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DNA Adenine Methylase Is Essential for Viability and Plays a Role in the Pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae*

STEVEN M. JULIO,[1] DOUGLAS M. HEITHOFF,[1] DANIELE PROVENZANO,[2] KARL E. KLOSE,[2] ROBERT L. SINSHEIMER,[1] DAVID A. LOW,[1] AND MICHAEL J. MAHAN[1]*

Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, California 93106,[1] and Department of Microbiology, University of Texas Health Science Center, San Antonio, Texas 78229-3900[2]

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Salmonella **strains that lack or overproduce DNA adenine methylase (Dam) elicit a protective immune response to different *Salmonella* species. To generate vaccines against other bacterial pathogens, the *dam* genes of *Yersinia pseudotuberculosis* and *Vibrio cholerae* were disrupted but found to be essential for viability. Overproduction of Dam significantly attenuated the virulence of these two pathogens, leading to, in *Yersinia*, the ectopic secretion of virulence proteins (*Yersinia* outer proteins) and a fully protective immune response in vaccinated hosts. Dysregulation of Dam activity may provide a means for the development of vaccines against varied bacterial pathogens.**

Salmonella DNA adenine methylase (Dam) mutants ectopically express multiple genes that are normally induced during infection (18, 20, 27). These Dam mutants are markedly attenuated but highly effective as live vaccines against murine typhoid fever (12, 20). DNA adenine methylases are highly conserved in many pathogens such as *Vibrio cholerae* (http://www.tigr.org), *Salmonella enterica* serovar Typhi (http://www.sanger.ac.uk), pathogenic *Escherichia coli* (2), *Yersinia pestis* (http://www.tigr.org), and *Haemophilus influenzae* (10). The goal of this study was to determine whether the findings regarding Dam's role in *Salmonella* pathogenesis could be extended to *V. cholerae* and *Yersinia pseudotuberculosis*, the causative agents of human cholera and gastroenteritis, respectively; additionally, *Y. pseudotuberculosis* causes a fatal bacteremia in mice. In contrast to *Salmonella*, which is a facultative intracellular parasite, both *Y. pseudotuberculosis* and *V. cholerae* are principally extracellular pathogens. *Yersinia* sp. pathogenesis is dependent upon virulence proteins called Yops (for Yersinia outer proteins) (6, 15, 37), which, upon host contact, are injected directly into the host cell cytoplasm, where they act as effectors to inhibit phagocytosis and proinflammatory cytokine release (31, 32, 36, 38). In contrast, *V. cholerae* is a mucosal pathogen that expresses virulence factors, including cholera toxin and toxin coregulated pilus, in the small intestine (9).

In this report, we show that Dam is essential for the viability of *Y. pseudotuberculosis* and *V. cholerae*. Overproduction of Dam was not lethal and attenuated the virulence of both pathogens. Additionally, Dam-overproducing strains of *Yersinia* ectopically secreted Yop virulence proteins in vitro and conferred full protection against *Yersinia* bacteremia in vaccinated hosts.

* Corresponding author. Mailing address: Dept. of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA 93106. Phone: (805) 893-7160. Fax: (805) 893-4724. E-mail: mahan@lifesci.ucsb.edu.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Derivatives of *Y. pseudotuberculosis* strain YPIIIpYV (obtained from Stanley Falkow) and *V. cholerae* strain 0395 (classical, Ogawa serotype; obtained from John Mekalanos) (Table 1) were grown overnight with shaking at 28 and 37°C, respectively. The following antibiotics were used at the indicated concentrations: for *V. cholerae*, kanamycin (50 μg/ml), ampicillin (50 μg/ml), tetracycline (1.2 μg/ml), and streptomycin (100 μg/ml); and for *Y. pseudotuberculosis*, kanamycin (50 μg/ml), ampicillin (50 μg/ml), tetracycline (5 μg/ml), and chloramphenicol (20 μg/ml).

Construction of genomic DNA libraries. Genomic DNA libraries for *Y. pseudotuberculosis* and *V. cholerae* were constructed using approximately 2 to 5 μg of genomic DNA that was partially digested with *Sau*3AI and size fractionated to 2.5 to 6 kb on a 0.8% agarose gel. The size-fractionated DNA was ligated into the vector pWKS30 (ampicillin resistant [Ap^r] [43]) that was previously cleaved with *Bam*HI. The recombinant plasmids were introduced into *E. coli* DH5α λ*pir* by electroporation; we then pooled the Ap^r recombinant clones, from which plasmid DNA was isolated.

