JOURNAL OF BACTERIOLOGY, Mar. 2007, p. 1556–1564 Vol. 189, No. 5
0021-9193/07/$08.00+0 doi:10.1128/JB.01580-06

Altered Levels of *Salmonella* DNA Adenine Methylase Are Associated with Defects in Gene Expression, Motility, Flagellar Synthesis, and Bile Resistance in the Pathogenic Strain 14028 but Not in the Laboratory Strain LT2[▽]

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Received 11 October 2006/Accepted 5 December 2006

Comparative genomic analysis has revealed limited strain diversity between *Salmonella* pathogenic and nonpathogenic isolates. Thus, some of the relative virulence and host-immune response disparities may be credited to differential gene regulation rather than gross differences in genomic content. Here we show that altered levels of *Salmonella* DNA adenine methylase (Dam) resulted in acute defects in virulence-associated gene expression, motility, flagellin synthesis, and bile resistance in the *Salmonella* pathogenic strain 14028 but not in avirulent laboratory strain LT2. The defects in motility exhibited by 14028 in response to altered Dam levels was not dependent on the presence of the regulatory protein, RpoS. The transitioning between flagellar types (phase variation) was also differentially regulated in 14028 versus LT2 in response to *dam* levels, resulting in distinct differences in flagellin expression states. These data suggest that differential gene regulation may contribute to the relative virulence disparities observed between *Salmonella* serovars that are closely related at the DNA level.

Salmonella enterica is a significant pathogen of reptiles, birds, and mammals and is an important food-borne pathogen of humans, wherein a wide variety of infections can occur, ranging from gastroenteritis to bacteremia and typhoid fever (53). More than 2,500 serovars of *S. enterica* have been identified and classified typically by serotyping, based on antigenic variation in the lipopolysaccharide (O-antigen) and phase 1 (H1) and phase 2 (H2) flagella (33, 56, 57). Although serotyping has been a versatile, convenient, and epidemiologically useful tool for classifying isolates, comparative genomic analysis has provided much of our insight regarding bacterial diversity, evolutionary relatedness, and pathogenicity between species and between serovars (10, 17, 27, 40, 79, 84).

Surprisingly, limited strain diversity has emerged from comparative genomic analyses between pathogenic *Salmonella* serovars (4, 84), as well as within pathogenic and nonpathogenic isolates of the same serovar (68; http://www.sanger.ac.uk). Accordingly, some of the relative differences in virulence may be attributed to differential gene regulation, which is not revealed by standard genomic comparisons (17). For example, the avirulent laboratory Typhimurium strain, LT2, harbors the principal pathogenicity islands and other known functions associated with virulence but remains defective in the ability to cause disease in animal models of infection (68; http://www.sanger.ac.uk). The principal known virulence difference at the genomic level between *Salmonella* pathogenic strains and avirulent laboratory strain LT2 resides within the alternative sigma factor *rpoS*, wherein replacement of the mutant $rpoS_{LT2}$ allele with that of an *rpoS* allele from a pathogenic strain results in a significant, but incomplete, restoration of virulence to LT2 (55, 97, 102). Since pathogenic Typhimurium strains and LT2 are closely related at the genomic level, some of the *rpoS*-independent virulence disparities may also be regulatory in nature.

DNA adenine methylase (Dam) is a regulatory protein that directly controls bacterial virulence gene expression (5, 11, 15, 48). In *Salmonella*, *dam* mutants ectopically express multiple genes that are preferentially expressed during infection, modulate host immune responses, are attenuated for virulence, and confer heightened immunity in vaccinated hosts (35, 44, 45, 62, 92). We show here that altered levels of Dam differentially affected several virulence-associated phenotypes, including bacterial virulence gene expression, motility, flagellar synthesis, bile resistance, and phase variation in *Salmonella* pathogenic strain 14028 compared to the avirulent laboratory strain, LT2.

MATERIALS AND METHODS

Bacterial strains, phage, and media. The *Salmonella* pathogenic strains used in the present study were derived from *S. enterica* serovar Typhimurium strain ATCC 14028 (CDC 6516-60), UK-1 (43), and F98 (6, 43); the pathogenic strains Typhimurium TY1212 and *S. enterica* O6,14,24:e,h- monophasic K00-670 (29, 30) were recovered from recent virulent calf and poultry outbreaks, respectively, and were obtained from the California Animal Health and Food Safety Laboratory; all *Salmonella* field isolates were obtained from U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS). Typhimurium avirulent laboratory strains derived from LT2 (25, 89) and LT7 (54, 89) were obtained from John Roth and Tom Cebula, respectively. Dam-overproducing (Dam^{OP}) strains contained *Escherichia coli dam* on a recombinant plasmid (pTP166) (67); introduction of pTP166 into all *Salmonella* isolates tested resulted in ~50- to 100-fold increased Dam activity (as observed in *E. coli* [59, 67]). *dam* derivatives contained a *dam102*::Mu*d*-Cm insertion or *dam*Δ*232*, a nonpolar in-frame deletion (45); dam^+ and *dam* mutant derivatives contained an empty plasmid vector, pTP166-Δ*dam*, in which the *dam* gene was removed from pTP166. *lacZ* transcriptional fusions to flagellar genes were obtained from Kelly Hughes and transduced into strains 14028 and LT2 (*flhC5213*::Mu*d*A [TH4314]; *fliA*::*lacZ*

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▽ Published ahead of print on 15 December 2006.

