DNA Adenine Methyltransferase Influences the Virulence of *Aeromonas hydrophila*

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Received 21 September 2005/Returned for modification 13 October 2005/Accepted 17 October 2005

Among the various virulence factors produced by *Aeromonas hydrophila*, a type II secretion system (T2SS)-secreted cytotoxic enterotoxin (Act) and the T3SS are crucial in the pathogenesis of *Aeromonas*-associated infections. Our laboratory molecularly characterized both Act and the T3SS from a diarrheal isolate, SSU of *A. hydrophila*, and defined the role of some regulatory genes in modulating the biological effects of Act. In this study, we cloned, sequenced, and expressed the DNA adenine methyltransferase gene of *A. hydrophila* SSU (*dam*) in a T7 promoter-based vector system using *Escherichia coli* ER2566 as a host strain, which could alter the virulence potential of *A. hydrophila*. Recombinant Dam, designated as M.AhySSUDam, was produced as a histidine-tagged fusion protein and purified from an *E. coli* cell lysate using nickel affinity chromatography. The purified Dam had methyltransferase activity, based on its ability to transfer a methyl group from S-adenosyl-l-methionine to N⁴-methyladenine-free lambda DNA and to methylate lambda DNA from digestion with DpnII but not against the DpnI restriction enzyme. The *dam* gene was essential for the viability of the bacterium, and overproduction of Dam in *A. hydrophila* SSU, using an arabinose-inducible, P_BAD promoter-based system, reduced the virulence of this pathogen. Specifically, overproduction of M.AhySSUDam decreased the motility of the bacterium by 58%. Likewise, the T3SS-associated cytotoxicity, as measured by the release of lactate dehydrogenase enzyme in murine macrophages infected with the Dam-overproducing strain, was diminished by 55% compared to that of a control *A. hydrophila* SSU strain harboring the pBAD vector alone. On the contrary, cytotoxic and hemolytic activities associated with Act as well as the protease activity in the culture supernatant of a Dam-overproducing strain were increased by 10-, 3-, and 2.4-fold, respectively, compared to those of the control *A. hydrophila* SSU strain. The Dam-overproducing strain was not lethal to mice (100% survival) when given by the intraperitoneal route at a dose twice that of the 50% lethal dose, which within 2 to 3 days killed 100% of the animals inoculated with the *A. hydrophila* control strain. Taken together, our data indicated alteration of *A. hydrophila* virulence by overproduction of Dam.

*Aeromonas hydrophila* is both a human and an animal pathogen, and the diseases associated with this bacterium in humans include gastroenteritis, wound infections, and septicemia (36, 88). In immuno-compromised individuals, some of the more serious complications associated with *A. hydrophila* infections are cellulitis, myonecrosis, and ecthyma gangrenosum, which often are fatal (2, 36). A variety of virulence factors/mechanisms, such as the surface layer, capsule, different forms of pili (e.g., type IV [Tap] and bundle-forming pili), toxins, quorum sensing, biofilm formation and, more recently, the type III secretion system (T3SS), were identified in various *Aeromonas* species that are involved in the pathogenesis of *Aeromonas* infections (10, 11, 40, 52, 75, 82, 89). Currently, the set of virulence factors crucial in causing human diseases is unknown in this pathogen and, therefore, the search for new virulence factors and their regulation must continue.