Construction of plasmids containing *dam* derived from *V. cholerae*, *Y. pseudotuberculosis*, and *E. coli*. Plasmid pWKS30Tc is a tetracycline-resistant derivative of pWKS30 (43). The *tet* gene and promoter from pBR322 were excised as an *Ava*I/*Eco*RI fragment, treated with the Klenow fragment to produce blunt ends, and cloned into plasmid pWKS30, which had been partially digested with *Bgl*II and *Ssp*I (removing the *bla* gene) and treated with the Klenow fragment to produce pWKS30Tc. The *V. cholerae dam* gene was cloned into the blunt-ended *Sma*I site of pWKS30Tc by ligating a 1.2-kb *Dra*III/*Eco*RI chromosomal DNA fragment containing *V. cholerae dam* that had been treated with the Klenow fragment, generating a pWKS30Tc plasmid harboring *V. cholerae dam*. The *Y. pseudotuberculosis dam* gene was cloned into the blunt-ended *Sma*I site of pWKS30Tc by ligating a 1.3-kb *Nco*I/*Pvu*II chromosomal DNA fragment containing *Y. pseudotuberculosis dam* that had been treated with the Klenow fragment, generating a pWKS30Tc plasmid harboring *Y. pseudotuberculosis dam*. The *E. coli dam* gene was cloned into the blunt-ended *Sma*I site of pWKS30Tc by ligating a 1.3-kb *Xba*I/*Pvu*II chromosomal DNA fragment containing *E. coli dam* that had been treated with the Klenow fragment, generating a pWKS30Tc plasmid harboring *E. coli dam*. Plasmid clones containing the putative *V. cholerae*, *Y. pseudotuberculosis*, and *E. coli dam* genes were introduced into an *E. coli dam* mutant strain. Recovered plasmids were found to be resistant to the methylation-sensitive restriction enzyme *Mbo*I, indicating that the recombinant clones encode Dam.

Plasmid pTP166Cm is a chloramphenicol-resistant derivative of pTP166 (Ap^r), which overproduces *E. coli dam* from a P_{tac} promoter (29). A blunt-ended 1.4-kb *Bsa*AI fragment containing the chloramphenicol resistance gene and its promoter from pACYC184 was ligated to a pTP166 plasmid derivative that had been digested with *Dra*I and *Aat*II (removing the *bla* gene) and treated with the Klenow fragment. The resulting chloramphenicol-resistant clone, pTP166Cm, overproduced *E. coli dam*, as evidenced by the DNA methylase assay (see below).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
Y. pseudotuberculosis		
YPIIIpYV	Wild type	Stanley Falkow
MT2294	*dam*::Kn + pTP166-Cm	This work
MT2357	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)]	This work
MT2358	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)] pWKS30-Tc + *Y. pseudotuberculosis dam*$^+$	This work
MT2359	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)] pWKS30-Tc + *E. coli dam*$^+$	This work
MT2360	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)] pWKS30-Tc	This work
V. cholerae		
O395	Wild type; classical	John Mekalanos
MT2284	*dam*::Kn pWKS30-Tc + *E. coli dam*$^+$	This work
MT2361	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)]	This work
MT2362	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)] pWKS30-Tc + *V. cholerae dam*$^+$	This work
MT2363	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)] pWKS30-Tc + *E. coli dam*$^+$	This work
MT2364	DUP[ϕ(*dam*::Kn)∗pCVD442∗(*dam*$^+$)] pWKS30-Tc	This work
S. enterica serovar Typhimurium		
MT2188	*damΔ232*	20
Plasmids		
pTP166-Cm	*E. coli dam* under *tac* promoter control; chloramphenicol-resistant (Ap^s) derivative of pTP166	29; this work
pWKS30-Tc	Tetracycline-resistant (Ap^s) derivative of pWKS30	43; this work

DNA sequencing and protein sequence analysis. The nucleotide sequences of the *dam* genes were determined using a Big Dye Terminator reaction kit, and samples were analyzed on a model 310 genetic analyzer (PE Biosystems). Protein sequence alignment was performed using the CLUSTAL W method available at the European Bioinformatics website (http://www2.ebi.ac.uk/clustalw/).

DNA adenine methylase assays. Dam activity was measured by a modified tritiated *S*-adenosylmethionine ([^{3}H]SAM) incorporation assay (13) wherein SAM serves as a methyl donor for DNA methylases. Briefly, cells from an overnight grown culture (10 ml) were collected by centrifugation, quick frozen under liquid nitrogen, washed once in 1× Tris-EDTA buffer (10 mM Tris HCl [pH 8.0]–1 mM EDTA [pH 8.0]), and resuspended in 10× Tris-EDTA. Lysozyme (0.05 mg) was added, and the cells were disrupted by sonication. Cell debris was removed by centrifugation, and the cell extracts were recovered. The total amount of protein in each cell extract was determined by a Bradford protein assay. GATC-specific methylase activity was quantified by adding 0.1 ml of cell extract to 7 μl of the following methylase reaction mixture: 0.015 mM SAM, 34 mM dithiothreitol, 0.5 μg of RNase A, 4 μg of bovine serum albumin, 5 μg of double-stranded GATC containing the DNA substrate (5′-CAGGATCCATGC GATCAACCGATCAAGGATCCAC-3′), and 0.55 μCi of [*methyl*-^{3}H]SAM. The reaction mixture was incubated for 1 h at 37°C, after which an excess of unlabeled SAM was added to reach a final concentration of 3.0 mM to stop the reaction. A 0.08-ml sample of each reaction mixture was transferred to Whatman DE81 filter paper and washed several times with 0.4 M NH_4HCO_3, followed by one wash with 95% ethyl alcohol. The filters were allowed to dry, and the amount of *methyl*-^{3}H incorporated onto the DNA substrate was determined by scintillation counting. Methylase activity in each cell extract was calculated as counts per minute of *methyl*-^{3}H per microgram of total protein in the cell extract.

