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Mutations within the Catalytic Motif of DNA Adenine Methyltransferase (Dam) of *Aeromonas hydrophila* Cause the Virulence of the Dam-Overproducing Strain To Revert to That of the Wild-Type Phenotype

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In this study, we demonstrated that the methyltransferase activity associated with Dam was essential for attenuation of *Aeromonas hydrophila* virulence. We mutated aspartic acid and tyrosine residues to alanine within the conserved DPPY catalytic motif of Dam and transformed the pBAD/$dam_{D/A}$, pBAD/$dam_{Y/A}$, and pBAD/dam_{AhSSU} (with the native *dam* gene) recombinant plasmids into the *Escherichia coli* GM33 (*dam*-deficient) strain. Genomic DNA (gDNA) isolated from either of the *E. coli* GM33 strains harboring the pBAD vector with the mutated *dam* gene was resistant to DpnI digestion and sensitive to DpnII restriction endonuclease cutting. These findings were contrary to those with the gDNA of *E. coli* GM33 strain containing the pBAD/dam_{AhSSU} plasmid, indicating nonmethylation of *E. coli* gDNA with mutated Dam. Overproduction of mutated Dam in *A. hydrophila* resulted in bacterial motility, hemolytic and cytotoxic activities associated with the cytotoxic enterotoxin (Act), and protease activity similar to that of the wild-type (WT) bacterium, which harbored the pBAD vector and served as a control strain. On the contrary, overproduction of native Dam resulted in decreased bacterial motility, increased Act-associated biological effects, and increased protease activity. Lactone production, an indicator of quorum sensing, was increased when the native *dam* gene was overexpressed, with its levels returning to that of the control strain when the *dam* gene was mutated. These effects of Dam appeared to be mediated through a regulatory glucose-inhibited division A protein. Infection of mice with the mutated Dam-overproducing strains resulted in mortality rates similar to those for the control strain, with 100% of the animals dying within 2 to 3 days with two 50% lethal doses (LD_{50}s) of the WT bacterium. Importantly, immunization of mice with a native-Dam-overproducing strain at the same LD_{50} did not result in any lethality and provided protection to animals after subsequent challenge with a lethal dose of the control strain.

Aeromonads are ubiquitous organisms which exist in aquatic environments, various types of foods, such as meat, fish and vegetables, and the intestines of apparently healthy humans with diarrhea (11). Among the various *Aeromonas* species, *Aeromonas hydrophila* is most commonly involved in human infections, such as septicemia and gastroenteritis (3, 7). The pathogenesis of *A. hydrophila* infection is complex and multifactorial and characterized by the involvement of a number of virulence factors (1).

Our laboratory recently extensively characterized a type 2 secretion system (T2SS)-secreted cytotoxic enterotoxin, Act, and two cytotonic enterotoxins, Alt (heat labile) and Ast (heat stable), from a diarrheal isolate, SSU, of *A. hydrophila* (31). We provided evidence that ferric uptake regulator (Fur) and glucose-inhibited division A protein (GidA) regulated Act levels at the transcriptional and translational levels, respectively (32, 33). All of these three enterotoxins contributed significantly to the causation of fluid secretion in both a mouse model and humans (1, 31). We also characterized a type 3 secretion system (T3SS) from the same *A. hydrophila* isolate and demonstrated that mutants deficient in the production of Act and an *Aeromonas* outer membrane protein B (AopB), which is an essential component of the T3SS needle structure, were avirulent in a mouse model (34).

More recently, we characterized the DNA adenine methyltransferase (MTase) (Dam), which methylates GATC sequences within the genomic DNA (gDNA) of *A. hydrophila* SSU strain (designated M.AhySSUDam based on the nomenclature of MTases), and reported its role in modulating the function of both T2SS- and T3SS-associated bacterial virulence (6). We provided evidence that Dam was required for the viability of *A. hydrophila*, and Dam overproduction reduced the T3SS-associated cytotoxicity and bacterial motility in in vitro models and a mouse model of lethality. In contrast, biological activities (e.g., hemolytic and cytotoxic) associated with Act and protease activity in bacterial culture supernatants were increased as a result of Dam overproduction (6).

Dam alters the interaction of some regulatory proteins with their designated gene targets by adding methyl groups to various sites along the cellular DNA and, in the process, effectively controlling the expression of those genes. Such changes

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can both modulate bacterial virulence and elicit protective immune responses in the host (23).

This study was undertaken as a continuation of our recently reported findings which demonstrated, by using an arabinose-inducible pBAD expression vector, that Dam overproduction in *A. hydrophila* attenuated the virulence of the bacterium in an in vivo model of pathogenesis (6). The intent of the present study was to show whether the decreased virulence of *A. hydrophila* following Dam overproduction could be attributed to a direct increase in the MTase activity associated with Dam. To address this question, we mutated aspartic acid (D) and tyrosine (Y) residues individually within the Dam catalytic DPPY motif (region IV) by in vitro site-directed mutagenesis based on earlier studies with various MTases (9, 29).

Most importantly, we showed that when mutated Dam with no MTase activity was overproduced from *A. hydrophila*, the latter exhibited a virulence similar to that found with the wild-type (WT) bacterium with pBAD vector alone, which served as a control strain, while overproduction of native Dam in *A. hydrophila* highly attenuated the virulence of this pathogen (6). Furthermore, we illustrated a link between the regulatory functions of the *dam* gene and the phenomenon of quorum sensing (QS). Our studies indicate that Dam enzyme overproduction led to an increase in lactone production, which returned to the levels seen in the control strain when the mutated Dam was overproduced. When overproduced from the pBAD vector in an *Escherichia coli* GM33 strain, additional site-directed mutants of Dam (e.g., dam_{D71A} and dam_{E76A}, in which mutations were not within the catalytic motif of Dam) exhibited an MTase activity similar to that of native Dam. Finally, we provided evidence that Dam operates via GidA in the regulation of *A. hydrophila* virulence.

The efficacy of Dam-based vaccines has been explored in other pathogens, such as *Salmonella enterica* serovar Typhimurium (5, 27) and *Yersinia pseudotuberculosis* (37). Our studies indicate that, in addition to causing no lethality in mice at two 50% lethal doses (LD_{50}s) of the WT bacterium (6), the Dam-overproducing *A. hydrophila* SSU strain conferred protection on animals subsequently challenged with a lethal dose of the control strain. The link between the activity of Dam and its role in virulence was demonstrated by our in vivo studies, in which we showed that derivatives of an *A. hydrophila* SSU strain with an intact *dam* gene on the chromosome but overexpressing a mutated version of the *dam* gene (encoding mutated Dam with no detectable MTase activity) in the pBAD vector killed mice at the same rate as did the control strain. However, when the native *dam* gene was overexpressed in *A. hydrophila* from the pBAD/dam_{AhSSU} plasmid, attenuation in bacterial virulence was noted as measured by an absence of animal lethality.

Taken together, our findings provide conclusive evidence that the MTase activity associated with Dam of *A. hydrophila* was required to attenuate bacterial virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. The *A. hydrophila* SSU and *E. coli* strains, as well as the plasmids used in this study, are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani (LB) broth and LB agar plates (6, 30). The medium was supplemented with L-arabinose (0.2%) when the native or mutated *dam* gene was overexpressed from the pBAD/dam_{AhSSU}, pBAD/$dam_{D/A}$, or pBAD/$dam_{Y/A}$ plasmid (Table 1) under the control of an *araBAD* promoter (10). The native *dam* gene from the SSU isolate of *A. hydrophila* was designated dam_{AhSSU}. The mutated *dam* gene, in which either the aspartic acid (D; amino acid position 181) or the tyrosine (Y; amino acid position 184) residue was changed to alanine (A), was designated $dam_{D/A}$ or $dam_{Y/A}$. The mutated *dam* gene, in which either the D (amino acid position 71) or the glutamic acid (E; amino acid position 76) (6) residue was changed to A, was designated dam_{D71A} or dam_{E76A}. The antibiotics ampicillin (Ap), tetracycline (Tc), and rifampin (Rif) were used at concentrations of 100, 12, and 200 μg/ml, respectively. All of the antibiotics and arabinose were obtained from Sigma, St. Louis, MO. *Taq* DNA polymerase, restriction endonucleases, and T4 DNA ligase were purchased from New England BioLabs, Beverly, MA. Oligonucleotides for PCR amplification of the dam_{AhSSU}, $dam_{D/A}$, $dam_{Y/A}$, dam_{D71A}, and dam_{E76A} genes and mutagenic oligonucleotides that were used for the Altered Sites in vitro mutagenesis system (Promega, Madison, WI) were ordered from Integrated DNA Technologies, Inc., Coralville, IA.