[TH5597]; *flgM5207*::Mu*dJ* [TH2507]; *fliC5050*::Mu*dJ* [TH1077]; *fljB5001*::Mu*dJ* [TH714]; *motA5457*::Mu*dJ* [TH3929]; and *cheY5458*::Mu*dJ* [TH3930]) (21, 39). *spvB*, *pmrB*, *mgtA*, *entF*, and *fdnG lacZ* transcriptional fusion strains were derived from in vivo expression technology (45, 65). A nonpolar in-frame deletion Δ*flgM8041* was constructed by using internal oligonucleotides that serve as PCR primers designed to construct an in-frame 240-bp deletion of defined *flgM* sequence, which was confirmed by sequencing. The $rpoS_{LT2}$ allele was introduced into virulent strain 14028 by standard allelic replacement, generating strain MT2892 (28). *rpoS1221*::Mu*dJ* was constructed by standard genetic methods (16).

The high-frequency generalized transducing bacteriophage P22 mutant HT105/1, *int-201* was used for all transductional crosses (90), and phage-free, phage-sensitive transductants were isolated as previously described (18). Unless otherwise specified, Luria-Bertani (LB) broth (25) was the laboratory media used in these studies. The final concentrations of antibiotics (Sigma) were as follows: ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), and carbenicillin (100 μg/ml).

Motility assays. *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of *Salmonella* were inoculated into the center of soft-agar motility plates (38), incubated for 7 h at 37°C, and the motility area (in square centimeters) was calculated by the formula πr^2, where r is the growth radius of the swarm. Motility assays were conducted in the presence of ampicillin to maintain the Dam-overproducing plasmid, pTP166 (67) in Dam^{OP} strains; *dam*$^+$ and *dam* mutant derivatives contained an empty plasmid vector, pTP166-Δ*dam*, in which the *dam* gene was removed from pTP166. FlhC$^-$ (*flhC5456*::Mu*dJ*) strains TH3928 and MT2425 were used as nonmotile controls (21). For each strain, the assay was performed in triplicate, and the average growth diameter of the swarm was determined (standard deviation of <10% of the mean).

Western blot analysis. Whole-cell protein extracts prepared from ~10^7 cells were processed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (~20 μg of protein/well), transferred to polyvinylidene difluoride (PVDF) membrane (Pierce), and probed with *Salmonella* primary antibody H antiserum i (anti-FliC) or H antiserum 1 complex (anti-FljB) for Typhimurium or H antiserum eh (anti-FliC) for *S. enterica* O6,14,24:e,h− monophasic (Difco); for *Salmonella* field isolates, *E. coli* flagella monoclonal antibody 15D8 (IgG1; BioVeris), which recognizes a conserved flagellar epitope that cross-reacts with other flagellum-expressing *Enterobacteriaceae*, was used as the primary antibody. Peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham Biosciences) and goat anti-mouse immunoglobulin G (Pierce) were used as secondary antibody for *Salmonella* specific and *Escherichia coli* nonspecific flagellar primary antibodies, respectively. Signal was detected by chemiluminescence using Supersignal West Femto maximum sensitivity substrate (Pierce), followed by exposure to film. Cultures used for Western analysis were grown in the presence of carbenicillin to maintain the Dam-overproducing plasmid, pTP166 (67) in Dam^{OP} strains; *dam*$^+$ and *dam* mutant derivatives contained an empty plasmid vector, pTP166-Δ*dam*, in which the *dam* gene was removed from pTP166. FlhC$^-$ (*flhC5456*::Mu*dJ*) strains TH3928 and MT2425 were used as nonflagellated controls (21).

β-galactosidase assays. *Salmonella* cultures containing *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of *lacZ* transcriptional fusions were grown for 16 h in Luria-Bertani medium (25) at 37°C (13, 14) or 30°C (Mu*dA*::*lacZ* fusions) and assayed for β-galactosidase activities as described previously (94). Dam-overproducing strains contained *E. coli dam* on a recombinant plasmid (pTP166) (67); *dam*$^+$ and *dam* mutant derivatives contained an empty plasmid vector, pTP166-Δ*dam*, in which the *dam* gene was removed from pTP166. Units refer to β-galactosidase activities (micromoles of *o*-nitrophenol [ONP] formed per minute per A_{600} unit per milliliter of cell suspension × 10^3). Values are an average of at least two triplicates performed on separate days; the standard deviation was <10% of the mean.

Bile sensitivity assays. Bile sensitivity assays were performed as a modification of methods described previously (101). *Salmonella* cultures containing *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of strains 14028 and LT2 were grown overnight in LB medium (25) at 37°C. Approximately 5 × 10^2 cells from overnight grown cultures were added to individual wells in 96-well Polystyrene microtiter plates (Becton Dickinson) containing 150 μl of LB medium with the indicated concentrations of ox bile (sodium choleate [Sigma]) and incubated for 16 h at 37°C without shaking. Growth was assessed by measurement of the optical density at 600 nm (OD_{600}). Assays were conducted in the presence of ampicillin to maintain the Dam-overproducing plasmid, pTP166 (67), in Dam^{OP} strains; *dam*$^+$ and *dam* mutant derivatives contained an empty plasmid vector, pTP166-Δ*dam*, in which the *dam* gene was removed from pTP166. The values given are an average of the OD_{600} values from at least three triplicates; the standard deviation was <20% of the mean. Values of <0.02 represent no detectable growth under the condition tested.