Virulence, colonization, and protection assays. (i) LD_{50} assay. For *Yersinia*, an assay was used to determine the lethal dose required to kill 50% of the animals (LD_{50}); this virulence assay was performed as described in reference 20. Briefly, mutant and wild-type *Yersinia* species grown overnight in Luria broth at 28°C with shaking were washed in 0.15 M NaCl, diluted in 0.2 ml of 0.2 M phosphate buffer (pH 8.0), and used to perorally infect BALB/c mice by gastrointubation. The protective capacity of Dam derivatives was determined by challenging immunized mice with the virulent parental strain. Mice were examined daily following challenge for morbidity and mortality. To determine the number of bacteria in host tissues, moribund mice were sacrificed and bacteria were recovered from host tissues and plated for colony counts. Host tissues assayed included Peyer's patches (the four Peyer's patches proximal to the ileal-cecal junction), mesenteric lymph nodes, and spleens.

(ii) Competitive index assay. The competitive index is the ratio of mutant to wild-type organisms recovered from host tissue after infection. For *Y. pseudotuberculosis* infection, 6- to 8-week-old BALB/c mice were gastrointubated with 8.0×10^8 cells of mutant organisms and 8.0×10^8 cells of wild-type organisms. After 7 days, mice were sacrificed, spleens were recovered and homogenized, and bacteria were enumerated by direct colony count as described previously (5). For *V. cholerae* infection, 5-day-old CD-1 suckling mice were coinoculated perorally with approximately 10^5 mutant organisms and 10^5 wild-type organisms; 24 h postinfection, mice were sacrificed and bacterial numbers were isolated from the intestine as described previously (7).

Nucleotide sequence accession numbers. The sequence data for *V. cholerae dam* and *Y. pseudotuberculosis dam* have been submitted to the DDBJ, EMBL, and GenBank databases under accession numbers AF274317 and AF274318, respectively.

RESULTS

Characterization of *dam* from *Y. pseudotuberculosis* and *V. cholerae*. To clone the *dam* gene from *Y. pseudotuberculosis* and *V. cholerae*, recombinant plasmids derived from a genomic DNA library constructed from both pathogens were screened for the ability to complement the 2 aminopurine (2-AP) sensitivity phenotype of an *S. enterica* serovar Typhimurium *dam* mutant strain (2-AP is a purine analog which is toxic to *dam* mutants [14]). A 1.3-kb *Nco*I/*Pvu*II DNA fragment from *Y. pseudotuberculosis* conferred 2-AP resistance to a *dam* mutant serovar Typhimurium strain and encoded DNA adenine methylase activity as evidenced by resistance of the recombinant plasmid (recovered from *dam* mutant *E. coli*) to digestion with the restriction enzyme *Mbo*I, which cleaves only nonmethylated GATC sequences. Sequence analysis revealed an open reading frame (ORF) encoding a putative 271-amino-acid protein exhibiting 71% identity to the entire *E. coli* Dam protein. Taken together, these data indicate that this ORF encoded *Y. pseudotuberculosis* Dam activity.

A 1.2-kb *Eco*RI/*Dra*III *V. cholerae* DNA fragment conferred 2-AP resistance to *dam* mutant serovar Typhimurium and encoded DNA adenine methylase activity as judged by resistance of the plasmid clone to *Mbo*I cleavage. Sequence analysis of

the insert DNA revealed an ORF encoding a 277-amino-acid protein which displays 63.5% identity over the entire *E. coli* Dam protein. The *V. cholerae dam* gene described in this study differs from a previously published *V. cholerae dam* sequence, which partially overlaps and is in the opposite orientation to that of the *dam* gene identified here and has only 30 to 35% identity over the entire *E. coli* Dam sequence (1). Moreover, a recombinant plasmid containing the previously identified *dam* gene was unable to confer 2-AP resistance to a *dam* mutant serovar Typhimurium strain, and when this plasmid was recovered from *dam* mutant *E. coli*, it was completely digested by *Mbo*I, indicating the lack of DNA methylase activity (data not shown). These data suggest that the *dam* gene identified in this study encodes *V. cholerae* DNA adenine methylase activity that is specific for GATC sequences.

***dam* is essential for viability in *Y. pseudotuberculosis and V. cholerae*.** Standard genetic procedures to remove the *dam* gene from *Y. pseudotuberculosis* and *V. cholerae* were unsuccessful, suggesting that, in contrast to *Salmonella* and *E. coli, dam* is essential for viability in *Yersinia* and *Vibrio*. To confirm the requirement of *dam* for growth, suicide plasmids containing *dam* deletion mutations were integrated into the native chromosomal *dam* locus of *Y. pseudotuberculosis* and *V. cholerae*. The chromosome integration event generates a duplication of the *dam* locus, in which one copy is dam^+ and one copy has a mutation (22). Essentiality (on rich medium) was demonstrated by showing that the generation of the *dam* mutation haploid state from the parental *dam* duplication is dependent upon the presence of a dam^+ gene provided in *trans*. The *dam* deletion structure in the chromosomes of *Y. pseudotuberculosis* and *V. cholerae* was confirmed by both PCR and Southern analysis according to the method of Julio et al. (22). Table 2 shows that *dam* mutant segregants of *Y. pseudotuberculosis* and *V. cholerae* were obtained only in the presence of a wild-type copy of *dam* provided in *trans*. These data indicate that *dam* is essential for viability in *Y. pseudotuberculosis* and in *V. cholerae*, similar to the essential role of the cell cycle-regulated methyltransferase CcrM in *Caulobacter crescentus, Rhizobium meliloti, Brucella abortus*, and *Agrobacterium tumefaciens* (23, 33, 35, 44).