Generation of mutated *dam* genes by site-directed mutagenesis. To obtain the pALTER-1/$dam_{D/A}$ and pALTER-1/$dam_{Y/A}$ plasmids (Tc^s and Ap^r) (Table 1) with a mutated *dam* gene, first, the PCR-amplified native dam_{AhSSU} gene was cloned into the pALTER-1 vector with *A. hydrophila* SSU gDNA and the *dam*N-EcoRI/*dam*C-BamHI primers (5′-CCGGAATTCATGAAAAAAACACGCGCTTTTTTAAA-3′/5′-CGCGGATCCTCAGCCGAGTGGCGCCAGTTCGGCGT-3′), with underlined bases representing the restriction enzyme sites. Subsequently, *E. coli* JM109 cells containing the pALTER-1/dam_{AhSSU} recombinant plasmid (Tc^r and Ap^s) were infected with the R408 helper phage, and the phagemid single-stranded DNA was isolated as described by the manufacturer. This DNA was used as a template for the mutagenesis reaction with the mutagenic *dam*D/A and *dam*Y/A primers (5′-GTCATCTATTGCGCTCCGCCCTATGCGCCGCTCTC-3′ and 5′-GTCATCTATTGCGATCCGCCCGCTGCGCCGCTCTC-3′). The underlined bases represented nucleotides that were changed, resulting in the mutations of D and Y to A (6). Each mutagenesis reaction was transformed into *E. coli* ES1301 *mutS*-competent cells. The plasmid DNA isolated from this bacterial strain was then transformed into the *E. coli* JM109 strain, and transformants were screened for tetracycline sensitivity. The mutations within the *dam* gene were confirmed by DNA sequence analysis. All procedures were performed according to Promega's protocol. Competent *E. coli* cells (JM109 and ES1301 *mutS*) were prepared using a Z-Competent *E. coli* transformation kit from Zymo Research, Orange, CA.

For generation of mutated dam_{D71A} and dam_{E76A} genes, we used the 5′-GCGGACGCCGGCCAGCTTCATCGCCGAGGCGCGCA-3′ and 5′-CGCCGGACAGCTTCATCGCCGCGGCGCGCAAGCTG-3′ mutagenic (D71A and E76A) primers, respectively. The underlined bases represent nucleotides that were changed, resulting in the mutations of D to A and E to A at amino acid positions 71 and 76 of Dam. Thus, by using an Altered Sites in vitro mutagenesis system, we mutated D, Y, and E residues to A within and outside the conserved DPPY catalytic motif of Dam.

Cloning of the mutated *dam* gene into the pBAD/Thio-E vector. To overexpress mutated *dam* genes, the pBAD/$dam_{D/A}$ and pBAD/$dam_{Y/A}$ plasmids were constructed in a manner similar to that for the pBAD/dam_{AhSSU} plasmid, as recently described (6), using the same primers, *dam*N-NcoI/*dam*C-PmeI (5′-ATGCCATGGATGAAAAAAACACGCGCTTTTTTAAAATGG-3′/5′-AGCTTTGTTTAAACGCCGAGTGGCGCCAGTTCGGCGTCGC-3′), with underlined bases representing restriction enzyme sites. We used pALTER-1/$dam_{D/A}$ and pALTER-1/$dam_{Y/A}$ plasmids as templates for PCRs. The PCR conditions were the same as those described recently (6), and the PCR products were digested with NcoI-PmeI restriction endonucleases and ligated to the pBAD/Thio-E vector cut with the compatible restriction enzymes. The ligation reaction was transformed into *E. coli* JM109-competent cells. The pBAD/dam_{D71A} and pBAD/dam_{E76A} plasmids were constructed similarly. The recombinant plasmids were then isolated from *E. coli*, and to generate *A. hydrophila* SSU with the mutated *dam* gene, the pBAD/$dam_{D/A}$, pBAD/$dam_{Y/A}$, pBAD/dam_{D71A}, and pBAD/dam_{E76A} plasmids were transformed into bacterial cells by electroporation, and these *A. hydrophila* SSU mutated strains were used for the study of bacterial virulence. The levels of native Dam versus mutated Dam production from the pBAD vector system after induction of appropriate *A. hydrophila* cultures with arabinose were analyzed by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) of the whole cells, and the gels were stained with Coomassie blue (6).

Dam activity. To examine MTase activity associated with the native or mutated Dam, the pBAD/$dam_{D/A}$, pBAD/$dam_{Y/A}$, pBAD/dam_{D71A}, and pBAD/dam_{E76A} plasmids as well as the pBAD/dam_{AhSSU} plasmid (with the native *dam* gene to serve as a positive control) were transformed into the *E. coli* GM33 (*dam*-deficient) strain (Table 1) (25). These cultures were grown overnight in the presence of arabinose at 37°C with shaking (180 rpm). The gDNA was then

TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)	Source or reference
A. hydrophila strains		
SSU		CDC, Atlanta, Ga.[a]
SSU-R	Rifampin-resistant (Rif^r) strain of *A. hydrophila* SSU	Laboratory stock
Control	*A. hydrophila* SSU-R harboring pBAD, Rif^r Ap^r	6
Dam-overproducing strain	*A. hydrophila* SSU-R harboring pBAD-*dam*$_{AhSSU}$, Rif^r Ap^r	6
D/A Dam mutant	*A. hydrophila* SSU-R harboring pBAD-*dam*$_{D/A}$, Rif^r Ap^r	This study
Y/A Dam mutant	*A. hydrophila* SSU-R harboring pBAD-*dam*$_{Y/A}$, Rif^r Ap^r	This study
M*gidA*	*gidA* isogenic mutant of *A. hydrophila* SSU-R, Rif^r Km^r	31
M*gidA*/pBAD	*gidA* isogenic mutant of *A. hydrophila* SSU-R harboring pBAD plasmid, Rif^r Km^r Ap^r	This study
M*gidA*/pBAD-*dam*$_{AhSSU}$	*gidA* isogenic mutant of *A. hydrophila* SSU-R harboring pBAD-*dam*$_{AhSSU}$ plasmid, Rif^r Km^r Ap^r	This study
E. coli strains		
ES1301 *mutS*	*lacZ53 mutS201*::Tn*5 thyA36 rha-5 metB1 deoC*	Promega
JM109	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* λ$^-$ Δ(*lac-proAB*) [F′ *traD36 proA*$^+$*B*$^+$ *laclZ*Δ*M15*]	Promega
GM33	F$^-$ *dam-3 sup-85* (Am)	25
C. violaceum CV026	Mini-Tn*5* mutant of ATCC 31532	26
Plasmids		
pALTER-1	Vector for the *dam*$_{AhSSU}$ gene cloning, Tc^r	Promega
pALTER-1/*dam*$_{AhSSU}$	*dam* gene of *A. hydrophila* cloned at the EcoRI/BamHI sites of pALTER-1 vector for mutagenesis, Tc^r	This study
pALTER-1/*dam*$_{D/A}$	pALTER-1 vector containing *dam*$_{D/A}$ mutation, Ap^r	This study
pALTER-1/*dam*$_{Y/A}$	pALTER-1 vector containing *dam*$_{Y/A}$ mutation, Ap^r	This study
pBAD/Thio-E	*araBAD* promoter expression vector, Ap^r	Invitrogen
pBAD	Control vector, Ap^r	6
pBAD/*dam*$_{AhSSU}$	*dam* gene of *A. hydrophila* cloned into pBAD/Thio-E, Ap^r	6
pBAD/*dam*$_{D/A}$	pBAD vector carrying *dam*$_{D/A}$ mutation, Ap^r	This study
pBAD/*dam*$_{Y/A}$	pBAD vector carrying *dam*$_{Y/A}$ mutation, Ap^r	This study
pBAD/*dam*$_{D71A}$	pBAD vector carrying *dam*$_{D71A}$ mutation, Ap^r	This study
pBAD/*dam*$_{E76A}$	pBAD vector carrying *dam*$_{E76A}$ mutation, Ap^r	This study