Flagellar-phase transition rates. The *fljB*::*lacZ* transition rates (per cell per generation) of $fljB_{On}$ to $fljB_{Off}$ and of $fljB_{Off}$ to $fljB_{On}$ were calculated from a single blue colony (Lac$^+$) or a single white colony (Lac$^-$) from *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of Typhimurium *fljB5001*::Mu*dJ* fusion strain (38) grown on minimal E medium agar (25) containing 0.2% glycerol and 40 to 80 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; US Biologicals)/ml. Colonies exhibiting a Lac$^+$ or Lac$^-$ phenotype (no sectors) were excised from the agar and plated to determine the total number of organisms in the colony and to score the Lac phenotype after incubation for 48 h at 37°C. Transition rates represent the weighted average of five independent colonies as described previously (8, 32). The transition rates were calculated by the formula $(M/N)/g$, where M/N is the ratio of Lac$^+$ or Lac$^-$ cells to total cells, and g is the number of generations of growth from a single cell to the total number of cells in the colony. The weighted average of the transition rates was calculated by the formula $[(M_1/g_1) + (M_2/g_2) + (M_n/g_n)]/(N_1 + N_2 + N_n)$, where M, N, and g are as described above and n depicts each individual transition rate calculation. In order to calculate the transition rates, the assumption was made that Lac$^+$ colonies arose from a single Lac$^+$ parent cell and Lac$^-$ colonies arose from a single Lac$^-$ parent cell.

RESULTS

Dam overproduction results in acute defects in motility and flagellar synthesis in *Salmonella* pathogenic strains but not in the avirulent laboratory strain LT2. Flagella are an important virulence factor for a wide variety of pathogens, engaging in required roles in bacterial adhesion to epithelial cell surfaces, colonization, biofilm formation, and invasion of host tissues (reviewed in reference 88). Although *Salmonella* flagellin and motility are dispensable in the mouse model (61, 91), there are several reports indicating that flagella are important for the establishment of *Salmonella* infection. *Salmonella* flagella are required for efficient attachment and transport through rabbit appendix M cells in vivo (66), for *Salmonella* invasiveness in a cell culture model, and for induction of polymorphonuclear leukocyte infiltration in a calf intestinal model of infection (91). In addition, *Salmonella* flagellins are principal antigens that are recognized by the innate immune system via flagellin pathogen associated molecular patterns (2, 51, 98), have the capacity to elicit different host responses (20), and are trafficked through eukaryotic cells in advance of infecting organisms (63).

To further understand the virulence disparity between the pathogenic strain 14028 and the relatively pathogenic strain LT2, we examined whether motility and flagellar synthesis were differentially regulated in response to altered Dam levels. Note that the growth rates of *dam* mutant and Dam^{OP} derivatives did not significantly differ from that of wild type. As was shown in another pathogenic strain (5), the lack of *dam* was associated with relatively mild defects in all *Salmonella* strains tested (Table 1). In contrast, Dam overproduction resulted in severe defects in motility (Fig. 1) and flagellar synthesis (Fig. 2) in 14028 and in three other pathogenic Typhimurium strains that have been associated with acute disease in livestock, as well as in one field isolate of Typhimurium var. Copenhagen that has been associated with asymptomatic colonization and/or persistence in chickens (Table 1). Growth under Dam^{OP} conditions did not significantly alter motility or flagellar synthesis in avirulent laboratory Typhimurium strain LT2 or LT7.

To assess whether such differential regulation extended to other *Salmonella* serovars, motility and flagellar synthesis were examined in two other pathogenic *Salmonella* serovars that are

TABLE 1. Motility and flagellar synthesis are defective in Dam^{OP} derivatives of *Salmonella* clinical isolates

Strain[a]	Serovar (serogroup)	Motility[b] (area in cm^2)			Flagellar synthesis[c] (mutant/wild-type)		
		dam$^+$	*dam* mutant	Dam^{OP}	*dam*$^+$	*dam* mutant	Dam^{OP}
MT2425 (FlhC$^-$)	Typhimurium (B)	0.031	0.031	0.031	0.0078	0.0078	0.0078
Laboratory strains							
LT2	Typhimurium (B)	11.0	2.3	10.1	1	1	1
LT7	Typhimurium (B)	17.3	2.3	12.3	1	1	1
Pathogenic strains							
ATCC 14028	Typhimurium (B)	16.3	5.9	0.34	1	1	0.0156
UK-1	Typhimurium (B)	16.8	5.1	0.38	1	1	0.0078
F98	Typhimurium (B)	17.5	5.9	0.72	1	1	0.0078
TY1212	Typhimurium (B)	14.9	ND	0.28	1	ND	0.0078
03-721	Newport (C2)	16.6	ND	1.81	1	ND	0.25
K00-670	O6,14,24:e,h− monophasic (H)	14.2	ND	0.31	1	ND	0.0156
Field isolates							
EPIMD142	Typhimurium var. Copenhagen (B)	14.51	ND	0.45	1	ND	0.0156
EPIMD144	Istanbul (C3)	16.61	ND	1.91	1	ND	0.0156
BL9W2FL	Thompson (C1)	15.19	ND	0.67	1	ND	0.0312
CH10W4WI	Montevideo (C1)	5.55	ND	0.34	1	ND	0.0156
NM 1-41	Kentucky (C3)	16.10	ND	0.41	1	ND	0.0312
NM 26-71	Anatum (E1)	16.10	ND	2.54	1	ND	0.0312
NM 27-07	Montevideo (C1)	9.23	ND	2.08	1	ND	0.0312
NM 25-06	Meleagridis (E1)	10.51	ND	0.83	1	ND	0.0312
NM 25-46	Sandiego (B)	17.78	ND	7.06	1	ND	1
NM 28-54	Cerro (K)	10.34	ND	0.83	1	ND	0.0625
NM 30-31	Paratyphi B var. Java (B)	17.56	ND	2.16	1	ND	0.0312