Our laboratory defined the role of three enterotoxins of a diarrheal isolate, SSU of *A. hydrophila*, namely, cytotoxic enterotoxin (Act) and cytotoxic enterotoxins (heat-labile [Alt] and heat-stable [Ast]), in causing gastroenteritis (1, 72), based on case-control human studies. The role of these enterotoxins in a mouse model of gastroenteritis was also shown by preparing specific enterotoxin gene-targeted mutants via marker exchange mutagenesis. Our studies indicated that Act that was secreted by the T2SS contributed maximally to fluid secretion, and this toxin also had hemolytic and cytotoxic activities, in addition to those associated with lethality in mice (27, 64, 72, 73). Further, we characterized the T3SS from an *A. hydrophila* SSU strain which contained 35 open reading frames (ORFs) (75). We recently noted minimal lethality (10%) in mice infected with the mutant of *A. hydrophila* SSU that had deletion of both the *act* and *Aeromonas* outer membrane protein B (*aopB*) genes at a dose two times the 50% lethal dose (LD₅₀). The *aopB* gene constitutes part of the T3SS and is involved in cytotoxicity of the host cell (75). This finding was compared to those in animals infected with *act* and *aopB* mutants alone (40 to 50% lethality) and thus signified that both the T2SS-associated Act and the T3SS played crucial roles in bacterial virulence (75). Further, we identified the role of the ferric uptake regulator (fur) and glucose-inhibited division gene (*gidA*) in modulating the biological effects of Act (73, 74). In the search for additional genes that could alter bacterial virulence, we now report characterization of the DNA adenine methyltransferase (Dam) from *A. hydrophila* SSU that modulates the function of both T3SS and T2SS-associated Act.

DNA methylation occurs in bacteria, plants, and mammals and, recently, methylated DNA was also reported in *Drosos...
**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td><strong>A. hydrophila</strong></td>
<td></td>
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<tr>
<td>SSU-R</td>
<td>Rifampin-resistant (Rif*) strain of <em>A. hydrophila</em> SSU</td>
<td>CDC, Atlanta, Ga.*</td>
</tr>
<tr>
<td>Dam-overproducing strain</td>
<td><em>A. hydrophila</em> SSU-R harboring plasmid pBAD-dam&lt;sub&gt;ASSU&lt;/sub&gt;; Rif* Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>Control strain</td>
<td><em>A. hydrophila</em> SSU-R harboring plasmid pBAD; Rif* Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>dam mutant</td>
<td>Chromosomal copy of dam gene was deleted from Dam-overproducing strain of <em>A. hydrophila</em> SSU-R (Δdam); Rif&lt;sup&gt;+&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>E. coli</strong></td>
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<td>TOP10</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcrA Δ(mrr-hsdRMS-mcrBC) 6810lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str&lt;sup&gt;R&lt;/sup&gt;) endA1 supG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thi hsdR17 (rK&lt;sup&gt;−&lt;/sup&gt;) relA1 supE44 Δ(lac-proAB) [F&lt;sup&gt;−&lt;/sup&gt; traD36 proAB lacP2ZΔM15]</td>
<td>Promega</td>
</tr>
<tr>
<td>ER2566</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; ΔrfaA1 ompF lacZ::T7gene1 galI sulA11 Δ(mcrC-mrr)114::IS10 (R9mcr-73-mini-Tn10-Tet&lt;sup&gt;R&lt;/sup&gt;) 2R(aro-210::Tn10) (Tet&lt;sup&gt;R&lt;/sup&gt;) endA1</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>SM10(apir)</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; thi-1 thr leu tonA lacY supE recA&lt;sup&gt;R&lt;/sup&gt;</td>
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<td><strong>Plasmids</strong></td>
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<td>pCR2.1</td>
<td>TA cloning vector; Ap&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<td>pCR2.1-dam</td>
<td>TA cloning vector pCR2.1 harboring a portion of the dam gene (400 bp) which was PCR amplified from <em>A. hydrophila</em> SSU gDNA; Ap&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pET-30a(+)</td>
<td>T7 promoter-based prokaryotic expression vector; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Novagen</td>
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<td>pET-30a(+) - dam&lt;sub&gt;ASSU&lt;/sub&gt;</td>
<td>dam gene of <em>A. hydrophila</em> cloned at NdeI/Xhol sites of pET-30a(+) vector for purification of DAM protein; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td>pBAD-Thio-E</td>
<td>Arabino-inducible arnBAD promoter-based prokaryotic expression vector; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<td>pBAD</td>
<td>Control vector, which was derived from pBAD-Thio-E by deleting the Ncol/PmeI fragment; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD-dam&lt;sub&gt;ASSU&lt;/sub&gt;</td>
<td>dam gene of <em>A. hydrophila</em> cloned at the Ncol/PmeI sites of pBAD-Thio-E vector for hyperexpression; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td>pKD4</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;; template plasmid for kanamycin cassette (Km1)</td>
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<tr>
<td>pUC4K</td>
<td>A plasmid containing 1.2-kb kanamycin cassette (Km2); Km&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMS197</td>
<td>A suicide vector; oriT oriT sacB; Te&lt;sup&gt;C&lt;/sup&gt;</td>
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<td>pMS197tpkm1down</td>
<td>Suicide vector pMS197 containing Km1 cassette flanked by upstream and downstream sequences of the dam gene; Km&lt;sup&gt;R&lt;/sup&gt; Te&lt;sup&gt;C&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pMS197tpkm1/Km2down</td>
<td>Suicide vector pMS197 containing Km cassettes (Km1 and Km2) flanked by upstream and downstream sequences of the dam gene; Km&lt;sup&gt;R&lt;/sup&gt; Te&lt;sup&gt;C&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pTP166</td>
<td>A plasmid containing <em>E. coli</em> dam gene; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>7</td>
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* CDC, Centers for Disease Control and Prevention.