Dam overproduction attenuates the virulence of *Y. pseudotuberculosis* and *V. cholerae*. To examine whether altered levels of Dam activity affected the virulence of *Y. pseudotuberculosis* and *V. cholerae*, recombinant plasmids that overproduced *E. coli* Dam were introduced into both pathogens, and the resulting strains were assayed for virulence. Note that loss of the Dam-overproducing plasmid in a dam^+ parental background would result in a virulent (wild-type) strain. Thus, these virulence studies were performed with *dam* mutant parental backgrounds since *dam* is essential for viability in *Y. pseudotuberculosis* and *V. cholerae* and loss of the Dam-overproducing plasmids in *dam* mutant backgrounds is lethal for both pathogens.

Overproduction of *E. coli* Dam from a recombinant plasmid in *Y. pseudotuberculosis* (MT2294) and *V. cholerae* (MT2284), resulting in 74- and 53-fold increases in Dam activity, respectively, was not lethal but significantly attenuated the virulence of these two pathogens. That is, Dam overproduction results in a >6,000-fold attenuation in a *Y. pseudotuberculosis* murine bacteremia infection model and a 5-fold defect ($P < 0.05$) in *V. cholerae* colonization in a suckling mouse model (Table 3). The attenuation in both organisms was not due to a general growth defect since the Dam-overproducing strains showed growth rates in vitro similar to that of the wild type (data not shown). Relevant to these findings, CcrM overproduction was recently shown to attenuate the intracellular replication of *B. abortus* in murine macrophages (35).

TABLE 2. Dam is essential for viability in *Y. pseudotuberculosis* and *V. cholerae*[a]

Strain	Plasmid	No. of colonies with the indicated genotype at the *dam* locus after excision of pCVD442	
		dam^+	Δ*dam*
Y. pseudotuberculosis			
MT2357	None	200	0
MT2358	dam^+ (*Y. pseudotuberculosis*)	135	44
MT2359	dam^+ (*E. coli*)	151	21
MT2360	Vector alone	200	0
V. cholerae			
MT2361	None	196	0
MT2362	dam^+ (*V. cholerae*)	153	47
MT2363	dam^+ (*E. coli*)	118	76
MT2364	Vector alone	194	0

[a] Integration of the suicide plasmid pCVD442 (8) into the *Y. pseudotuberculosis* or *V. cholerae* chromosome results in a duplication of the *dam* locus wherein one copy contains the wild-type *dam* gene and the other copy contains *dam* with a deletion associated with a kanamycin resistance marker (22). Selection for the excision of pCVD442 on plates containing 10% sucrose result in either dam^+ or *dam* mutant (Δ*dam*::Kn) segregants (8). The *dam* mutant segregants of *Y. pseudotuberculosis* or *V. cholerae* are associated with chromosomal internal deletions of 314 or 324 bp of the *dam* sequence, respectively. For plasmid complementation experiments, pWKS30-Tc (43) was used to provide the *dam* gene from *Y. pseudotuberculosis, V. cholerae*, or *E. coli* as indicated.

Dam-overproducing *Y. pseudotuberculosis* ectopically secretes Yops. To understand the mechanism of *Yersinia* virulence attenuation, we questioned whether Dam-overproducing *Y. pseudotuberculosis* showed altered secretion of Yops. Yops, which play essential roles in *Yersinia* virulence, are normally under strict regulatory control by the low calcium response, whereby Yop secretion occurs in vitro only at 37°C under conditions of low calcium (40). However, overproduction of Dam in *Y. pseudotuberculosis* resulted in the relaxation of the temperature, but not the low-calcium, dependence of Yop secretion (Fig. 1). These data indicate that Dam participates in the environmental regulation of the secretion of *Yersinia* virulence proteins.

Dam-overproducing *Y. pseudotuberculosis* strains confer protective immune responses in mice. Because Dam-overproducing *Y. pseudotuberculosis* strains were attenuated for virulence, we determined whether they could serve as live attenuated vaccines against murine bacteremia. BALB/c mice were perorally immunized via gastrointubation with a dose of 9.3×10^9 cells of the *Y. pseudotuberculosis* Dam-overproducing strain MT2294, as described in footnote *a* of Table 2. Eight weeks later, the immunized mice were challenged perorally with 2.6×10^{10} wild-type *Y. pseudotuberculosis* cells. All (13 of 13) mice immunized with Dam-overproducing *Y. pseudotuberculosis* survived a wild-type *Yersinia* challenge of >1,000-fold above

TABLE 3. Dam overproduction confers a virulence defect in *Y. pseudotuberculosis* and *V. cholerae*

Strain	Relevant genotype[a]	Oral LD_{50} ratio[b] (mutant/wild type)	Competitive index[c]
MT2294	Dam^{OP} *Y. pseudotuberculosis*	>6,000	$<10^{-4}$
MT2284	Dam^{OP} *V. cholerae*	ND	0.218