[a] *Salmonella* pathogenic strains used in this study were derived from Typhimurium strain ATCC 14028 (CDC 6516-60), UK-1 (43); F98 (6, 43); Typhimurium TY1212 and O6,14,24:e,h− monophasic K00-670 (29, 30) were recovered from recent virulent calf and poultry outbreaks, respectively, and were obtained from the California Animal Health and Food Safety Laboratory; Newport 03-721 was recovered from a recent calf outbreak and was obtained from Veterinary Medical Teaching Hospital Microbiology Lab at the University of California, Davis. All *Salmonella* field isolates were obtained from the USDA-ARS. Typhimurium avirulent laboratory strains were derived from LT2 (25, 89) and LT7 (54, 89). ND, not determined.

[b] *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of *Salmonella* were inoculated into the center of soft agar motility plates (38) and incubated for 7 h at 37°C, and the motility area of the swarm was determined. For each strain, the assay was performed in triplicate and the average growth diameter of the swarm was determined; the standard deviation was <10% of the mean. FlhC$^-$ (*flhC5456*::Mu*dJ*) strain MT2425 was used as a nonmotile control (21).

[c] Whole-cell protein extracts prepared from ~10^7 cells were processed by SDS-PAGE (~20 μg of protein/well), transferred to PVDF membrane (Pierce), and probed with *Salmonella* primary antibody H antiserum i (anti-FliC) or H antiserum 1 complex (anti-FljB) for Typhimurium or H antiserum eh (anti-FliC) for *S. enterica* O6,14,24:e,h− monophasic (Difco); for *Salmonella* field isolates, *E. coli* flagellum monoclonal antibody 15D8 (IgG1; BioVeris) was used as a primary antibody. Signal was detected as described in Materials and Methods. Units refer to relative mutant/wild-type levels of flagellin determined by Western analysis using flagellar antibodies. Protein extracts of *dam*$^+$ strains were diluted 4- to 128-fold before the signal was equal to that observed in Dam^{OP} cells; values given are representative of at least three independent Western blots. FlhC$^-$ (*flhC5456*::Mu*dJ*) strain MT2425 was used as a nonmotile control (21).

associated with acute disease in chickens and cattle and in ten field isolates that are associated with asymptomatic colonization and/or persistence without acute disease manifestation in these animals. Similar to Typhimurium, growth under Dam^{OP} conditions resulted in defects in motility and flagellar synthesis in nearly all (11 of 12) of the non-Typhimurium pathogenic and field isolates tested (Table 1). These data suggest that the differential regulation of motility and flagellar synthesis in re-

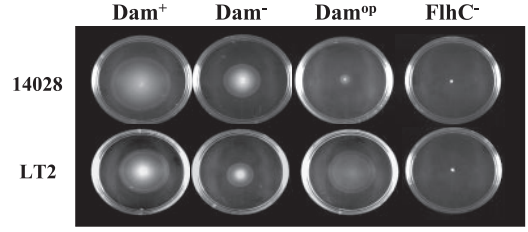

FIG. 1. Dam overproduction (Dam^{OP}) results in motility defects in *Salmonella* strain 14028 but not in strain LT2. *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of the Typhimurium pathogenic strain, 14028, and the avirulent laboratory strain, LT2, were inoculated into the center of soft agar motility plates (38). Motility was assessed by measuring the growth diameter of the swarm after 7 h of incubation at 37°C. Cells recovered from the outermost motility zone of the swarm generated by Dam^{OP} cells were shown to be motile escape mutants selected during the assay (data not shown). FlhC$^-$ (*flhC5456*::Mu*dJ*) strains TH3928 and MT2425 were used as nonmotile controls (21).

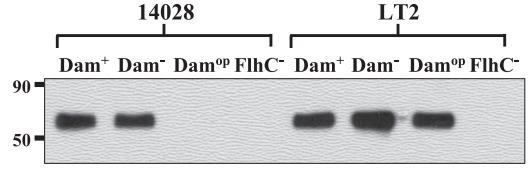

FIG. 2. Dam overproduction results in flagellar synthesis defects in *Salmonella* strain 14028 but not in strain LT2. Whole-cell protein extracts corresponding to ~10^7 *Salmonella* cells from *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of the Typhimurium pathogenic strain, 14028, and the avirulent laboratory strain, LT2, were subjected to SDS-PAGE and transferred to a PVDF membrane. Membranes were probed with *Salmonella* H antiserum 1 complex (anti-FljB). Signal was detected as described in Materials and Methods. Extracts of *dam*$^+$ and *dam* mutant 14028 and LT2 strains were diluted 64-fold before the flagellar signal was undetectable. FlhC$^-$ (*flhC5456*::Mu*dJ*) strains TH3928 (LT2 background) and MT2425 (14028 background) were used as nonflagellated controls (21).