[a] CDC, Centers for Disease Control and Prevention.

isolated and subjected to digestion with DpnI (which cuts methylated DNA) and DpnII (which cuts nonmethylated DNA) restriction enzymes. As a negative control, we used gDNA isolated from the original *E. coli* GM33 (*dam*-deficient) strain. Dam activity for some Dam mutants was also confirmed by transfer of the ^{3}H-*methyl* group from [*methyl*-^{3}H]AdoMet (*S*-adenosyl-L-methionine) by Dam onto N^6-methyladenine-free lambda DNA as described previously (6).

Measurement of motility, Act-associated biological activities, and protease activity. We determined the motility of *A. hydrophila* SSU strains carrying the pBAD vector alone, pBAD/*dam*$_{AhSSU}$, pBAD/*dam*$_{D/A}$, or the pBAD/*dam*$_{Y/A}$ plasmid, as recently described, using LB agar (0.35%) plates (6). Likewise, the culture supernatants from bacteria were used to measure Act-associated hemolytic and cytotoxic activities as well as the protease activity (6). The hemolytic activity was measured by examining the release of hemoglobin (optical density at 540 nm [OD_{540}]) from rabbit erythrocytes (3%), while cytotoxic activity associated with Act was determined by measuring the percentage of lactate dehydrogenase (LDH) enzyme released from RAW264.7 murine macrophages treated with bacterial culture supernatants for 2 h with a CytoTox96 kit from Promega (6). The protease activity was measured by examining the release of blue color (OD_{595}) from Hide azure powder. The enzyme-linked immunosorbent assay (ELISA) for the detection of Act antigen levels in the culture supernatants was performed as described previously (6).

Lactone production. To detect acylhomoserine lactones (AHLs), we used the WT *A. hydrophila* SSU strain (a positive control), *A. hydrophila* strains containing the pBAD, pBAD/*dam*$_{AhSSU}$, pBAD/*dam*$_{D/A}$, or pBAD/*dam*$_{Y/A}$ plasmid, and the *gidA* isogenic mutant of *A. hydrophila* (32) harboring the pBAD or the pBAD/*dam*$_{AhSSU}$ plasmid. We employed the method of McClean et al. (26) with modifications and used the *Chromobacterium violaceum* CV026 strain for lactone production (Table 1). This method allowed us to quantitate the amount of lactone produced, compared to the original qualitative method for the detection of lactones (36). Briefly, *Aeromonas* strains were grown overnight in LB medium (pH 7.0) with appropriate antibiotics at 37°C, and the *C. violaceum* CV026 strain was grown in LB broth for 24 h at 28°C with shaking (150 rpm). The test strains of *A. hydrophila* were reinoculated in the morning using 3 ml of the LB medium with arabinose and incubated for 4 h (an optimal time interval that was standardized empirically). The OD_{600} and the number of CFU were determined for each culture, which was centrifuged at 13,000 × *g* for 10 min. The culture supernatants were then filter sterilized (0.22 μm). Subsequently, the *C. violaceum* culture was mixed with the supernatant from each of the *A. hydrophila* cultures at a 1:10 ratio and incubated for 24 h at 28°C with shaking. The cultures were centrifuged and pellets suspended in 50% ethanol and 1% SDS solution. The mixture was vortexed, and the bacterial sediment was centrifuged. The deep blue color in the supernatant was measured at OD_{590} using a VERSA$_{max}$ reader (Molecular Devices Corporation, Sunnyvale, CA). The use of *C. violaceum* CV026 culture for lactone production is by far the best for *Aeromonas* and *Pseudomonas* species and was discussed in our recent publication (34) as well as in other studies (26, 36). *C. violaceum* specifically detects AHLs with *N*-acyl side chains of four to eight carbons, especially BHLs (*N*-[butanoyl]-L-homoserine lactones), which are produced predominantly by *Aeromonas* species.

Animal experiments. Eight-week-old female Swiss Webster mice (Taconic Farms, CA) were used to determine whether animals immunized with the *A. hydrophila* Dam-overproducing strain would protect mice against lethal infection with the WT *A. hydrophila* harboring the pBAD plasmid alone. Forty mice (group 1) were inoculated with Dulbecco's phosphate-buffered saline (DPBS) and served as controls. A second group (10 mice) was infected intraperitoneally (i.p.) with 1×10^7 CFU of WT *A. hydrophila* (with pBAD vector alone), and group 3 (40 mice) was infected with 1×10^7 CFU of an *A. hydrophila* (pBAD/*dam*$_{AhSSU}$) strain that overproduced Dam. Animals were observed for mortality for 1 week. The animals were provided with 0.2% arabinose and Ap (40 mg/kg of body weight/day) in their drinking water over the first 2 to 3 days to retain the plasmid in bacteria. At 1, 3, 5, and 6 weeks postinoculation, mice ($n = 10$) in groups 1 and 3 were challenged with two LD_{50}s (1×10^7 CFU) of the WT *A.*

hydrophila SSU strain containing pBAD vector alone without the *dam* gene and observed for mortality.

To define whether mutations within the catalytic domain of Dam affected lethality in mice, we infected another four groups of mice (each group contained 10 mice) i.p. with a lethal dose (5×10^6 to 1×10^7 CFU) of the WT *A. hydrophila* (pBAD), the Dam-overproducing strain (pBAD/dam_{AhSSU}), and the mutated-Dam-overproducing strains (with pBAD/$dam_{D/A}$ or pBAD/$dam_{Y/A}$ plasmid). Mice were observed daily for 2 weeks for mortality.

Statistical analysis. Wherever appropriate, the data were analyzed using Student's *t* test or the Fisher exact test, and *P* values of ≤ 0.05 were considered significant. At least three independent experiments were performed for statistical analysis of data.

RESULTS

To define precisely the role of Dam activity in the virulence potential of *A. hydrophila*, we constructed *A. hydrophila* strains in which the mutated *dam* gene was expressed from the pBAD vector system. All MTases possess a similar catalytic domain and D/NPPY/F sequence, which represents one of nine described motifs (I to VIII and X) and is the common conserved catalytic motif in region IV of adenine-N^6 and cytosine-C^4 DNA MTases (21, 29, 43). Residues located in motifs IV (Asp^{193} and Tyr^{196}), V (Asp^{211}), VI (Ser^{229} and Trp^{231}), and VIII (Tyr^{258}) of EcoRV adenine-N^6-methyltransferase (Dam) are involved in catalysis, some of them presumably in the binding of the flipped target base, because mutations in these residues fail to significantly interfere with DNA and AdoMet binding but strongly reduce catalysis (29). To verify that alteration of amino acid residues within or outside the conserved catalytic DPPY active domain abolishes the MTase activity of *A. hydrophila*, we examined *E. coli* GM33 strains containing pBAD/*dam* plasmids with different amino acid mutations within the *dam* gene.

