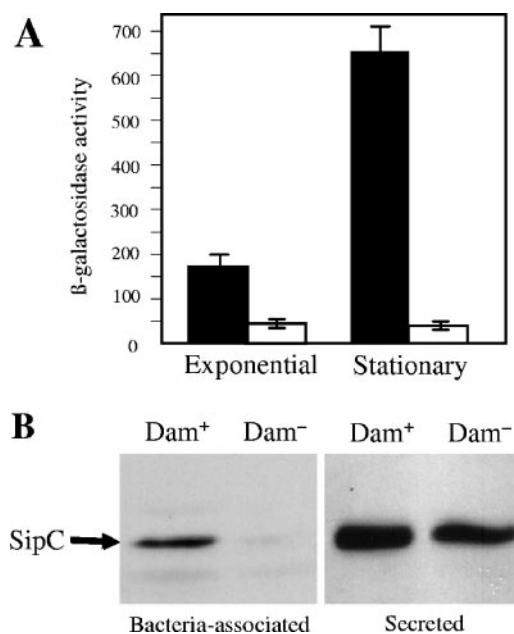


FIG. 4. (A) β -Galactosidase activities of a *sipC::lac* transcriptional fusion in Dam⁺ (black bars) and Dam⁻ (white bars) hosts. The strains used were EE638 (Dam⁺) and SV5202 (Dam⁻). The data shown are the means and standard deviations of four independent experiments. (B) Levels of SipC protein in membrane and supernatant (secreted) protein extracts prepared from isogenic Dam⁺ and Dam⁻ strains (SL1344 and SV4203, respectively). In all samples, the amount of extract loaded corresponded to the equivalent of 2×10^7 CFU. SipC protein was identified by Western blotting with a polyclonal anti-SipC antibody.

this purpose, *hilA::lac*, *hilC::lac*, *invF::lac*, *sipB::lac*, *sicA::lac*, and *prgH::lac* fusions were constructed. As expected (56), all fusions were expressed at low levels in exponential-phase cultures and at higher levels in stationary-phase cultures (note the different scale in the top panel of Fig. 5 than in the other two panels). Assays were also carried out under so-called “optimal conditions” for SPI-1 expression (static cultures in LB medium supplemented with 0.3 M NaCl [56]). The differences found between Dam⁺ and Dam⁻ hosts were as follows. (i) In exponential cultures, all fusions but *prgH::lac* were expressed at lower levels in a Dam⁻ background, as previously found for *sipC* (compare Fig. 4 and 5). (ii) All fusions, including *prgH*, showed Dam-dependent regulation in both stationary-phase and static cultures, and the differences between Dam⁺ and Dam⁻ hosts were larger than in exponential-phase cultures (Fig. 5).

These results suggest that the *hilA*, *hilC*, *invF*, and *sipB* genes (and, to a lesser extent, *sicA*) may undergo Dam-dependent regulation in a manner similar to *sipC* (compare Fig. 4 and 5). A slightly different expression pattern was found for *prgH*, which showed Dam-dependent regulation only in stationary-phase cultures. These observations confirm the occurrence of Dam-dependent regulation in seven genes of SPI-1 and suggest the possibility that expression of the whole island might be under Dam control, albeit with slight differences from gene to gene.

Regulation of motility and chemotaxis. We discovered that two flagellar structural genes (*fliC* and *fliD*) were more highly