TABLE 2. Differential regulation of motility exhibited by 14028 and LT2 grown under Dam^{OP} conditions was not dependent on the presence of RpoS or mutant allele of *rpoS*

Relevant strain and genotype	Motility[a] (area in cm^2)		
	dam$^+$	*dam* mutant	Dam^{OP}
14028	16.4	4.1	0.50
14028 *rpoS1221*::Mu*dJ*	14.2	3.4	0.31
14028 $rpoS_{LT2}$	18.2	6.6	0.58
LT2	11.1	2.4	11.7
LT2 *rpoS1221*::Mu*dJ*	8.5	1.8	8.1

[a] *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of *Salmonella* were inoculated into the center of soft agar motility plates (38) and incubated for 7 h at 37°C, and the motility area of the swarm was determined. For each strain, the assay was performed in triplicate, and the average growth diameter of the swarm was determined; the standard deviation was <10% of the mean.

TABLE 3. Dam overproduction leads to enhanced bile sensitivity in *Salmonella* sp. strain 14028 but not in LT2

Bile content (%)	OD_{600}[a]					
	14028			LT2		
	MT2461 *dam*$^+$	MT2462 *dam* mutant	MT2128 Dam^{OP}	MT2582 *dam*$^+$	MT2583 *dam* mutant	MT2584 Dam^{OP}
5	0.097	<0.02	<0.02	0.084	<0.02	0.074
4.5	0.125	<0.02	0.048	0.100	<0.02	0.093
4	0.170	0.084	0.069	0.128	0.036	0.112
3	0.228	0.156	0.127	0.168	0.117	0.159
0	0.299	0.267	0.257	0.264	0.249	0.247

[a] *Salmonella* cultures containing *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of strains 14028 and LT2 were grown for 16 h in Luria-Bertani media (25). Approximately $5 \infty 10^2$ of the overnight grown cells were added to individual microtiter wells containing LB in addition to the listed final concentration of ox bile (sodium choleate). Cells were incubated for an additional 16 h without shaking at 37°C. Values given are an average of OD_{600} values from at least three triplicates; the standard deviation was <20% of the mean. Values of <0.02 represent no detectable growth under the condition tested.

sponse to Dam levels extends to other *Salmonella* serovars, including pathogenic isolates as well as field isolates that are associated with asymptomatic colonization or persistence.

The defect in motility exhibited by pathogenic strain 14028 in response to altered Dam levels is not dependent on the presence of RpoS. The alternative sigma factor, RpoS, is involved in *Salmonella* virulence and virulence-associated gene expression (34). In addition, allelic replacement of *rpoS* from a pathogenic strain results in the partial restoration of virulence to LT2 (55, 97, 102). Here we examined whether the differential regulation of motility under Dam^{OP} conditions was dependent on the presence of RpoS. An *rpoS* mutation (*rpoS1221*::Mu*dJ*) did not significantly affect the motility of strain 14028 or LT2 (Table 2), a finding consistent with the observation that the lack of RpoS results in only mild defects in flagellin production in a pathogenic strain (1). However, neither *rpoS* mutation nor the introduction of the $rpoS_{LT2}$ allele into strain 14028 alleviated the acute motility defect inherent to 14028 Dam^{OP} cells, although a mild derepression of flagellin synthesis was observed (Table 2 and data not shown). In addition, sequence analysis of avirulent Typhimurium strain, LT7, revealed a wild-type *rpoS*, indicating that the inability of LT7 to respond to Dam overproduction (Table 1) was not attributable to a mutant *rpoS* allele. Taken together, these data indicate that differential regulation of motility exhibited by 14028 and LT2 in response to altered Dam levels was not dependent on the presence of RpoS or a mutant allele of *rpoS*.

Dam overproduction leads to enhanced bile sensitivity in pathogenic strain 14028 but not in strain LT2. Enteric bacteria are inherently resistant to bile and utilize bile concentrations as a signal for the temporal and spatial production of virulence factors and for the induction of other adaptive mechanisms, including multidrug resistance (41, 78). Bile has been shown to repress *Salmonella* flagellar gene expression and motility (81). In addition, mutants that lack or overproduce *dam* are highly sensitive to bile (44, 82). Here we examined whether bile sensitivity was differentially regulated in pathogenic strain 14028 and LT2 in response to altered Dam levels. Although the lack of *dam* was associated with bile sensitivity in both strains, growth under Dam^{OP} conditions resulted in enhanced bile sensitivity specifically in 14028 cells over a range of physiologically relevant bile concentrations (3 to 5%) (41); the bile sensitivity of LT2 Dam^{OP} cells did not significantly differ from that exhibited by wild type (*dam*$^+$) (Table 3). Thus, growth under Dam^{OP} conditions is associated with acute defects in motility, flagellar synthesis, and bile resistance in pathogenic strain 14028 but not in the laboratory strain LT2.

***Salmonella* gene expression is differentially regulated in *dam* mutant derivatives of strains 14028 and LT2.** Greater than 40 genes are required for the proper morphogenic development of a functional flagellum, and they are classified with respect to the timing of their expression as early, middle, and late genes (reviewed in reference 19). Here we examined whether altered levels of the Dam regulatory protein differentially affected the transcription of Typhimurium flagellar genes (21, 38, 39), which encode products that contribute to pathogenicity and the elicitation of host immune responses (2, 22, 50, 86) in pathogenic strain 14028 versus laboratory strain LT2. Although the lack of *dam* did not significantly affect flagellar gene expression in either strain, Dam overproduction in pathogenic strain 14028 resulted in a 2- to 25-fold reduction in the transcription of early and/or middle regulatory genes (*flhC*, *flgM*, and *fliA*) and late structural genes encoding FliC and FljB flagellins, a motor-force-generating protein (MotA), and a chemosensory protein (CheY) with respect to wild-type (*dam*$^+$) levels (Fig. 3A). In contrast, Dam overproduction in avirulent laboratory strain LT2 did not significantly affect the transcription of these genes compared to that observed in wild type (*dam*$^+$). These data indicate that flagellar gene expression is differentially regulated in 14028 versus LT2 in response to Dam^{OP} conditions.