The mutated Dam in the DPPY motif did not possess MTase activity. To test the GATC DNA methylation activity of the mutated *A. hydrophila* Dam enzyme, we used the *E. coli* GM33 (*dam*-deficient) strain (Table 1), which is deficient in N^6-adenine MTase activity (chromosomal *dam* gene mutation of the WT *E. coli* GM1 strain) (25). The pBAD/$dam_{D/A}$ and pBAD/$dam_{Y/A}$ plasmids were transformed into Z-competent *E. coli* GM33 cells, and isolated gDNA from these strains was digested with DpnI, which digests methylated DNA, and the DpnII restriction endonuclease, which cuts nonmethylated DNA. As controls, we prepared gDNA from the *E. coli* GM33 strain, which either did not harbor any plasmid or harbored the pBAD vector alone. The gDNA from the *E. coli* GM33 strain containing the pBAD/dam_{AhSSU} plasmid, which encoded native and functionally active Dam, was also used as a positive control. As shown in Fig. 1A, the gDNA isolated from either the *E. coli* GM33 (pBAD/$dam_{D/A}$) or the *E. coli* GM33 (pBAD/$dam_{Y/A}$) strain grown in the presence of arabinose was resistant to DpnI (which cuts methylated DNA) digestion (Fig. 1A, lanes 10 and 12) and sensitive to DpnII (which cuts nonmethylated DNA) restriction endonuclease cutting (Fig. 1A, lanes 11 and 13). These data confirmed that the gDNA from these *E. coli* GM33 strains, with mutated, overproduced *A. hydrophila* Dam, was not methylated. These data were similar to those obtained when the gDNA of the *E. coli* GM33 (*dam*-deficient) strain was digested with DpnI and DpnII restriction enzymes (Fig. 1A, lanes 3 and 4), indicating the nonmethylated status of the gDNA in this strain. A similar digestion pattern with enzymes DpnI and DpnII was noted when gDNA from the *E. coli* GM33 strain with pBAD vector alone was used (Fig. 1A, lanes 6 and 7). The additional lower molecular bands in the vicinity of the gDNA (Fig. 1A, lanes 5, 6, 9, 10, and 12)

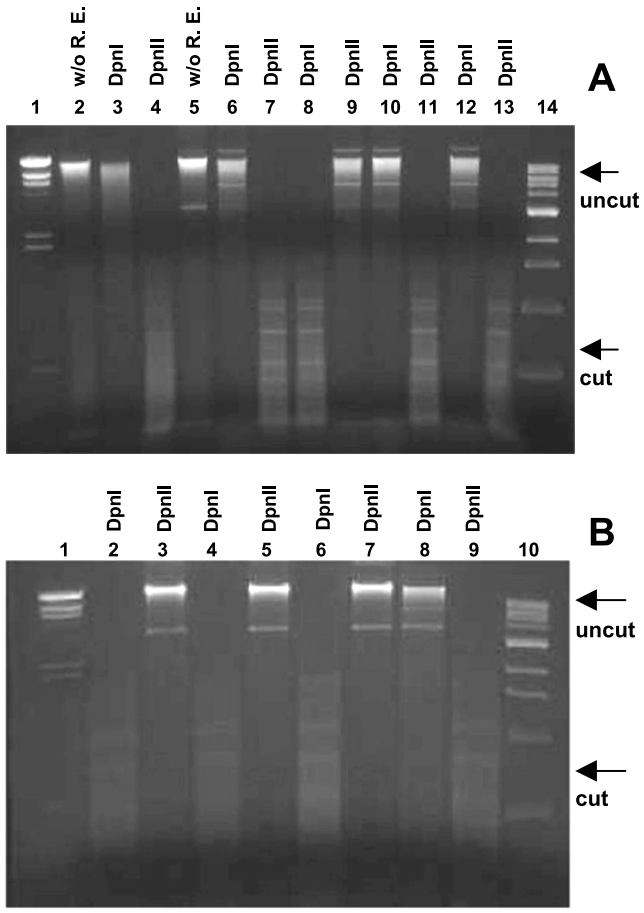

FIG. 1. DpnI and DpnII cleavage patterns of gDNA isolated from the *E. coli* GM33 (*dam*-deficient) strain containing pBAD plasmid with the native or mutated *dam* gene. (A) Lane 1, lambda DNA/HindIII markers (Promega); lanes 2, 3, and 4, gDNA from the *E. coli* GM33 strain (without any plasmid as a control) either without any restriction enzyme digestion (w/o R. E.) (lane 2) or treated with DpnI (lane 3) or DpnII (lane 4); lanes 5, 6, and 7, gDNA from *E. coli* GM33 containing the pBAD plasmid alone either with no enzyme digestion (w/o R. E.) (lane 5) or treated with DpnI (lane 6) or DpnII (lane 7); lanes 8 and 9, gDNA from *E. coli* GM33 containing the pBAD/dam_{AhSSU} recombinant plasmid treated with DpnI or DpnII enzymes; lanes 10 and 11, gDNA from the *E. coli* GM33 strain with the pBAD/$dam_{D/A}$ plasmid treated with DpnI or DpnII; lanes 12 and 13, gDNA from *E. coli* GM33 strain carrying the pBAD/$dam_{Y/A}$ plasmid treated with DpnI and DpnII, respectively; and lane 14, 1-kb DNA ladder (New England BioLabs). (B) Lanes 1 and 10, lambda DNA/HindIII markers (Promega) and 1-kb DNA ladder (New England BioLabs), respectively; lanes 2 and 3, gDNA from *E. coli* GM33 containing the pBAD/dam_{AhSSU} recombinant plasmid treated with DpnI or DpnII enzymes; lanes 4 and 5, gDNA from the *E. coli* GM33 strain with the pBAD/dam_{D71A} plasmid treated with DpnI or DpnII; lanes 6 and 7, gDNA from the *E. coli* GM33 strain carrying the pBAD/dam_{E76A} plasmid treated with DpnI and DpnII, respectively; lanes 8 and 9, gDNA from the *E. coli* GM33 strain with the pBAD/$dam_{D/A}$ plasmid treated with DpnI or DpnII. All cultures which were used for the isolation of gDNA were grown in the presence of arabinose. The gDNA was digested at 37°C for 1 h and subjected to 1% agarose gel electrophoresis. The gels were visualized under UV light after being stained with ethidium bromide.

represented the plasmid DNA. Opposite cleavage patterns were noted when gDNA from the *E. coli* GM33 strain with the pBAD/dam_{AhSSU} plasmid was digested with DpnI and DpnII enzymes. Isolated gDNA from this strain was sensitive to DpnI (Fig. 1A, lane 8) and resistant to DpnII (Fig. 1A, lane 9) digestion. These data signified that the overproduction of native Dam from pBAD/dam_{AhSSU} plasmid in the *E. coli* GM33 strain methylated its gDNA, while the mutated Dam could not, thus indicating that mutations of D and Y residues within the DPPY motif resulted in no detectable Dam activity.

However, production of MTase from pBAD/dam_{D71A} and pBAD/dam_{E76A} plasmids in the *E. coli* GM33 strain methylated its gDNA, showing the expected phenotype of native Dam (expressed from the pBADdam_{AhSSU} plasmid), i.e., digestion with DpnI but not with DpnII (Fig. 1B, lanes 4, 5, 6, and 7 versus lanes 2 and 3), while mutated Dam from the pBAD/$dam_{D/A}$ plasmid exhibited the opposite pattern (digestion with DpnII but not DpnI) (Fig. 1B, lanes 8 and 9), demonstrating an original Dam$^-$ phenotype for the *E. coli* GM33 strain. These data indicate that mutations of amino acid residues outside the catalytic motif of Dam did not affect MTase activity associated with Dam.