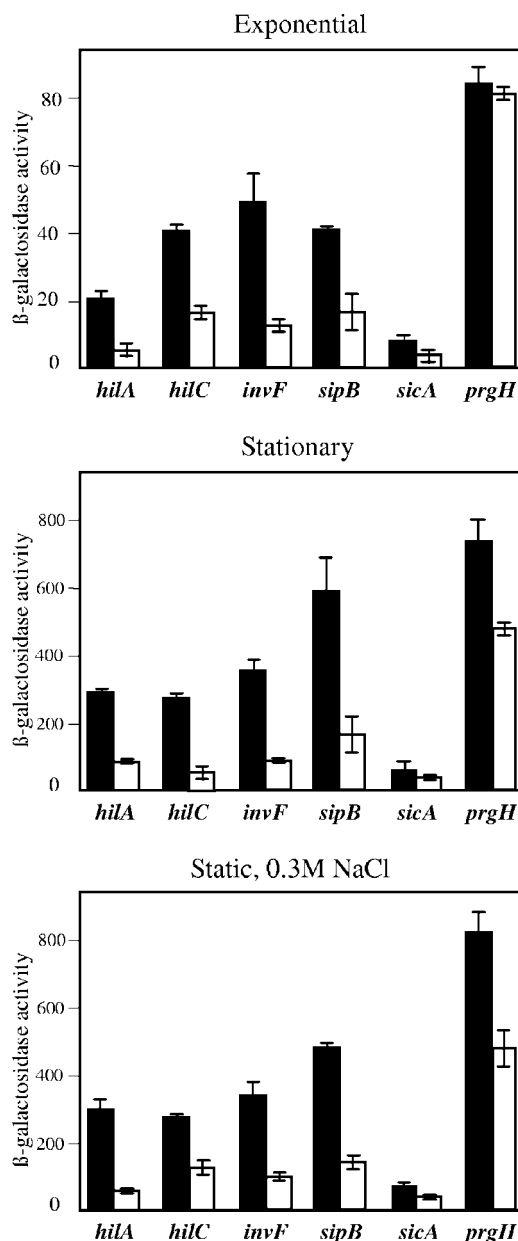


FIG. 5. β -Galactosidase activities of SPI-1 transcriptional fusions in Dam⁺ (black bars) and Dam⁻ (white bars) hosts. The Dam⁺ strains used were SV5246 (*hilA::lac*), SV5248 (*hilC::lac*), SV5258 (*invF::lac*), SV5254 (*sipB::lac*), SV5252 (*sicA::lac*), and SV5262 (*prgH::lac*). Their Dam⁻ derivatives were SV5247, SV5249, SV5259, SV5255, SV5453, and SV5263, respectively. The data shown are the means and standard deviations of four independent experiments.

expressed in a Dam⁻ background (Table 2 and data not shown), suggesting that these loci are repressed by Dam methylation. Use of a transcriptional *fliC::lac* fusion confirmed the occurrence of repression by Dam methylation and raised the possibility of activation by GATC hemimethylation: (i) in the wild-type, *fliC* expression decreased in stationary-phase cultures; (ii) a *dam* mutation caused derepression of *fliC* in both exponential- and stationary-phase cultures (Fig. 6).

The observation that certain flagellar genes were Dam re-

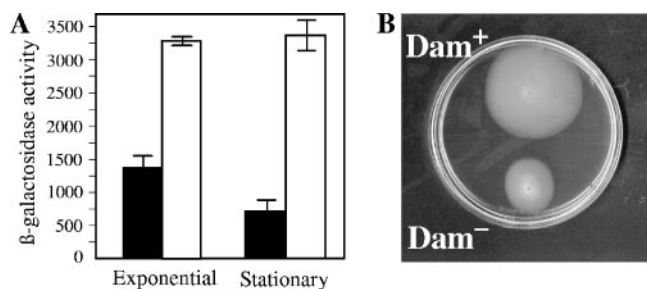


FIG. 6. (A) β -Galactosidase activities of a *fliC::lac* transcriptional fusion in Dam^+ (black bars) and Dam^- (white bars) hosts. The strains used to monitor *fliC* expression were SV5197 (Dam^+) and SV5198 (Dam^-). The data shown are the means and standard deviations of eight independent experiments. (B) Growth of strains SL1344 (Dam^+) and SV4203 (Dam^- ; isogenic) on motility agar.