[a] Bacterial strains are derivatives of *Y. pseudotuberculosis* strain YPIIIpYV and *V. cholerae* strain O395. Dam-overproducing (Dam^{OP}) strains of *Y. pseudotuberculosis* (MT2294) and *V. cholerae* (MT2284) contain *E. coli dam* on chloramphenicol- and tetracycline-resistant derivatives of the high-copy-number recombinant plasmid pTP166 (29) and the medium-copy-number plasmid pWKS30 (43), respectively, in Dam mutant (Δ*dam*::Kn) genetic backgrounds. (Dam overproduction from the high-copy-number plasmid pTP166-Cm in *V. cholerae* was deleterious to *V. cholerae* cell growth as evidenced by a 50% decrease in the rate of growth on rich medium.) Since *dam* is essential for viability in *Y. pseudotuberculosis* and *V. cholerae*, the loss of the Dam-overproducing plasmids in *dam* mutant backgrounds is lethal for both pathogens.

[b] The oral LD_{50} ratio (the LD_{50} of the Dam-overproducing strain divided by the LD_{50} of wild-type bacteria) was determined by infecting 18 BALB/c mice with 1.56×10^{11} cells of the *Y. pseudotuberculosis* Dam-overproducing strain MT2294 as described previously (20); 18 of 18 mice survived this challenge dose, and no visible signs of infection were observed. The peroral LD_{50} of wild-type *Y. pseudotuberculosis* (2.5×10^7) was determined by Monack et al. (30). ND, not determined.

[c] For *Y. pseudotuberculosis* infection, six BALB/c mice were gastrointubated with 8.0×10^8 *Yersinia dam* mutant cells containing the Dam-overproducing plasmid from strain MT2294 and 8.0×10^8 cells of the wild type. After 7 days, mice were sacrificed, spleens were recovered and homogenized, and bacteria were enumerated by direct colony counting as described previously (5). Of the 10^6 to 10^7 *Yersinia* organisms recovered from each of six spleens, none contained the Dam-overproducing plasmid. For *V. cholerae* infection, six CD-1 mice were gastrointubated with a 1:1 ratio of Dam-overproducing cells (MT2284) to wild-type cells; 24 h postinfection, mice were sacrificed and bacterial numbers were determined from the intestine as described previously (7). The attenuation conferred was significant according to the two-tailed Fisher exact test ($P < 0.05$).

the LD_{50}. None of 14 unimmunized mice survived the challenge dose. The protection conferred was significant according to the two-tailed Fisher exact test ($P < 0.05$). Mice immunized with the *Yersinia* Dam-overproducing strain were cleared of the vaccine between day 5 and day 21 and thus Dam-overproducing *Y. pseudotuberculosis* cells were not present at the time of challenge (S. M. Julio et al., submitted for publication). Moreover, vaccinated animals blocked the proliferation of virulent (Dam^+) *Yersinia* in Peyer's patches, mesenteric lymph nodes, and spleens and cleared these virulent bacteria by 21 days postinfection (Fig. 2). These data suggest that mice vaccinated with *Yersinia* Dam-overproducing strains hinder the proliferation of virulent *Yersinia* in mucosal and systemic tissues, similar to what has been observed in mice vaccinated with *Salmonella* Dam mutants (19).

DISCUSSION

DNA adenine methylase plays a pivotal role in many bacterial functions, including the replication, repair, transposition, and segregation of chromosomal DNA (26, 28). Additionally, in *Salmonella*, Dam is a global regulator of bacterial gene expression and plays a critical role in virulence, and mutants with altered levels of Dam activity elicit protective immune responses to murine typhoid fever (12, 20). Here we explored the role of Dam in the pathogenesis of two other enteric bacteria, *Y. pseudotuberculosis* and *V. cholerae*. In contrast to results of studies performed with *E. coli* and *Salmonella* (4, 28), Dam was found to be essential for viability in *Yersinia* and *Vibrio*. Dam overproduction attenuated the virulence of *Y. pseudotuberculosis* and *V. cholerae*, leading to, in *Yersinia*, a fully protective immune response in vaccinated hosts. Since mutations in Dam attenuate the virulence of several diverse pathogens, the role of DNA methylation in virulence may emerge as a common theme in bacterial pathogenesis.

Dam's essential role in the viability of *Y. pseudotuberculosis* and *V. cholerae*, which are members of the gamma subdivision of proteobacteria, parallels the essential role of CcrM (cell cycle-regulated methyltransferase) for the viability of several proteobacteria of the alpha subdivision, including *C. crescentus, R. meliloti, B. abortus*, and *A. tumefaciens* (23, 35, 39, 44). Both Dam and CcrM catalyze the transfer of a methyl group from SAM to the N-6 position of adenine at specific target sequences within DNA. However, the target sequences of these two enzymes are different: Dam methylates GATC sequences, and CcrM methylates GANTC sequences. Moreover, both the catalytic and SAM binding domains of Dam and CcrM are arranged in a different linear order. For these reasons, Dam and CcrM belong to different methyltransferase groups. Despite these differences, DNA adenine methylation may exert its effects on diverse bacteria via its role as a global regulator of gene expression. That is, Dam regulates many (>20) *Salmonella* genes that are specifically induced during infection (20), and CcrM autoregulates *ccrM* transcription and has been implicated in the regulation of a number of genes involved in normal cell cycle progression (33, 34). Thus, the role of DNA methylation in regulating gene expression may explain, in part, Dam's function in many cellular processes of diverse bacteria.