To demonstrate production of native and mutated Dam proteins, we performed SDS-PAGE with *E. coli* whole cells containing tested recombinant plasmids with the *dam* gene under the *araBAD* promoter (data not shown). The gels were visualized after being stained and destained. All of the mutants tested produced similar levels of Dam after induction with arabinose, as noted, with *E. coli* overproducing native Dam. These data suggested possibly no or minimal alteration in the conformation of the mutated Dam after site-directed mutagenesis that could affect MTase activity. We expected minimal conformational changes, as similar amino acid residues were mutated in other MTases with no significant alterations in the levels of the mutated protein produced. Further, these mutated MTases behaved biochemically in a manner predicted from their structural models (18, 29, 43).

The inability of mutated Dam in the DPPY motif (pBAD/$dam_{D/A}$ and pBAD/$dam_{Y/A}$) to transfer methyl groups from [*methyl*-^{3}H]AdoMet to N^6-methyladenine-free lambda DNA was confirmed quantitatively by using a radioactivity incorporation assay (Fig. 2). As noted from Fig. 2, cell extracts from the *E. coli* GM33 strain with native Dam (pBAD/dam_{AhSSU}) showed a concentration-dependent increase in MTase activity. The MTase activity associated with the mutated Dam in the *E. coli* cell extracts was similar to that in *E. coli* with pBAD vector alone.

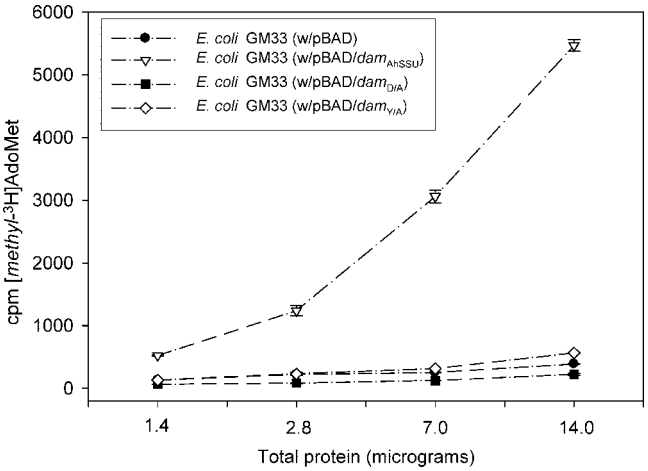

FIG. 2. MTase activity associated with native and mutated Dam of *A. hydrophila* SSU in the *E. coli* GM33 strain by the AdoMet assay. The cell extracts from *E. coli* strains containing pBAD plasmid (as a control), the pBAD/dam_{AhSSU} recombinant plasmid, pBAD/$dam_{D/A}$, and the pBAD/$dam_{Y/A}$ plasmid were prepared, and the ability of Dam to transfer *methyl*-^{3}H groups from AdoMet to N^6-methyladenine-free lambda DNA was measured (6). Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted. The increase in MTase activity in the cell extracts of *E. coli* overproducing native Dam was statistically significant at all of the protein concentrations according to Student's *t* test, compared to the activity in *E. coli* cell extracts overproducing mutated Dam. cpm, counts per minute.

Overproduction of inactive mutated Dam in *A. hydrophila* SSU causes its virulence level to revert to that of WT *A. hydrophila*. In our recently published studies, we showed that overproduction of native Dam in *A. hydrophila* altered its virulence potential (6). In particular, our data demonstrated that native Dam overproduction decreased the motility and the T3SS-associated cytotoxicity of the bacterium and increased the biological activities associated with Act and protease production.

To define the role of Dam (both enzymatically active and enzymatically inactive forms) overproduced from *A. hydrophila* SSU in terms of its effect on overall bacterial virulence, we used arabinose-induced native and mutated *dam* genes that were expressed from the *araBAD* promoter in vector pBAD. We used *A. hydrophila* SSU with pBAD vector alone as a control. Based on SDS-PAGE and the stained gels, the levels of native and mutated Dam, when Dam was overproduced from the pBAD vector after arabinose induction, were similar (data not shown). As noted from Fig. 3, the native Dam-overproducing strain was much less motile (by 57%) than the control strain ($P = 0.0008$), confirming our previous data (6). When the *dam* gene was mutated, the bacterium showed a significantly higher motility than *A. hydrophila* expressing the native *dam* gene, although it did not reach the level for WT *A. hydrophila* with the pBAD vector alone ($P = 0.05$). These data might indicate some residual MTase activity associated with mutated Dam. The hemolytic activity associated with Act of *A. hydrophila* was significantly increased (at least 14-fold) when the native *dam* gene was overexpressed compared to that of the control *A. hydrophila* strain with the pBAD vector alone. The hemolytic activity in the culture filtrates of *A. hydrophila* with mutated Dam was similar to that in the control strain (Fig. 4A).

We also examined Act-associated cytotoxicity (as measured by the percentage of release of LDH enzyme) from RAW264.7 murine macrophages. As shown in Fig. 4B, compared to the control strain, a threefold increase in the release of LDH from macrophages treated with the culture filtrates of the *A. hydrophila* strain that overproduced native Dam was noted. However, the level of LDH release from macrophages was decreased significantly and comparable to that for the *A. hydrophila* (pBAD) control strain when the *dam* gene was mutated. These data were further confirmed by performing ELISA on the culture supernatants of the above-mentioned *A.*

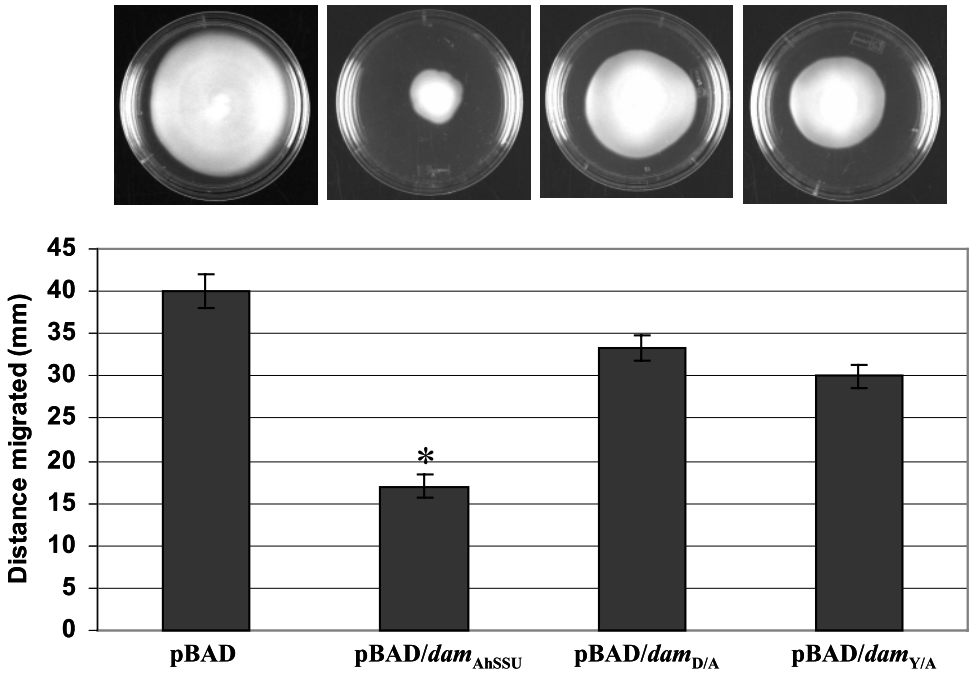

FIG. 3. (Top) *A. hydrophila* with overproducing mutated Dam (with pBAD/$dam_{D/A}$ or pBAD/$dam_{Y/A}$ plasmid) exhibits a motility phenotype similar to that of the *A. hydrophila* SSU strain containing pBAD vector alone. The motility of the *A. hydrophila* strain with pBAD/dam_{AhSSU} plasmid was significantly reduced. The strains were grown in the presence of arabinose. (Bottom) The bar graph demonstrates the distances in millimeters by which these strains migrated from the points of inoculation in soft agar plates. Data from three independent experiments were plotted with standard deviations. An asterisk denotes statistical significance by Student's *t* test ($P = 0.0008$).

hydrophila strains and using antibodies specific to Act. As noted from Fig. 5A, the native Dam-overproducing strain produced much higher Act levels than the control strain. However, when the *dam* gene was mutated, the Act levels produced by the mutated *A. hydrophila* strains were similar to those for the control strain, clearly indicating the necessity of MTase activity in Act production. Finally, we noted a similar pattern of increased protease production when native Dam was overproduced, compared to the protease production in the culture supernatants of *A. hydrophila* SSU strains in which the *dam* gene was mutated as well as that in the control strain (Fig. 5B).