pressed suggested that unregulated expression of flagellar genes might impair motility in Dam^- strains. Furthermore, reduced motility has been described in Dam^- mutants of *E. coli* (45). Hence, the motilities of Dam^+ and Dam^- isogenic strains (SL1344 and SV4203, respectively) were compared. The growth rates of these strains were different: on average, the doubling time of the Dam^- strain in liquid motility medium was 10 to 15% higher (data not shown). However, reduced halo formation on motility plates (an example is shown in Fig. 6) suggests that Dam^- strains of *S. enterica* do have a motility defect, probably similar to that described in *E. coli* (45). At first sight, it may seem surprising that overexpression of structural flagellar genes can result in reduced motility. However, the complexity and the hierarchical structure of the flagellar regulon is known to require tight and coordinated regulation of gene expression. For instance, transcription of the filament genes *fliC* and *fljB* does not take place until a flagellar motor is available (11). It is thus conceivable that unregulated expression of flagellar genes in a Dam^- background (affecting *fliC* and perhaps other loci) (Table 2 and data not shown) can interfere with the orderly process of flagellar assembly.

DISCUSSION

Genes regulated by Dam methylation were first described 2 decades ago in *E. coli* and more recently in *Salmonella enterica* (37, 40, 66). The current list of Dam-regulated loci includes genes involved in DNA replication, transposition of insertion elements, metabolism, synthesis of fimbrial and nonfimbrial adhesins, and conjugal-plasmid transfer (66). When Dam^- mutants of *Salmonella enterica* were found to be avirulent in the mouse model (16, 21), the possibility that Dam methylation might also regulate the expression of virulence-related genes was considered. The basis for this hypothesis was the observation that *Salmonella* Dam^- mutants displayed multiple defects during animal infection, suggesting that virulence attenuation was multifactorial (16, 21). Furthermore, a relevant phenotype of *Salmonella* Dam^- mutants, inefficient invasion of epithelial-cell cultures, was found to be correlated with altered secretion patterns of invasion proteins (21). Based on these antecedents, we undertook the study of transcriptional profiles in Dam^+ and Dam^- strains of *Salmonella enterica* serovar Typhimurium

in the hope of identifying *Salmonella* virulence genes regulated by Dam methylation.

Transcriptomic analyses of Dam^+ and Dam^- strains were carried out in LB medium. The reason for this choice was that most virulence defects so far described in *Salmonella* Dam^- mutants affect the intestinal stage of infection (16, 21), and LB medium is believed to mimic (to a certain extent) the extracellular environment typical of the intestinal lumen. RNA isolation for transcriptomic analysis was performed in exponential cultures, to ensure that cells contained both methylated and hemimethylated DNA; the DNA of nondividing cells is mostly methylated. Under these conditions, transcriptomic analysis identified 176 *Salmonella* genes that showed differential expression in Dam^+ and Dam^- hosts. These Dam-regulated loci fell into two classes. (i) Genes that were up-regulated in Dam^- mutants, indicating that Dam methylation represses their expression. This class was the most abundant (139 genes), as was observed in *E. coli* (36). Relevant examples of Dam-repressed loci are the fimbrial genes *stdA*, *stdB*, and *stdC*; the flagellar genes *fliC* and *fliD*; the chemotaxis gene *cheR*; a group of genes belonging to the SOS regulon; and an even larger number of genes of the ST64B, Gifsy-1, and Fels-1 prophages of *S. enterica* strain SL1344 (Table 2 and supplemental data available at <http://alojamientos.us.es/genbac>). (ii) Genes that were down-regulated in Dam^- mutants, indicating that Dam methylation activates their expression. Relevant members of this class, which included 37 loci, are *sipC* and other invasion genes of pathogenicity island 1 and the Braun lipoprotein gene, *lppB* (Table 2 and data not shown).

The finding of the SOS regulon as a major subclass among Dam-repressed genes in *S. enterica* agrees with a report for *E. coli* (36). In fact, constitutive SOS induction is a well-known trait of Dam^- strains in both *E. coli* and *Salmonella* (40, 60, 66). The effect of Dam methylation on SOS gene expression is, however, indirect: in the absence of Dam-directed strand discrimination, the mismatch repair MutHLS complex produces double-strand DNA breaks, and SOS induction occurs (18). An exception is the cell division gene *sulA*, whose expression is regulated directly by Dam methylation and also responds to SOS control (40).