DNA methyltransferases (MTases) catalyze methylation of either the cytosine residues at the C-5 or N-4 position or at the N-6 position of an adenine residue within the DNA (85). Specifically, Dam exerts its function by DNA methylation at adenine residues in GATC sequences (59). Methylation is a postreplicative process, and the newly replicated DNA is methylated only on the parental strand. Therefore, this hemimethylated DNA is distinct from the rest of the chromosomal DNA. The hemimethylated status of newly synthesized DNA provides a timeframe during which cellular processes, such as DNA replication (43, 55, 68) and repair of mismatched bases as well as alteration of gene expression (50), occur. During DNA replication, the GATC hemimethylated sites have a high affinity for SeqA protein, which is a negative regulator of replication initiation and essential for sequestration, a process that blocks secondary replication initiation events (46, 83). Likewise, during methyl-directed mismatch repair, mutH binds to hemimethylated DNA and cleaves the nonmethylated strand (3, 51). Finally, activation of a promoter upon hemimethylation could result in a burst of transcription shortly after passage of the replication fork, thereby linking gene expression to the cell cycle. Indeed, in the case of the Tn10 transposase gene, an increase in the promoter activity was directly demonstrated when DNA was in the hemimethylated state (55).

In addition to the above-mentioned functions of the hemimethylated DNA, GATC methylation can also influence gene expression. For example, the methylated GATC sequences in gene promoter regions can alter the affinity of regulatory protein(s) to DNA target sites. Conversely, regulatory proteins may bind nonmethylated DNAs with high affinity, thus protecting specific DNA sequences from methylation, resulting in the formation of DNA methylation patterns (DMPs) (32, 45, 51). One of the best-studied examples for regulation of gene expression by DMPs is the pyelonephritis-associated pilus (pap) operon of uropathogenic *Escherichia coli*. The DMPs influence the binding of the regulatory proteins Lrp and PapI to the *papB4* pilin promoter, which correlates with the on and off stages of pilus expression in this bacterium (33, 45).

Dam methylation has received significant interest recently because of its impact on the virulence of several pathogens, such as *Vibrio cholerae* (38, 49), *Salmonella enterica* serovar Typhimurium (19, 20, 28, 57), pathogenic *E. coli* strains (12, 43), *Yersinia pseudotuberculosis* (4, 39), *Yersinia enterocolitica* (25), *Haemophilus influenzae* (9, 90), and others. However, in *Shigella flexneri*, the dam mutants showed no attenuation of...
The role of DNA methylation has been reported in the viability of V. cholerae, Y. pseudotuberculosis, and Y. enterocolitica (25, 38). Further, it was shown that Dam overproduction led to attenuation in the virulence of these pathogens (25, 38). Dam was required for virulence in Pasteurella multocida, known to cause bovine respiratory disease (14). Recently, the gene encoding Dam was cloned and sequenced from Actinobacillus actinomycetemcomitans, which is implicated in causing human periodontal diseases (21). Therefore, Dam presents the exciting possibility that it may play a role in the virulence of a broad range of pathogens and, thus, further investigation is merited.