Quorum sensing and Dam activity. QS, the ability of bacteria to communicate and coordinate behavior via signaling molecules, has been shown in several pathogenic microorganisms (15, 17, 35). We described a correlation between the T3SS and Act production in *A. hydrophila* with lactones (34) and provided evidence that mutations in the *act* and *aopB* genes resulted in reduced lactone production. To determine whether Dam overproduction altered lactone production, which is a signaling molecule of QS, we noted that the Dam-overproducing strain with the native *dam* gene produced significantly more lactones than the control strain (Fig. 4C). However, when the mutated *dam* gene (in the pBAD/$dam_{D/A}$ or the pBAD/$dam_{Y/A}$ plasmid) was overexpressed, the lactone production level was similar to that of the *A. hydrophila* (pBAD) control strain (Fig. 4C). To eliminate any possible nonspecific contribution from protein overproduction, using an arabinose-inducible pBAD vector, we included the original *A. hydrophila* SSU strain (without any plasmid) as an additional control, whose lactone production level was similar to that of the *A. hydrophila* SSU strain with the pBAD vector alone (data not shown). These control cultures were the most appropriate for these studies, as they also ruled out any influence on lactone production by other virulence factors produced by *A. hydrophila* SSU that are present in the culture filtrates (8).

Regulation of Act and lactone production in *A. hydrophila* SSU by Dam occurs via GidA. We demonstrated previously that Act production was significantly reduced when the *gidA* gene was deleted from *A. hydrophila*, an effect which could be complemented (32). Based on the upstream sequence of the *gidA* structural gene, multiple GATC sequences (a total of 15) that potentially could be methylated by Dam, resulting in increased Act production, were detected within the putative promoter region by using a software program from Softberry, Inc. Indeed, we noted that the hemolytic activity in the culture filtrate of the WT *A. hydrophila* strain harboring the pBAD/dam_{AhSSU} plasmid was considerably greater than that of the *A. hydrophila* control strain harboring the pBAD vector alone (Fig. 4A). As expected, truncation of the *gidA* gene resulted in a reduced level of hemoglobin release from erythrocytes (Fig. 4A). More interestingly, when the native *dam* gene was overexpressed in the *gidA* mutant, no further increase in the hemolytic activity (compared to that of the *gidA* mutant with pBAD vector alone) was noted (Fig. 4A). Similar findings were observed when we examined cytotoxic activity (as a percentage of LDH release) associated with Act in the culture filtrates of the native Dam-overproducing WT and the *gidA* mutant of *A. hydrophila* (Fig. 4B). As noted from Fig. 4B, overproduction of Dam in WT *A. hydrophila* increased Act-associated cell toxicity compared to that in the control strain. The cytotoxicity was

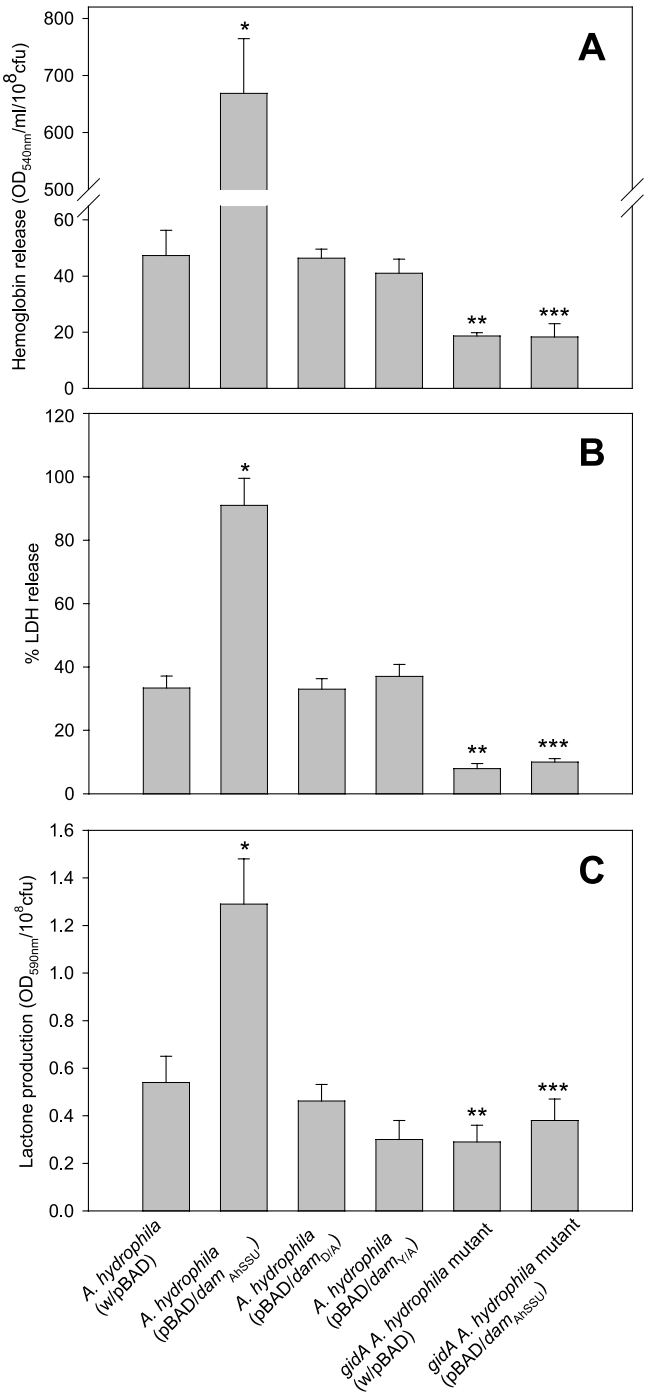

FIG. 4. Overproduction of mutated Dam decreases biological activities of T2SS-associated Act and lactone production to the level of the WT bacterium (*A. hydrophila* with or without the pBAD plasmid). A regulatory GidA protein is crucial in Dam-associated alteration in biological activities of Act and lactone production. (A and B) Act-associated hemolytic and cytotoxic activities in the culture supernatants of *A. hydrophila* strains containing pBAD plasmid alone and pBAD/dam_{AhSSU}, pBAD/$dam_{D/A}$, and pBAD/$dam_{Y/A}$ plasmids were measured by the release of hemoglobin from rabbit erythrocytes and LDH enzyme from macrophages, respectively (6). (C) Overproduction of native Dam (the *A. hydrophila* SSU strain with pBAD/dam_{AhSSU} plasmid) but not the mutated Dam (the *A. hydrophila* SSU strain with the pBAD/$dam_{D/A}$ or the pBAD/$dam_{Y/A}$ plasmid) increases lactone production. The *gidA* mutant of *A. hydrophila* does not respond to overproduction of native Dam with increasing activities of T2SS-associated Act and lactone production. The strains were grown in the presence of arabinose. Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted. The data were normalized to 1×10^8 CFU to account for any differences in the growth rates of the various bacterial strains used. *, statistically significant difference between the *A. hydrophila* control strain and the Dam-overproducing strain of WT *A. hydrophila* by Student's *t* test. **, statistically significant difference between the WT control and the *gidA* mutant control strain of *A. hydrophila*. ***, statistically significant difference between the native Dam-overproducing WT strain and the *gidA* mutant of *A. hydrophila* overproducing native Dam.