Genes belonging to *Salmonella* prophages ST64B, Gifsy-1, and Fels-1 showed increased expression in Dam^- mutants, suggesting that these prophages undergo increased rates of passage from lysogeny to the lytic cycle in the absence of Dam methylation. Spontaneous prophage induction is well known in *E. coli* Dam^- mutants and seems to be an indirect consequence of SOS induction (40). Evidence exists that the increased expression of *Salmonella* ST64B, Gifsy-1, and Fels-1 prophage genes detected in a Dam^- background may be caused by SOS activity: a recent study has described SOS-dependent prophage induction of the ST64B prophage (1), and similar observations have been carried out for the Gifsy-1 and Fels-1 prophages (A. Serna, L. Bossi, and J. Casadesús, unpublished data).

The most spectacular example of repression by Dam methylation detected in our study involved the *std* fimbrial operon. In Dam^- mutants, the *stdA* mRNA level was found to increase 140-fold (Table 2 and data not shown). Smaller but significant mRNA increases were also found for the downstream genes *stdB*, *stdC*, STM3026, and STM3025 (Table 2, Fig. 2, and data

not shown). The marked decrease in mRNA content detected in the distal genes of the *std* operon may reflect natural polarity, a phenomenon frequently observed in polycistronic units, or different stabilities of specific mRNA regions. Whatever the case, the 140-fold difference in *std* operon expression observed between Dam⁺ and Dam⁻ strains of *S. enterica* is, to our knowledge, the largest effect of Dam methylation on gene expression ever described. Western analysis of StdA protein contents in Dam⁺ and Dam⁻ strains confirmed that Std fimbriae are not produced in the wild type while large amounts are synthesized in Dam⁻ mutants (Fig. 3). Hence, Dam methylation seems to be a key factor for regulation of the *std* operon, which is known to be tightly repressed outside the intestinal lumen (28).

Especially relevant examples of virulence loci activated by Dam methylation are genes of pathogenicity island I (Table 2 and Fig. 4 and 5). The need for Dam methylation to activate the expression of SPI-1 genes seems to provide a straightforward explanation for the reduced secretion of SPI-1 effectors typical of *Salmonella* Dam⁻ mutants and for their deficient interaction with the intestinal epithelium (16). Furthermore, Dam⁻ mutants of *S. enterica* show reduced motility and altered expression patterns of flagellar genes (Fig. 6), as previously found in *E. coli* (45). Because flagella and chemotactic proteins are involved in the interaction between *Salmonella* and the intestinal epithelium (30, 52, 57), reduced motility might contribute to attenuation of Dam⁻ strains upon oral infection of mice. An additional factor that might cause reduced motility in the absence of Dam methylation is lowered transcription of the *lppB* gene (15). Decreased synthesis of the *lppB* gene product, Braun lipoprotein (15, 55), might also cause deficient anchoring of peptidoglycan to the outer membrane, which has been postulated as a possible cause of envelope instability in Dam⁻ mutants (48). Leakage of proteins and release of membrane vesicles may contribute to the ability of *S. enterica* Dam⁻ mutants to elicit strong immune responses in animals, a trait that has been successfully exploited for the design of vaccines (22).

The molecular mechanisms that regulate the virulence genes described above remain to be investigated. In the case of the *stdABC* operon, clustering of GATC sites upstream from the promoter suggests the possibility of transcriptional control by a *trans*-acting regulator sensitive to the methylation state of the clustered GATCs, a mechanism found in other Dam-regulated genes (66). The complex regulatory patterns of both SPI-1 (29, 34) and the flagellar-gene network (11) means that it is premature to suggest mechanisms for Dam-dependent regulation at this stage. Classical examples of gene regulation by Dam methylation involve control of transcription initiation (35, 37, 40, 66). However, the possibility that Dam methylation controls the elongation of certain transcripts has been considered (49). An intriguing case of postranscriptional regulation mediated by Dam methylation has been also presented (3), suggesting the existence of Dam-dependent cell functions involved in mRNA turnover, mRNA translation, or protein degradation. This view is supported by the example of FinP, a small regulatory RNA involved in translational regulation whose synthesis is regulated by Dam methylation (9).