In this paper, we showed that a diarrheal isolate, SSU of A. hydrophila, harbored the dam gene and that its overexpression attenuated bacterial virulence, specifically that of T3SS-associated cytotoxicity, motility, and virulence in a mouse lethality model. This is the first report of characterization of the dam gene from Aeromonas species. According to the recent nomenclature for methyltransferases and their genes, we denoted the A. hydrophila SSU strain DNA adenine MTase as M.AhSSU.Dam and the dam gene as dam<sub>AhSSU</sub> (66).

Materials and Methods

Reagents. Restriction enzymes, Taq DNA polymerase, T4 ligase, and N<sup>6</sup>-methyladenine-free lambda DNA were purchased from New England BioLabs, Beverly, MA. Proteinase K and unlabeled S-adenosyl-l-methionine (AdoMet) were obtained from Sigma, St. Louis, MO. We purchased [methyl-<sup>3</sup>H]AdoMet (15 Ci/mmol; 1 mCi/ml) from New England Nuclear, Boston, MA. ProBond resin charged with nickel was from Invitrogen, Carlsbad, CA.

DNA and RNA manipulations. Plasmid DNA was isolated using a QiAprep Spin Miniprep kit (QIAGEN, Valencia, CA). A. hydrophila genomic DNA (gDNA) for sequencing was isolated using the published protocol with some modifications (63) or by utilizing a DNAeasy tissue kit (QIAGEN) for PCR assays. DNA fragments were purified using a PCR purification kit or gel extraction kit (QIAquick, QIAGEN, Valencia, CA). Plasmid DNA was isolated using a QiAprep Spin Miniprep kit (QIAGEN, Valencia, CA).

DNA sequencing of gDNA to obtain coding region of the dam gene

Southern blot analysis.

Primers and PCR assays. The primers used for various experiments are indicated in Table 2 and were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR assays were performed with 100 ng of gDNA and 50 ng of plasmid DNA. The PCR program used was as follows: 94°C for 5 min (denaturation) followed by 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 to 3 min (depending on the size of the amplified DNA fragment). The PCR products were then held at 72°C for 2 min and stored at 4°C or at −20°C until they were subsequently run on a 0.8% agarose gel. We performed PCR assays from single bacterial colonies in TEP buffer (10 mMTris-HCl, pH 8.0, 1 mM EDTA, and 50 μg/ml proteinase K). Briefly, a single colony was removed from the LB agar plate and resuspended in 12 μl of the TEP buffer. The mixture was incubated first at 55°C and then at 85°C for 15 min. After incubation on ice for 1 min, cellular debris was removed and 3 μl of the clarified extract was used in a PCR assay.

Southern blot analysis. The digested gDNA of A. hydrophila or the PCR products were transferred to a nylon membrane (Gibco BRL, Gaithersburg, MD) and baked at 80°C in a vacuum oven for 2 h. The blots were prehybridized virulence (34). Overall, data seem to support the hypothesis that Dam could globally alter virulence gene expression in gram-negative bacteria (31, 45).
for 6 h and hybridized overnight under low-stringency conditions in a solution containing 30% formamide, 1 mM EDTA, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt’s solution, 100 μg/ml salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS) at 42°C (69). For hybridization, a [32P]-labeled E. coli dam gene probe (the 854-bp XbaI-PvuII fragment of pTP166 plasmid) was used (7). The membranes were washed at 54°C with 5× SSC plus 0.1% SDS for 40 min and then in 4× SSC plus 0.1% SDS for another 40 min. This analysis identified the initial 400-bp fragment of the dam gene, which was used to obtain the full-length dam AhSSU gene.

Fosmid library construction. A. hydrophila gDNA (5 μg) was sheared to generate fragments in the 25- to 40-kb size range. The sheared DNA was end-repaired to generate blunt ends and size selected using a 1% low-melting-point agarose gel. The size-selected DNA was then ligated to the dephosphorylated blunt-ended pEpiFOS-5 Fosmid vector (Epicenter, Madison, WI) and packaged using MaxPlax Lambda packaging extracts. E. coli EPI100 plating cells (Epicenter) were used as the host. Fosmid colonies were lifted onto nylon filters from LB agar plates containing 12.5 μg/ml chloramphenicol (Cm). The gene encoding Cmr was carried by the fosmid vector. The filters were screened with the [32P]-labeled 400-bp dam gene probe by colony blot hybridization. Positive colonies were identified and grown overnight in LB medium with Cm and 10 mM MgSO4 for DNA isolation.