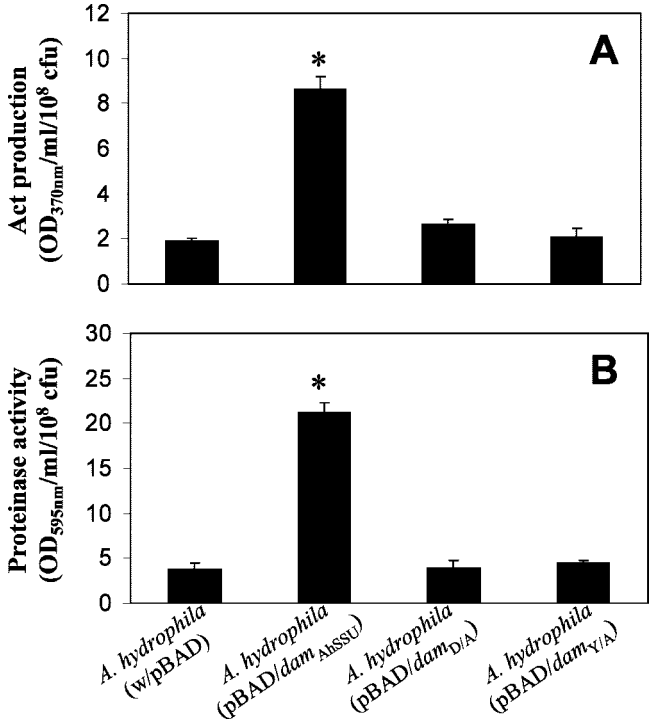

FIG. 5. Overproduction of mutated Dam decreases Act antigen levels and protease activities to those of the WT *A. hydrophila* strain. (A) Production of Act based on ELISA with the culture supernatants of the *A. hydrophila* strain with pBAD vector only, the pBAD/dam_{AhSSU} recombinant plasmid, and the pBAD/$dam_{D/A}$ or the pBAD/$dam_{Y/A}$ plasmid. (B) Protease activity in the culture supernatants of *A. hydrophila* strains with pBAD, pBAD/dam_{AhSSU}, pBAD/$dam_{D/A}$, or pBAD/$dam_{Y/A}$ plasmid, as measured by the hydrolysis of Hide azure powder. The strains were grown in LB medium with arabinose. Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted. The data were normalized to 1×10^8 CFU to account for any differences in the growth rates of the various bacterial strains used. Asterisks denote statistically significant values by Student's *t* test.

decreased in the *gidA* mutant with the pBAD vector compared to that in the WT *A. hydrophila* control strain. Overproduction of native Dam in the *gidA* mutant exhibited no further changes in cytotoxicity (Fig. 4B).

Finally, overproduction of native Dam in the *gidA* isogenic mutant failed to increase lactone production compared to that in WT *A. hydrophila* expressing the native *dam* gene (Fig. 4C). As expected, a significant increase in lactone production in WT *A.*

TABLE 2. Immunization of mice with the native Dam-overproducing strain of *A. hydrophila* provides protection against subsequent challenge with the lethal dose of WT *A. hydrophila* SSU

Period of immunization with the native Dam overproducer[a] (no. of wks)	No. of survivals/total no. of animals infected		
	Over the period of immunization	After immunization and challenge with two LD_{50}s of WT *A. hydrophila*[b]	For control animals given DPBS and then challenged with two LD_{50}s of WT *A. hydrophila*[b]
1	10/10	10/10	0/10
3	10/10	10/10	0/10
5	10/10	10/10	0/10
6	10/10	10/10	0/10

[a] Animals were immunized with 1×10^7 CFU of the Dam-overproducing strain.
[b] Animals were challenged with 1×10^7 CFU of WT *A. hydrophila*, which represented approximately two LD_{50}s.

hydrophila overexpressing the native *dam* gene, compared to that in the *A. hydrophila* control strain, was noted. Taken together, these data indicate the involvement of GidA in Dam-associated biological functions. We confirmed that Dam activity was indeed increased in the *gidA* mutant harboring the pBAD/dam_{AhSSU} plasmid after arabinose induction, as measured by the transfer of methyl groups from [*methyl*-^{3}H]AdoMet to N^6-methyladenine-free lambda DNA (data not shown).

Protection of mice immunized with the Dam-overproducing strain against WT *A. hydrophila* challenge. As indicated in our previous studies, the native Dam-overproducing strain of *A. hydrophila* SSU was less virulent and attenuated in a mouse infection model (6). To further examine whether this strain might immunize mice to subsequent *Aeromonas* infection, animals were infected i.p. with the native-Dam-overproducing bacteria at a dose of 1×10^7 CFU. Mice immunized with Dam-overproducing bacteria were completely protected when they were subsequently challenged at 1 to 6 weeks postimmunization with WT *A. hydrophila* containing pBAD vector alone, indicating possibly the role of both cell-mediated and humoral immune responses in animal protection (Table 2). Since immune responses to this pathogen are largely unknown, our future studies will examine in detail the immunological basis of protection by Dam overproduction in a mouse model of infection. The nonimmunized group of mice, given only DPBS, died within 2 days after subsequent challenge with the lethal dose of the *A. hydrophila* control strain.

On the other hand, inoculation of mice with the mutated-Dam-overproducing strains indicated that such mutants (D/A and Y/A) induced lethality in mice similar to that of the control *A. hydrophila* (i.e., WT bacterium with pBAD vector alone), while the *A. hydrophila* strain with the native overproduced Dam did not cause any mortality at the same dose (Fig. 6). Our data indicate that mutations in the conserved catalytic DPPY motif of M.AhySSUDam indeed caused the phenotype of the Dam-overproducing strain to revert to that of the WT bacterium.

DISCUSSION

We demonstrated previously that Dam is crucial for the viability of *A. hydrophila* and that it affects bacterial virulence by altering the biological activities associated with type 2- and type 3-secreted proteins. In addition, Dam overproduction in *A. hydrophila* attenuated bacterial virulence in a mouse infection model (6).

Functional roles for strongly or moderately conserved amino acid residues were investigated for some MTases which can modify DNA by two classes of these enzymes, C-MTases modifying cytosines at the C-5 position, and N-MTases transferring a methyl group to adenine-N^6 or cytosine-N^4 positions (24, 42). The notion that region IV, which contains the DPPY motif, is possibly involved in enzymatic activity was described with regard to the phage T4 Dam, *E. coli* DNA N^6-adenine MTases (e.g., EcoRV Dam), and the BcgI restriction modification system (9, 14, 20, 21, 29, 42).

In this study, we generated two M.AhySSUDam mutants in the DPPY motif (D/A and Y/A), tested them for the ability to methylate gDNA of the *dam*-deficient *E. coli* GM33 strain, and evaluated the virulence of the *A. hydrophila* SSU strain with overproduced native and mutated Dam. Our data indicate that mutations in the DPPY motif of Dam resulted in no detectable MTase activity (Fig. 1 and 2).

We recently investigated the role of overproduced Dam in the pathogenesis of *A. hydrophila* (6). In this study, we examined the effect of overproduced but mutated Dam in the DPPY motif (region IV), which is responsible for the catalytic and substrate (AdoMet and DNA) binding activities of MTase relative to the virulence of the bacterium. Our data clearly indicate that the motility of the *A. hydrophila* SSU strain, as well as Act-associated biological activities, was directly linked to Dam activity. We also provided evidence that those Dam mutants with an altered catalytic site affected MTase activity, as mutations in two other tested amino acid residues outside the catalytic domain exhibited MTase activities similar to that of the native Dam. Further, the levels of overproduced native and mutated Dam in both *E. coli* and *A. hydrophila* strains were essentially similar.