DNA methylation plays a role in the virulence of a wide range of pathogens, including *Salmonella* spp. and *B. abortus* (which causes fetal-calf abortion) via Dam and CcrM activity, respectively (20, 26, 35). Dam also plays a role in *Salmonella* invasion, M-cell cytotoxicity, and the secretion of *Salmonella*

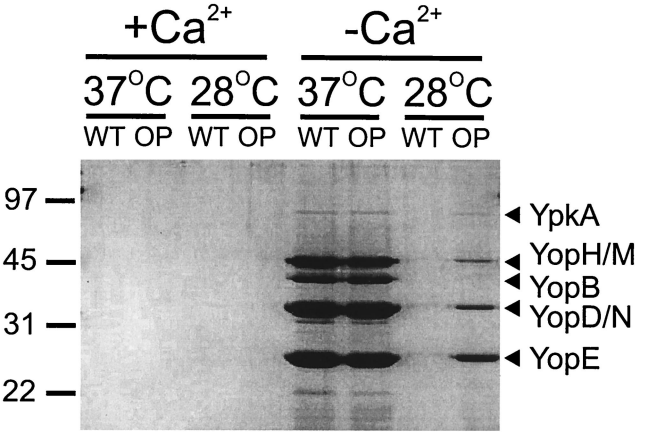

FIG. 1. Dam overproducer *Y. pseudotuberculosis* strains ectopically secrete Yops. Proteins were isolated from culture supernatants from wild-type (WT) and Dam overproducer (OP) *Yersinia* strains grown at the indicated temperatures in the presence ($+Ca^{2+}$) or absence ($-Ca^{2+}$) of calcium according to the method of Forsberg et al. (11). Cells were separated from the culture supernatant by centrifugation, supernatant proteins were precipitated with ammonium sulfate, and proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining. Yops were identified according to the method of Hakansson et al. (16). Numbers refer to molecular weight standards (in thousands).

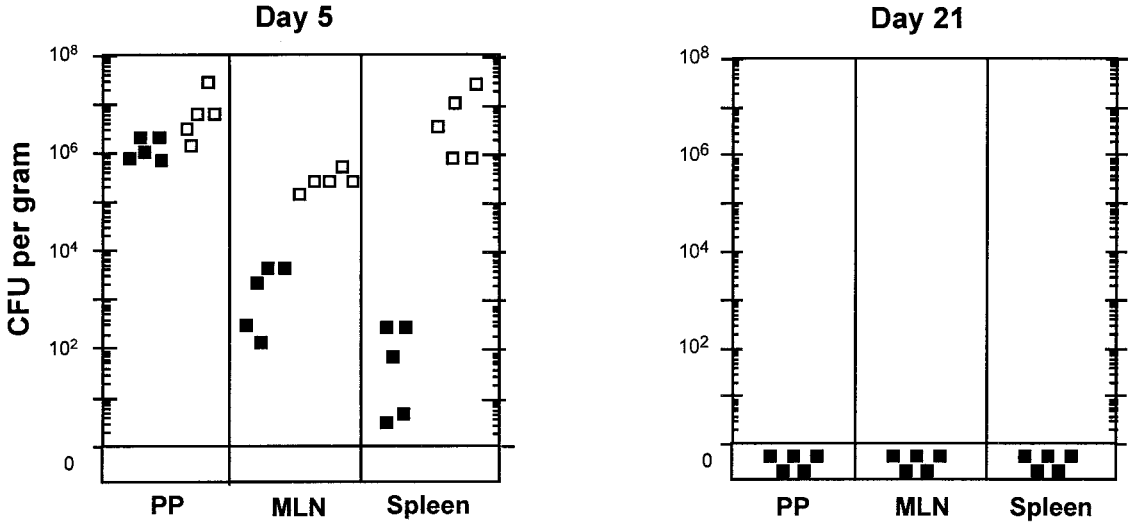

FIG. 2. Immunization with Dam overproducer *Yersinia* strains blocks the proliferation of virulent *Y. pseudotuberculosis* in mucosal and systemic tissues in mice. Virulent *Y. pseudotuberculosis* cells (3.2×10^9) were orally administered to nonvaccinated BALB/c mice (open boxes) or to mice vaccinated with 7.6×10^9 Dam overproducer *Y. pseudotuberculosis* cells (closed boxes). Vaccinated mice were orally challenged 8 weeks postimmunization. The fate of the virulent organisms was determined in host tissues at the times indicated after challenge. All nonvaccinated mice were dead by day 10. Mice immunized with the *Yersinia* Dam-overproducing strain were cleared of the vaccine between day 5 and day 21 and thus Dam-overproducing *Y. pseudotuberculosis* cells were not present at the time of challenge (Julio et al., submitted). PP, Peyer's patches; MLN, mesenteric lymph nodes.

virulence-associated proteins (12). Here we show that overproduction of Dam completely attenuated *Y. pseudotuberculosis* virulence and resulted in the export of Yops under conditions not normally permissive for secretion. Specifically, Dam overproduction relaxed the temperature dependence but not the low calcium dependence of Yop secretion, suggesting that Dam contributes to the strict environmental regulation governing the synthesis and/or secretion of *Yersinia* virulence proteins. Such altered protein secretion may attenuate the virulence of *Y. pseudotuberculosis*, as has been suggested for *Salmonella* (12). Moreover, the ectopic secretion of immunogens may contribute to the heightened immunity in hosts vaccinated with Dam mutants of *Yersinia* or *Salmonella* (19, 20).