The list of Dam-regulated genes of *S. enterica* identified in this study is inevitably incomplete, because the choice of con-

ditions to compare expression in Dam⁺ and Dam⁻ hosts can be expected to influence the outcome of transcriptomic screens. Dam methylation is not a direct regulator of gene expression but a mechanism that controls DNA-protein interactions (66), and the levels of many transcriptional regulators vary depending on physiological and environmental conditions. For instance, the oxygen concentration has a significant influence on the regulation of certain *E. coli* genes by Dam methylation (45). Hence, additional Dam-regulated genes may be discovered when transcriptomic analyses are carried out under growth conditions other than LB-grown exponential cultures.

The finding that specific virulence defects of Dam⁻ mutants can be correlated with altered gene expression patterns does not imply that gene regulation is the only virulence-related role of Dam methylation in *Salmonella*. Note that Dam methylation is also required for strand discrimination by the MutHLS system, which repairs bacterial DNA damage induced by host-synthesized compounds during animal infection (47). Hence, the avirulent phenotype of *Salmonella* Dam⁻ mutants must be viewed as a combination of defects in both DNA repair and virulence gene expression.

ACKNOWLEDGMENTS

This work was supported by grants BIO2004-3455-CO2-02 and GEN2003-20234-CO6-03 (to J.C.) and BIO2004-3455-CO2-01 and GEN2003-20234-CO6-01 (to F.G.D.P.) from the Spanish Ministry of Education and Science and the European Regional Fund and the Core Strategic Grant from the BBSRC to J.C.D.H. The EU Marie Curie Training Site grant QLK2-GH-01-60081-09 supported the visit of R.B. to the Institute of Food Research.

We thank Andreas Bäumlér for the anti-StdA antibody, Matthew Rolfe for assisting with data analysis, Kelly Hughes for providing bacterial strains, and David Low for valuable discussions. Plasmid pIZ999 was constructed by Ana I. Prieto. Preliminary experiments on the regulation of the fimbrial operon *std* by Dam methylation were carried out by Eva Camacho and Ana Serna.

REFERENCES

- Alonso, A., M. G. Pucciarelli, N. Figueroa-Bossi, and F. García-del-Portillo. 2005. Increased excision of the *Salmonella* prophage ST64B caused by a deficiency in Dam methylase. *J. Bacteriol.* **187**:7901–7911.
- Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee. 1996. Co-ordinate expression of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by the control of *hilA* expression. *Mol. Microbiol.* **22**:703–714.
- Bell, D. C., and C. G. Cupples. 2001. Very-short-patch repair in *Escherichia coli* requires the Dam adenine methylase. *J. Bacteriol.* **183**:3631–3635.
- Blattner, F. R., G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277**:1453–1474.
- Braun, R. W., and A. Wright. 1986. DNA methylation differentially enhances the expression of one of the two *dnaA* promoters in vivo and in vitro. *Mol. Gen. Genet.* **202**:246–250.
- Bölker, M., and R. Kahmann. 1989. The *Escherichia coli* protein OxyR discriminates between methylated and unmethylated states of the phage Mu *mom* promoter. *EMBO J.* **8**:2403–2410.
- Camacho, E. M., and J. Casadesús. 2002. Conjugal transfer of the virulence plasmid of *Salmonella enterica* is regulated by the leucine-responsive regulatory protein and DNA adenine methylation. *Mol. Microbiol.* **44**:1589–1598.
- Camacho, E. M., and J. Casadesús. 2005. Regulation of *traJ* transcription in the *Salmonella* virulence plasmid by strand-specific DNA adenine hemimethylation. *Mol. Microbiol.* **57**:1700–1718.
- Camacho, E. M., A. Serna, C. Madrid, S. Marques, R. Fernandez, F. de la Cruz, A. Juarez, and J. Casadesús. 2005. Regulation of *finP* transcription by DNA adenine methylation in the virulence plasmid of *Salmonella enterica*. *J. Bacteriol.* **187**:5691–5699.
- Cano, D. A., G. Domínguez-Bernal, A. Tierrez, F. García-del-Portillo, and J. Casadesús. 2002. Regulation of capsule synthesis and cell motility in *Salmonella enterica* by the essential gene *igaA*. *Genetics* **162**:1513–1523.