Dam represses the expression of several *Salmonella* genes that are preferentially expressed during infection (designated as in vivo-induced genes [*ivi*]) in strain 14028 (45, 65). In addition, microarray analysis of another Typhimurium pathogenic strain indicates that many genes are either activated or repressed in response to *dam* (5). To determine whether differential regulation affected genes other than those of the flagellar regulon, we assessed whether the lack of *dam* differentially affected *ivi* gene expression in strain 14028 versus strain LT2. As reported earlier (45), the lack of *dam* resulted in the derepression of several *ivi* genes in strain 14028, includ-

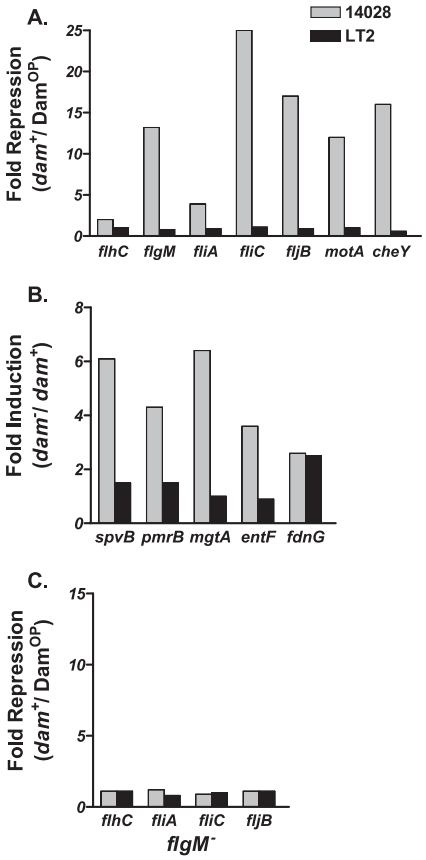

FIG. 3. Altered levels of Dam result in acute differences in bacterial gene expression in strain 14028 compared to strain LT2. (A) *Salmonella* cultures containing *dam*$^+$ and Dam^{OP} derivatives of flagellar gene transcriptional *lacZ* fusions in strain 14028 and strain LT2 were grown for 16 h in Luria-Bertani media (25) and assayed for β-galactosidase activity as described previously (94). Units reflect the relative fold repression (*dam*$^+$/Dam^{OP}). *B*. *dam*$^+$ and *dam* mutant derivatives of strains 14028 and LT2 containing *spvB*, *pmrB*, *mgtA*, *entF*, and *fdnG lacZ* transcriptional fusions were cultured and assayed as described above. Units reflect the relative fold induction (*dam* mutant/*dam*$^+$). *C*. *dam*$^+$ and Dam^{OP} derivatives of *flgM* mutant and *flgM*$^+$ isolates of strains 14028 and LT2 containing flagellar transcriptional fusions were cultured and assayed as described above. Units reflect the relative fold repression (*dam*$^+$/Dam^{OP}).

ing *spvB*, encoding an actin cytotoxin (58); *pmrB*, involved in resistance to antimicrobial peptides (87); and *mgtA* and *entF*, involved in the transport of magnesium and iron, respectively (31, 36) (Fig. 3B and data not shown). In contrast, only 5 of 26 *ivi* genes that were previously shown to be *dam* regulated in strain 14028 were derepressed in *dam* mutant strains of LT2, one of which is *fdnG*, encoding formate dehydrogenase, involved in anaerobic metabolism (96). These data indicate that differential regulation observed between strains 14028 and LT2 in response to altered Dam levels is not limited to genes of the flagellar regulon.

***flgM* contributes to the differential gene regulation observed between strains 14028 and LT2 in response to altered Dam levels.** To further understand the molecular basis of flagellar differential regulation displayed by 14028 and LT2 in response to growth under Dam^{OP} conditions, we assessed the role of FlgM, a negative regulator of flagellar gene expression (37, 38).

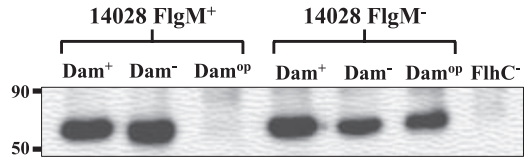

FIG. 4. Differential regulation of the strain 14028 flagellar synthesis in response to altered Dam levels occurs in a FlgM-dependent fashion. Whole-cell protein extracts corresponding to ~10^7 *Salmonella* cells from *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of Δ*flgM* and *flgM*$^+$ Typhimurium pathogenic strain 14028 were subjected to SDS-PAGE and transferred to PVDF membrane. Membranes were probed with *Salmonella* primary antibody H antiserum i (anti-FliC). Signal was detected as described in Materials and Methods. Extracts of *dam*$^+$ and *dam* mutant derivatives of Δ*flgM* 14028 were diluted fourfold to obtain a FliC signal intensity similar to that observed in Dam^{OP} derivatives. FlhC$^-$ (*flhC5456*::Mu*dJ*) strain MT2425 was used as a nonflagellated control (21).