The role of Dam in virulence and in the elicitation of protective immune responses may rely on its capacity as a global regulator of gene expression (19, 20, 24–26). Insights into the regulatory role of Dam have resulted from studies involving the *E. coli* pyelonephritis-associated pilus (*pap*) operon, which encodes pili that are required for infection of the urinary tract. The expression of *pap* genes is reversibly switched between the unexpressed state and the expressed state by a methylation-sensitive process termed phase variation (42). The reversible transition from non-pilus expressing to pilus expressing may allow the bacteria to attach and detach from urogenital tissues, enabling initial colonization and infection of the bladder and subsequent colonization and infection of the kidney, causing cystitis and pyelonephritis, respectively. Dam target sites (GATC sequences) in the *pap* promoter are protected from methylation by the binding of regulatory proteins at or near these sites, forming specific DNA methylation patterns analogous to what has been observed in eukaryotes (3, 17, 21, 41).

DNA methylation can modulate gene expression by altering the affinity of regulatory proteins for DNA, and, conversely, regulatory proteins can bind to nonmethylated Dam target sites, protecting these sites from methylation. Dysregulation of Dam activity can disable the ability of a pathogen to cause disease via aberrant virulence gene expression and contribute to the heightened immunity in vaccinated hosts through the ectopic production of an expanded repertoire of potential antigens. While a concern of this approach is that Dam overproducer strains can revert to wild-type virulence by mutation, insertion of multiple nontandem copies of Dam-overproducing cassettes in the chromosome should reduce the likelihood of this undesired scenario. Because the Dam methylase is essential for bacterial virulence or viability in multiple gram-negative pathogens (24, 26), Dam inhibitors are a promising target for antimicrobial drug development.

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REFERENCES

1. **Bandyopadhyay, R., and J. Das.** 1994. The DNA adenine methyltransferase-encoding gene (*dam*) of *Vibrio cholerae*. Gene **140:**67–71.
2. **Blattner, F. R., G. Plunkett III, 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.
3. **Braaten, B. A., X. Nou, L. S. Kaltenbach, and D. A. Low.** 1994. Methylation patterns in pap regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. Cell **76:**577–588.
4. **Casadesus, J., and J. Torreblanca.** 1996. Methylation-related epigenetic signals in bacterial DNA, p. 141–153. *In* V. E. A. Russo, R. A. Martienssen, and A. D. Riggs (ed.), Epigenetic mechanisms of gene regulation. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
5. **Conner, C. P., D. M. Heithoff, S. M. Julio, R. L. Sinsheimer, and M. J.**