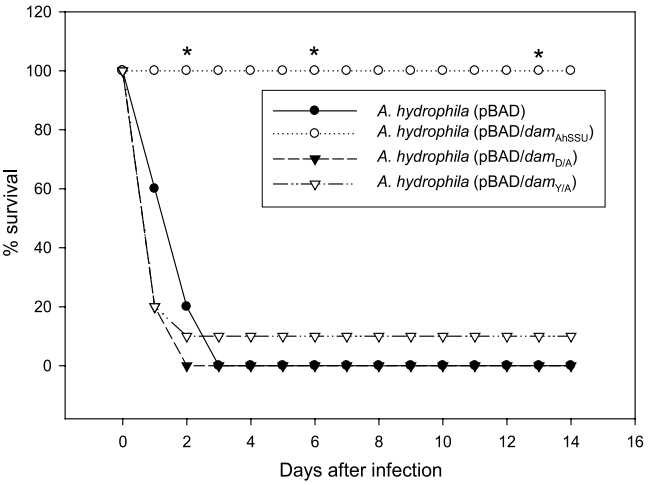

FIG. 6. *A. hydrophila* with mutated Dam is highly virulent in a mouse model. Mutants of *A. hydrophila* with pBAD/$dam_{D/A}$ or pBAD/$dam_{Y/A}$ plasmid led to mouse mortality within 48 h, similar to what was found for the WT *A. hydrophila* strain harboring only the pBAD vector. The Dam-overproducing *A. hydrophila* strain with pBAD/dam_{AhSSU} plasmid (with native Dam) fully protected mice. Asterisks denote statistically significant values by Fisher's exact test.

Bacteria that use QS produce and secrete certain signaling compounds, autoinducers (AIs), or pheromones, such as AHLs. It is known that culture supernatants from both *A. hydrophila* and *Aeromonas salmonicida* activate a range of biosensors responsive to AHLs, and the genes for a QS signal generator and a response regulator were cloned from each of the above-mentioned *Aeromonas* species and termed *ahyRI* and *asaRI*, respectively (36). Recently, we revealed that lactone production levels were decreased in the *act* (32%) and *aopB* (64%) mutants compared to that of the WT *A. hydrophila* SSU strain (34). The effect of Dam activity on lactone production is demonstrated in Fig. 4C, showing that the levels increased when the native *dam* gene was overexpressed and returned to that of the WT bacterium when the mutated *dam* genes were overexpressed. Since we previously demonstrated that the *act* mutant produced fewer lactones (34), our data indicate that when Act production was increased due to native Dam overproduction, lactone production was also increased, and this was indeed the case (Fig. 4A to C). However, one should not overlook the possible effect of AdoMet-dependent Dam on QS.

Interestingly, AdoMet (a source of methyl groups for MTases) is a direct precursor of the *Vibrio fischeri* AI (12). Hanzelka and Greenberg (12) showed that AdoMet is required for AI synthesis by LuxI in amino acid-starved *E. coli* mutants. Investigations of the in vivo source of the acyl chain and homoserine lactone components of AI synthesized by the LuxI homolog, TraI, showed that decreased levels of intracellular AdoMet caused by expression of bacteriophage T3 AdoMet hydrolase resulted in a marked reduction in AI synthesis. These data provided direct in vivo evidence that the homoserine lactone ring of LuxI family autoinducers is derived from AdoMet (39). In our future studies, this interesting correlation between Dam activity and lactone production as it relates to QS will be pursued.

The role that Dam plays in the regulation of bacterial virulence is only beginning to be understood (23), and studies have shown that adenine methylation can either directly or indirectly alter the interaction of regulatory proteins with DNA (4). Dam can act as a de novo methylase by methylating both nonmethylated and hemimethylated GATC sites (38) and plays a pivotal role in the control of gene expression by the formation of DNA methylation patterns (40). A typical example is the pyelonephritis-associated pilus (*pap*) operon of uropathogenic *E. coli* (16). The DNA methylation patterns influence the binding of the regulatory proteins Lrp and PapI to the *papBA* pilin promoter, which correlates with the ON and OFF stages of pilus expression in this bacterium.

In our previous studies, we demonstrated a direct role for GidA in the regulation of Act production (32). GidA was initially thought to be involved in chromosome replication and cell division; however, recent studies implicated GidA in a number of biological and pathogenic processes, suggesting a global regulatory role for GidA (41). It has been reported that GATC methylation sites are unevenly distributed in the genome of *E. coli*, and the *oriC* (chromosome origin of replication) has a GATC-rich region (2). These observations are in agreement with the role of Dam in chromosomal DNA replication. GidA is a FAD (flavin adenine dinucleotide) binding protein and might act as a sensor for the redox state of cells in a manner similar to that of the flavoproteins, e.g., aerotaxis signal transducer in *E. coli* (41). Importantly, we noted multiple DNA methylation sites within the putative promoter region of the *gidA* gene.

In a recent study using *E. coli* gene arrays, it was noted that those genes which were located proximal to the *oriC* gene were transcribed with enhanced frequency in a Dam-overproducing *E. coli* strain (22). The position of the *gidA* gene in *A. hydrophila* is immediately downstream of the *oriC* gene, indicating that the expression of the *gidA* gene could possibly be upregulated in the Dam-overproducing strain. Therefore, it is plausible that Dam might regulate gene expression through a global regulator, GidA. This scenario matches with our data indicating that the expression of the *act* gene was downregulated in the *gidA* isogenic mutant but upregulated in the native Dam-overproducing strain of *A. hydrophila*.

To test this possibility, we overproduced native Dam in a *gidA* isogenic mutant and demonstrated that increased MTase activity was unable to induce increased production of Act and lactones in the absence of GidA (Fig. 4A to C and 5A). These studies provided the first evidence of an intermediate protein that could be involved in the mechanism for the influence of Dam on bacterial virulence. These studies also suggested that different virulence genes may share a common regulatory mechanism whose activity is dependent on Dam methylation. Examination of the putative promoter region of the *act* gene indicated two potential GATC sequences which could be methylated by Dam. However, since Act production remained unaltered in the *gidA* mutant control strain versus that in the *gidA* mutant that overproduced native Dam, our future studies will focus on the potential role of GATC sequences of *gidA* in the alteration of virulence factor production by *A. hydrophila*.

Overproduction of Dam significantly attenuated the virulence of *Y. pseudotuberculosis* and provided a fully protective immune response in the vaccinated hosts (19). We provided evidence that the Dam-overproducing strain with pBAD/dam_{AhSSU} plasmid was avirulent in a mouse model compared to the WT bacterium with pBAD vector alone (6). Dysregulation of Dam activity can disable the ability of a pathogen to cause disease via aberrant virulence gene expression and contribute to heightened immunity in vaccinated hosts through the ectopic production of an expanded repertoire of potential antigens (19). While a concern with this approach is that virulence in Dam-overproducing strains can revert to WT levels by mutation, the insertion of multiple, nontandem copies of Dam-overproducing cassettes into the chromosome should reduce the likelihood of this undesired scenario (19). In this study, we showed that mutated Dam without MTase activity exhibited a virulence phenotype similar to that of the WT *A. hydrophila* SSU strain in both the in vitro and in vivo models. Further, immunization of mice with the Dam-overproducing strain provided protection to animals challenged with the lethal dose of WT *A. hydrophila.* Dam represents a possible target for vaccine development because the *dam* gene is highly conserved among a large majority of pathogenic bacteria and across lower and higher eukaryotes (13, 28). Thus, Dam is a global control factor for gene expression and overproduction of Dam results in altered virulence in bacterial pathogens.

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