flgM mutation in Dam^{OP} 14028 cells resulted in the derepression of all (four of four) flagellar genes tested under Dam^{OP} conditions (compared to the relative flagellar gene expression in *flgM*$^+$ [Fig. 3A] versus *flgM* mutant [Fig. 3C] cells). Accordingly, the *flgM* mutation partially relieved the defects in FliC and FljB synthesis (Fig. 4) and the associated defect in motility inherent to Dam^{OP} 14028 cells relative to that observed in *dam*$^+$ Δ*flgM* 14028 cells (data not shown). Although *flgM* mutation does not fully restore flagellar synthesis and motility to wild-types levels under Dam^{OP} conditions, these data indicate that significant aspects of flagellar differential gene regulation exhibited by 14028 relative to LT2 occur in an FlgM-dependent fashion.

***Salmonella* flagellar-phase variation is differentially affected in Dam-overproducing derivatives of strains 14028 and LT2.** Typhimurium strains oscillate between two flagellar expression states, consisting of either FliC (H1) or FljB (H2) flagellin subunits—a process termed phase variation (9, 93). The frequency of switching between flagellar types and magnitude of flagellar synthesis can be modulated by environmental and genetic signals, so that the pool of infecting organisms can be comprised of antigenically distinct populations that are altered in their capacity for virulence and elicitation of host immune responses (reviewed in reference 19 and 100). The flagellar-phase transition rate is controlled by a reversible genetic switch comprising the site-specific inversion of a promoter fragment that results in the mutually exclusive expression of either FliC or FljB (9, 93).

To assess whether altered Dam levels differentially affected flagellar phase variation, transition rates of *fljB*$_{On}$ to *fljB*$_{Off}$ and *fljB*$_{Off}$ to *fljB*$_{On}$ expression states were evaluated in pathogenic and nonpathogenic strains. In agreement with previous reports (38, 50), both 14028 and LT2 *dam*$^+$ strains favored the *fljB*$_{Off}$ expression state; i.e., the ratio of *fljB*$_{On}$ to *fljB*$_{Off}$/*fljB*$_{Off}$ to *fljB*$_{On}$ was >1.0 (Table 4). However, under Dam^{OP} conditions, the inherent bias toward the *fljB*$_{Off}$ expression state was increased from 3.8- to 8.0-fold in strain 14028 and decreased from 2.5- to 1.3-fold in LT2 relative to the transition rates observed in the respective *dam*$^+$ strains. Due to the reversible nature of the phase-variable switch, the increased and decreased frequency of the *fljB*$_{Off}$ expression state was also accompanied by a concomitant increased and decreased fre-

TABLE 4. Flagellar-phase transition rates are differentially affected in Dam^{OP} derivatives of strains 14028 and LT2

Strain[a]	Genotype	Switching frequency (10^{-3})		*fljB*$_{On}$ to *fljB*$_{Off}$/ *fljB*$_{Off}$ to *fljB*$_{On}$
		fljB$_{On}$ to *fljB*$_{Off}$	*fljB*$_{Off}$ to *fljB*$_{On}$	
14028	*dam*$^+$	1.69	0.446	3.8
	dam mutant	2.79	0.820	3.4
	Dam^{OP}	1.90	0.238	8.0
LT2	*dam*$^+$	1.19	0.469	2.5
	dam mutant	2.23	0.463	4.8
	Dam^{OP}	1.65	1.26	1.3

[a] *fljB*::*lacZ* transition rates (per cell per generation) of *fljB*$_{On}$ to *fljB*$_{Off}$ and *fljB*$_{Off}$ to *fljB*$_{On}$ were calculated from a single blue colony (Lac$^+$) or a single white colony (Lac$^-$) from *dam*$^+$, *dam* mutant, and Dam^{OP} derivatives of *fljB5001*::Mu*dJ* fusions (38) in Typhimurium pathogenic strain, 14028, and avirulent laboratory strain, LT2, grown on minimal E medium agar (25) containing 0.2% glycerol and 40 to 80 μg of X-Gal/ml. Colonies exhibiting a Lac$^+$ or Lac$^-$ phenotype (no sectors) were excised from the agar and plated to determine the total number of organisms in the colony and to score the Lac phenotype after incubation for 48 h at 37°C. Transition rates represent the weighted average of five independent colonies as described in Materials and Methods.

quency of the *fliC*$_{On}$ expression state in 14028 and LT2, respectively (data not shown). Thus, phase variation was differentially regulated in 14028 and LT2 in response to altered Dam levels, resulting in distinct differences in flagellin expression states.

DISCUSSION

The fundamental principles that distinguish a pathogenic serovar from a nonpathogenic serovar are often obscure since some nonpathogenic serovars contain virulence genes that could encode the capacity to enter into, replicate within, and persist at host sites that are inaccessible to commensal species. However, many nonpathogenic strains remain impaired for these virulence activities and cannot sustain a productive infection. Pathogenicity is further complicated by the fact that, among pathogenic isolates, some strains are capable of asymptomatic colonization or persistence in a particular animal species while causing acute disease in another animal species (84). In the present study, we show that altered Dam levels differentially affected virulence-associated bacterial gene expression, as well as flagellar synthesis, bacterial motility, bile resistance, and phase variation, in pathogenic strain 14028 compared to the closely related avirulent laboratory strain LT2. These data suggest that significant aspects of pathogenicity may be attributed to differential gene regulation rather than to major differences in genomic content.