Mahan. 1998. Differential patterns of acquired virulence genes distinguish Salmonella strains. Proc. Natl. Acad. Sci. USA **95:**4641–4645.
6. **Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier.** 1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol. Mol. Biol. Rev. **62:**1315–1352.
7. **Correa, N. E., C. M. Lauriano, R. McGee, and K. E. Klose.** 2000. Phosphorylation of the flagellar regulatory protein FlrC is necessary for *Vibrio cholerae* motility and enhanced colonization. Mol. Microbiol. **35:**743–755.
8. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
9. **Faruque, S. M., M. J. Albert, and J. J. Mekalanos.** 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. **62:**1301–1314.
10. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
11. **Forsberg, A., I. Bolin, L. Norlander, and H. Wolf-Watz.** 1987. Molecular cloning and expression of calcium-regulated, plasmid-coded proteins of *Y. pseudotuberculosis*. Microb. Pathog. **2:**123–137.
12. **Garcia-Del Portillo, F., M. G. Pucciarelli, and J. Casadesus.** 1999. DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. Proc. Natl. Acad. Sci. USA **96:**11578–11583.
13. **Geier, G. E., and P. Modrich.** 1979. Recognition sequence of the dam methylase of *Escherichia coli* K12 and mode of cleavage of *Dpn* I endonuclease. J. Biol. Chem. **254:**1408–1413.
14. **Glickman, B., P. van den Elsen, and M. Radman.** 1978. Induced mutagenesis in *dam*$^-$ mutants of *Escherichia coli*: a role for 6-methyladenine residues in mutation avoidance. Mol. Gen. Genet. **163:**307–312.
15. **Guan, K. L., and J. E. Dixon.** 1990. Protein tyrosine phosphatase activity of an essential virulence determinant in Yersinia. Science **249:**553–556.
16. **Hakansson, S., K. Schesser, C. Persson, E. E. Galyov, R. Rosqvist, F. Homble, and H. Wolf-Watz.** 1996. The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. EMBO J. **15:**5812–5823.
17. **Hale, W. B., M. W. van der Woude, and D. A. Low.** 1994. Analysis of nonmethylated GATC sites in the *Escherichia coli* chromosome and identification of sites that are differentially methylated in response to environmental stimuli. J. Bacteriol. **176:**3438–3441.
18. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by in vivo gene expression. Proc. Natl. Acad. Sci. USA **94:**934–939.
19. **Heithoff, D. M., E. Y. 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.
20. **Heithoff, D. M., 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.
21. **Hendrich, B., and A. Bird.** 2000. Mammalian methyltransferases and methyl-CpG-binding domains: proteins involved in DNA methylation. Curr. Top. Microbiol. Immunol. **249:**55–74.
22. **Julio, S. M., C. P. Conner, D. M. Heithoff, and M. J. Mahan.** 1998. Directed formation of chromosomal deletions in *Salmonella* typhimurium: targeting of specific genes induced during infection. Mol. Gen. Genet. **258:**178–181.
23. **Kahng, L. S., and L. Shapiro.** 2001. The CcrM DNA methyltransferase of *Agrobacterium tumefaciens* is essential, and its activity is cell cycle regulated. J. Bacteriol. **183:**3065–3075.
24. **Low, D. A., N. J. Weyland, and M. J. Mahan.** 2001. Roles of DNA methylation in regulating bacterial gene expression and virulence. Infect. Immun. **69:**7197–7204.
25. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assessment of bacterial pathogenesis by analysis of gene expression in the host. Annu. Rev. Genet. **34:**139–164.
26. **Mahan, M. J., and D. A. Low.** 2001. DNA methylation regulates bacterial gene expression and virulence. ASM News **67:**356–361.
27. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
28. **Marinus, M. G.** 1996. Methylation of DNA, edition VIII, p. 782–791. *In* F. C. Neidhardt, R. Curtiss III, 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, 2nd ed. ASM Press, Washington, D.C.
29. **Marinus, M. G., A. Poteete, and J. A. Arraj.** 1984. Correlation of DNA adenine methylase activity with spontaneous mutability in *Escherichia coli* K-12. Gene **28:**123–125.
30. **Monack, D. M., J. Mecsas, D. Bouley, and S. Falkow.** 1998. Yersinia-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. J. Exp. Med. **188:**2127–2137.
31. **Palmer, L. E., S. Hobbie, J. E. Galan, and J. B. Bliska.** 1998. YopJ of *Yersinia pseudotuberculosis* is required for the inhibition of macrophage TNF-alpha production and downregulation of the MAP kinases p38 and JNK. Mol. Microbiol. **27:**953–965.
32. **Persson, C., R. Nordfelth, A. Holmstrom, S. Hakansson, R. Rosqvist, and H. Wolf-Watz.** 1995. Cell-surface-bound Yersinia translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Mol. Microbiol. **18:**135–150.
33. **Reisenauer, A., L. S. Kahng, S. McCollum, and L. Shapiro.** 1999. Bacterial DNA methylation: a cell cycle regulator? J. Bacteriol. **181:**5135–5139.
34. **Reisenauer, A., K. Quon, and L. Shapiro.** 1999. The CtrA response regulator mediates temporal control of gene expression during the *Caulobacter* cell cycle. J. Bacteriol. **181:**2430–2439.
35. **Robertson, G. T., A. Reisenauer, R. Wright, R. B. Jensen, A. Jensen, L. Shapiro, and R. M. Roop II.** 2000. The *Brucella abortus* CcrM DNA methyltransferase is essential for viability, and its overexpression attenuates intracellular replication in murine macrophages. J. Bacteriol. **182:**3482–3489.
36. **Rosqvist, R., I. Bölin, and H. Wolf-Watz.** 1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. Infect. Immun. **56:**2139–2143.
37. **Rosqvist, R., A. Forsberg, M. Rimpilainen, T. Bergman, and H. Wolf-Watz.** 1990. The cytotoxic protein YopE of Yersinia obstructs the primary host defence. Mol. Microbiol. **4:**657–667.
38. **Rosqvist, R., K. E. Magnusson, and H. Wolf-Watz.** 1994. Target cell contact triggers expression and polarized transfer of Yersinia YopE cytotoxin into mammalian cells. EMBO J. **13:**964–972.
39. **Stephens, C., A. Reisenauer, R. Wright, and L. Shapiro.** 1996. A cell cycle-regulated bacterial DNA methyltransferase is essential for viability. Proc. Natl. Acad. Sci. USA **93:**1210–1214.
40. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca2+ in the Yersinia low-Ca2+ response. Mol. Microbiol. **8:**1005–1010.
41. **Tavazoie, S., and G. M. Church.** 1998. Quantitative whole-genome analysis of DNA-protein interactions by *in vivo* methylase protection in *E. coli*. Nat. Biotechnol. **16:**566–571.
42. **van der Woude, M., B. Braaten, and D. Low.** 1996. Epigenetic phase variation of the *pap* operon in *Escherichia coli*. Trends Microbiol. **4:**5–9.
43. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195–199.
44. **Wright, R., C. Stephens, and L. Shapiro.** 1997. The CcrM DNA methyltransferase is widespread in the alpha subdivision of proteobacteria, and its essential functions are conserved in *Rhizobium meliloti* and *Caulobacter crescentus*. J. Bacteriol. **179:**5869–5877.

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