11. Chilcott, G. S., and K. T. Hughes. 2000. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* **64**:694–708.
12. Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **90**:6640–6645.
13. Ellermeier, C. D., A. Janakiraman, and J. M. Slauch. 2002. Construction of targeted single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene* **290**:153–161.
14. Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. D. Hinton. 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol. Microbiol.* **47**:103–118.
15. Fadl, A. A., J. Sha, G. R. Klimpel, J. P. Olano, D. W. Niesel, and A. K. Chopra. 2005. Murein lipoprotein is a critical outer membrane component involved in *Salmonella enterica* serovar Typhimurium systemic infection. *Infect. Immun.* **73**:1081–1096.
16. García-del-Portillo, F., M. G. Pucciarelli, and J. Casadesús. 1999. DNA adenine methylase mutants of *Salmonella typhimurium* are deficient in protein secretion, cell invasion and M cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* **96**:11584–11588.
17. Gillen, K. L., and K. T. Hughes. 1991. Negative regulatory loci coupling flagellar synthesis to flagellar assembly in *Salmonella typhimurium*. *J. Bacteriol.* **173**:2301–2310.
18. Glickman, B., P. van der Eisen, and M. Radman. 1978. Induced mutagenesis in Dam⁻ mutants of *Escherichia coli*: a role for 6-methyladenine residues in mutation avoidance. *Mol. Gen. Genet.* **167**:307–312.
19. Goodman, A. L., and S. Lory. 2004. Analysis of regulatory networks in *Pseudomonas aeruginosa* by genome-wide transcriptional profiling. *Curr. Opin. Microbiol.* **7**:39–44.
20. Haagmans, W., and M. van der Woude. 2000. Phase variation of Ag43 in *Escherichia coli*: Dam-dependent methylation abrogates OxyR binding and OxyR-mediated repression of transcription. *Mol. Microbiol.* **35**:877–887.
21. Heithoff, D., R. L. Sinsheimer, D. A. Low, and M. J. Mahan. 1999. An essential role for DNA adenine methylation in bacterial virulence. *Science* **284**:967–970.
22. Heithoff, D. M., E. I. Enioutina, R. A. Daynes, R. L. Sinsheimer, D. A. Low, and M. J. Mahan. 2001. *Salmonella* DNA adenine methylase mutants confer cross-protective immunity. *Infect. Immun.* **69**:6725–6730.
23. Henderson, L. R., and P. Owen. 1999. The major phase-variable outer membrane protein of *Escherichia coli* structurally resembles the immunoglobulin A1 protease class of exported protein and is regulated by a novel mechanism involving Dam and OxyR. *J. Bacteriol.* **181**:2132–2141.
24. Hernday, A., M. Krabbe, B. Braaten, and D. Low. 2002. Self-perpetuating epigenetic pili switches in bacteria. *Proc. Natl. Acad. Sci. USA* **99**:16470–16576.
25. Hinton, J. C. D., I. Hautefort, S. Eriksson, A. Thompson, and M. Rhen. 2004. Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection. *Curr. Opin. Microbiol.* **7**:277–282.
26. Hoiseth, S. K., and B. A. Stocker. 1981. Aromatic-dependent *Salmonella enterica* are non-virulent and effective as live vaccines. *Nature* **291**:238–239.
27. Hsieh, T. 2001. Molecular mechanisms of DNA mismatch repair. *Mutat. Res.* **486**:71–87.
28. Humphries, A. D., M. Raffatellu, S. Winter, E. H. Weening, R. A. Kingsley, R. Droleskey, S. Zhang, K. Figueiredo, S. Khare, J. Nunes, L. G. Adams, R. M. Tsois, and A. J. Bäuml. 2003. The use of flow cytometry to detect expression of subunits encoded by the 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. *Mol. Microbiol.* **28**:1357–1376.
29. Jones, B. D. 2005. *Salmonella* invasion gene regulation: a story of environmental awareness. *J. Microbiol.* **43**:110–117.
30. Jones, B. D., C. A. Lee, and S. Falkow. 1992. Invasion by *Salmonella typhimurium* is affected by the direction of flagellar rotation. *Infect. Immun.* **60**:2475–2480.
31. Kaniga, K., D. Tollinger, and J. E. Galan. 1995. Identification of two targets of the type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella typhimurium* that have homology to the *Shigella* IpaD and IpaA proteins. *J. Bacteriol.* **177**:7078–7085.
32. Kelly, A., M. D. Goldberg, R. K. Carroll, V. Danino, J. C. Hinton, and C. J. Dorman. 2004. A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. *Microbiology* **150**:2037–2053.
33. Kücherer, C., H. Lother, K. Kölling, M. A. Schauzu, and W. Messer. 1986. Regulation of transcription of the chromosomal *dnaA* gene of *Escherichia coli*. *Mol. Gen. Genet.* **205**:115–121.
34. Lostroh, C. P., and C. A. Lee. 2001. The *Salmonella* pathogenicity island-1 type III secretion system. *Microbes Infect.* **3**:1281–1291.
35. Low, D. A., N. J. Weyand, and M. J. Mahan. 2001. Roles of DNA adenine methylation in regulating gene expression and virulence. *Infect. Immun.* **69**:7197–7204.