Dam methylation coordinates many cellular processes, including gene expression, DNA mismatch repair, chromosomal replication, and nucleoid structure (15, 60, 62). *dam* expression is increased in bacterial cells grown under log-phase conditions in vitro, presumably to keep pace with the need of rapidly dividing cells to maintain the appropriate methylation state. Such growth rate control may be a reflection of a pathogen's life cycle whereby, for example, *E. coli* is thought to grow more rapidly in the colon than outside the host (60, 83). The capacity of pathogenic strain 14028 to sharply decrease flagellar synthesis, motility, and bile resistance in response to altered Dam levels (Tables 1 and 3) may mimic the in vivo condition, wherein bile-mediated repression of flagellin upon entry into the intestine may be favorable until *Salmonella* transits through the mucus layer to colonize the epithelial surface (3, 23, 24, 41, 80). Since the Dam-dependent differential regulation exhibited by 14028 and LT2 extends to a wide variety of genes (Fig. 3b), the integration of environmental cues into bacterial regulatory networks that are critical to pathogenicity (26, 64, 69, 72) may not be operational, or may be operational to the same extent, in nonpathogenic strains such as LT2. Consistent with this suggestion, allelic differences in the *Salmonella* regulatory protein, RpoS, have been associated with the avirulence phenotype of LT2 (55, 97, 102). However, the differential regulation of motility exhibited by pathogenic strain 14028 and LT2 under Dam^{OP} conditions is not dependent on the presence of RpoS, indicating that other functions contribute to the regulatory differences inherent to these closely related strains. Differential gene regulation may also contribute to some of the virulence, host range, and disease manifestation disparities exhibited within and between closely related pathogenic serovars. Indeed, the *fliC* gene of another pathogenic Typhimurium strain is also regulated by *dam* but in a reciprocal fashion from that exhibited by strain 14028 (5).

The transitioning between Typhimurium flagellar types (phase variation) comprises a reversible genetic switch, involving the site-specific inversion of a promoter fragment, which controls the expression of FliC and FljB flagellins such that an individual cell is limited to one specific type at any given time (9, 93). In response to Dam overproduction, pathogenic strains exhibit an enhanced bias toward the FliC expression state (Table 4). The capacity of Dam levels to influence flagellar expression states in vitro may reflect a mechanism by which pathogenic strains are able to augment the frequency of FliC expressing cells, which have a selective advantage over FljB-expressing cells in animal models of typhoid fever (50). Thus, the capacity to alter *dam* levels can result in marked differences in flagellin expression states.

Insights into the possible mechanism by which Dam contributes to differential gene regulation come from the regulatory analysis of the uropathogenic *E. coli pap* operon, which encodes pili that are essential for urinary tract infections (11, 15, 47–49). The regulatory mechanism involves the formation of heritable DNA methylation patterns (11, 42, 85, 99) that control gene expression by modulating the binding of regulatory proteins, similar to what has been observed in eukaryotes (7, 46, 52). Since epigenetic regulatory mechanisms involve DNA modifications (methylation) that do not alter the DNA sequence, the progeny expression state can be readily reversed to that of the parent once the selection stimulus imposed upon the progeny cells is removed. Thus, bacterial pathogens may utilize epigenetic control of specific virulence functions as a reversible and heritable mechanism by which to engender variability to the infecting population. Epigenetic modifications are not subject to the same constraints as genetic mutations that are, by nature, relatively stable and perhaps more restricted in their ability to respond to evolutionary pressures. For example, through host Toll-like receptor 5, the innate immune system targets a conserved site on flagellin that is essential for bacterial motility, precluding mutations that result in a nonfunctional flagellum (73, 95). Taken together, the

present study suggests that significant aspects of pathogenicity may be attributable to differential gene regulation, perhaps via epigenetic modifications (DNA methylation) that may enhance microbial fitness by the augmentation of diversity at the phenotypic level without a concomitant augmentation of diversity at the genomic level.

Differential gene regulation coupled with classical genetic mutation may be vital to microbial fitness within the host since bacterial infections often originate from clonal expansion of a single cell (71, 75). Thus, pathogenic bacteria must generate diversity to adapt to host polymorphisms and immune clearance mechanisms, enabling them to evade immune defenses and to gain access to new sites within its natural host(s) (74, 76, 77). This is achieved by the generation of genetic variants with altered antigenic properties (antigenic variation) that arise by either classical genetic mutation or by gene regulatory mechanisms that facilitate the transitioning between expressed and unexpressed states (12, 24, 70) (e.g., phase variation of type 1 or type 2 flagella [100]). Such genetic plasticity may also have a profound effect on the emergence and/or evolution of pathogenic serovars as selective pressures give rise to genetic variants that may have altered virulence properties, e.g., maintaining the ability to cause acute disease in a given natural animal host while acquiring the ability to cause acute disease or asymptomatic colonization or persistence in a new animal host.

ACKNOWLEDGMENTS

We thank C. Samuel and D. Morse for critically reviewing the manuscript, K. Hughes for kindly providing strains, and R. Werlin for technical assistance with the bile assay.

This study was supported by the G. Harold and Leila Y. Mathers Foundation; by National Institutes of Health grants AI 61399-01 and AI 59242-04A1; and by National Research Initiative of the USDA Cooperative State Research, Education, and Extension Service grant (2004-04574) (to M.J.M.).

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