36. Løbner-Olesen, A., M. G. Marinus, and F. G. Hanssen. 2003. Role of SeqA and Dam in *Escherichia coli* gene expression: a global/microarray analysis. *Proc. Natl. Acad. Sci. USA* **100**:4672–4677.
37. Løbner-Olesen, A., O. Skovgaard, and M. G. Marinus. 2005. Dam methylation: coordinating cellular processes. *Curr. Opin. Microbiol.* **8**:154–160.
38. Mangan, M. W., S. Lucchini, V. Danino, T. O. Croinin, J. C. Hinton, and C. J. Dorman. 2006. The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **59**:1831–1847.
39. Marinus, M. G. 1985. DNA methylation influences *trpR* promoter activity in *Escherichia coli*. *Mol. Gen. Genet.* **200**:185–186.
40. Marinus, M. G. 1996. Methylation of DNA, p. 782–791. In F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
41. McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**:852–856.
42. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. Morgan, E., J. D. Campbell, S. C. Rowe, J. Bispham, M. P. Stevens, A. J. Bowen, P. A. Barrow, D. J. Maskell, and T. S. Wallis. 2004. Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **54**:994–1010.
44. Nicholson, B., and D. Low. 2000. DNA methylation-dependent regulation of Pfl expression in *Salmonella typhimurium*. *Mol. Microbiol.* **35**:728–742.
45. Oshima, T., C. Wada, Y. Kawagoe, T. Ara, M. Maeda, Y. Masuda, S. Hiraga, and H. Mori. 2002. Genome-wide analysis of deoxyadenosine methyltransferase-mediated control of gene expression in *Escherichia coli*. *Mol. Microbiol.* **45**:673–695.
46. Polaczek, P., K. Kwan, and J. L. Campbell. 1998. GATC motifs may alter the conformation of DNA depending on sequence context and N⁶-adenine methylation status: possible implications for DNA-protein recognition. *Mol. Gen. Genet.* **258**:448–493.
47. Prieto, A. I., F. Ramos-Morales, and J. Casadesús. 2004. Bile-induced DNA damage in *Salmonella enterica*. *Genetics* **168**:1787–1794.
48. Pucciarelli, M. G., A. I. Prieto, J. Casadesús, and F. García-del-Portillo. 2002. Envelope instability in DNA adenine methylase mutants of *Salmonella enterica*. *Microbiology* **148**:1171–1182.
49. Riva, A., M. O. Delorme, T. Chevalier, N. Guilhot, C. Hénaut, and A. Hénaut. 2004. Characterization of the GATC regulatory network in *E. coli*. *BMC Genomics* **5**:48–58.
50. Robbins-Manke, J. L., Z. Z. Zdraveski, M. Marinus, and J. M. Essigman. 2005. Analysis of global gene expression and double-strand-break formation in DNA adenine methyltransferase- and mismatch repair-deficient *Escherichia coli*. *J. Bacteriol.* **187**:7027–7037.
51. Roberts, D., B. C. Hoopes, W. R. McClure, and N. Kleckner. 1985. IS10 transposition is regulated by DNA adenine methylation. *Cell* **43**:117–130.
52. Robertson, J. M., N. H. McKenzie, M. Duncan, E. Allen-Vercoe, M. J. Woodward, H. J. Flint, and G. Grant. 2003. Lack of flagella disadvantages *Salmonella enterica* serovar Enteritidis during the early stages of infection in the rat. *J. Med. Microbiol.* **52**:91–99.
53. Schagger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **100**:368–379.
54. Schmieger, H. 1972. Phage P22 mutants with increased or decreased transducing abilities. *Mol. Gen. Genet.* **119**:75–88.
55. Sha, J., A. Fadl, G. R. Klimpel, D. W. Niesel, V. L. Popov, and A. K. Chopra. 2004. The two murein lipoproteins of *Salmonella enterica* serovar Typhimurium contribute to the virulence of the organism. *Infect. Immun.* **72**:3987–4003.
56. Song, M., H. J. Kim, E. Y. Kim, M. Shin, H. C. Lee, Y. Hong, J. H. Rhee, H. Yoon, S. Ryu, S. Lim, and H. E. Choy. 2004. ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1. *J. Biol. Chem.* **279**:34183–34190.
57. Stecher, B., S. Hapfelmeier, C. Müller, M. Kremer, T. Stallmach, and W. D. Hardt. 2004. Flagella and chemotaxis are required for efficient induction of *Salmonella enterica* serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect. Immun.* **72**:4138–4150.
58. Thompson, A., G. Rowley, M. Alston, V. Danino, and J. C. D. Hinton. 2006. *Salmonella* transcriptomics: relating regulons, stimulons and regulatory networks to the process of infection. *Curr. Opin. Microbiol.* **9**:109–116.
59. Torreblanca, J. 1999. Ph.D. thesis. University of Seville, Seville, Spain.
60. Torreblanca, J., and J. Casadesús. 1996. DNA adenine methylase mutants of *Salmonella typhimurium* and a novel Dam-regulated locus. *Genetics* **144**:15–26.
61. Torreblanca, J., S. Marques, and J. Casadesús. 1999. Synthesis of FinP RNA by plasmids F and pSLT is regulated by DNA adenine methylation. *Genetics* **152**:31–45.

62. **van der Velden, A. W. M., A. J. Bäumlér, R. M. Tsolis, and F. Heffron.** 1998. Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. *Infect. Immun.* **66**:2803–2808.
63. **Waldron, D. E., P. Owen, and C. J. Dorman.** 2002. Competitive interaction of the OxyR DNA-binding protein and the Dam methylase at the antigen 43 gene regulatory region in *Escherichia coli*. *Mol. Microbiol.* **44**:509–520.
64. **Wang, Q., J. G. Frye, M. McClelland, and R. M. Harshey.** 2004. Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. *Mol. Microbiol.* **52**:169–187.
65. **Weening, E. H., J. D. Barker, M. C. Laarakker, A. D. Humphries, R. M. Tsolis, and A. J. Bäumlér.** 2005. The *Salmonella enterica* serotype Typhimurium *lpf*, *bef*, *stb*, *stc*, *std*, and *sth* fimbrial operons are required for intestinal persistence in mice. *Infect. Immun.* **73**:3358–3366.
66. **Wion, D., and J. Casadesús.** 2006. N⁶-methyl-adenine: an epigenetic signal for DNA-protein interactions. *Nat. Rev. Microbiol.* **4**:183–192.
67. **Zheng, M., X. Wang, L. J. Templeton, D. R. Smulski, R. A. LaRossa, and G. Storz.** 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J. Bacteriol.* **183**:4562–4570.