Colony blot hybridization. Nylon membrane filters used for the colony blots were removed from the agar plates and processed as described elsewhere (69). The filters were dried and baked at 80°C for 2 h. Filters were prewashed with 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, and 0.1% SDS at 42°C to remove cell debris, prehybridized, and then hybridized with the [α-32P]dCTP-labeled 400-bp dam gene probe under high-stringency conditions. The filters were prehybridized using Quickhyb solution at 68°C as described by the manufacturer (Stratagene, La Jolla, CA) and hybridized with 32P-labeled probe. After 2 to 3 h, the filters were washed with 2× SSC, 0.1% SDS for 40 min and 1× SSC, 0.1% SDS for another 40 min at 68°C and exposed to autoradiograph film overnight at room temperature.

Primer extension analysis. The primer extension system kit from Promega (Madison, WI) was used to determine the presumptive transcriptional start site of the dam AhSSU gene. Primers (1 μM each) complementary and downstream to the initiation codon of the dam AhSSU gene (Table 2 and Fig. 1) were end labeled with 30 Ci of [α-32P]dATP (ICN). For each extension reaction mixture, 1 μM of labeled primer was mixed with 50 μg of RNA, heated to 58°C for 20 min, and cooled to room temperature. Subsequently, the mixture was incubated with 1 U of avian myeloblastosis virus reverse transcriptase (Promega). After 30 min of incubation, the products were mixed with loading buffer, heated at 90°C for 10 min, and analyzed by running on a denaturing polyacrylamide gel containing 8% acrylamide (19:1 acrylamide-bis) and 7 M urea as described in the protocol (Promega). The length of the cDNA on the gel reflected the number of bases between the labeled nucleotide of the primer and the 5′ end of the RNA and was compared with that of the labeled markers, which ranged in size from 24 to 311 bp.
Transformation of E. coli and A. hydrophila. Competent E. coli cells for transformation were prepared using the Z-Competent E. coli transformation kit from Zymo Research, Orange, CA. For electroporation, A. hydrophila SSU (50 ml) was grown until early log phase and resuspended in 300 ml of mM sucrose (sequentially in the same volume, in a 0.5 volume, and finally in a 0.01 volume) (77) and electroporated with the DNA (1 to 3 μg) by using GenePulser Xcell in 0.2-cm gap cuvettes (Bio-Rad, Hercules, CA).

Purification of DNA adenine methyltransferase. PCR amplified from A. hydrophila SSU gDNA, the dam\xa0\textsubscript{SSU} gene was cloned into the pET-30a (+) T7 promoter-based expression vector (Novagen, San Diego, CA) using primers with Ndel and XhoI restriction enzyme sites (Table 2). To overexpress the dam gene in E. coli, the recombinant plasmid was transformed into the E. coli ER256 strain, which harbored the T7 RNA polymerase gene under the control of the lac promoter. The E. coli(pET-30a-dam\xa0\textsubscript{SSU}) culture was grown in 500 ml of the LB medium with kanamycin (30 μg/ml) as an optical density at 600 nm (OD\textsubscript{600}) of 0.4 to 0.6 before induction with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. The bacterial cells were harvested, resuspended in 10 ml of appropriate buffer (Tris-HCl, pH 8.0, 100 mM NaiCl, 1 mM MgCl\textsubscript{2}, and 5% glycerol), and disrupted by sonication. The cell lysate was passed through ProBond resin (3-m1 bed volume in a 5-ml column) charged with nickel according to the manufacturer’s recommendations. The resin was washed with a five-column volume of the wash buffer, followed by elution of the recombinant protein with a concentration of 1 M NaCl (1.7 mM) in the purity of Dam was examined by SDS–12% polyacrylamide gel electrophoresis followed by Coomassie blue staining of the gel. After dialysis and concentration, purified MAHySSUDam was used to determine enzymatic activity by the DNA adenine methylation assays as described below.

Cloning of the dam gene under the araBAD promoter. To regulate dam expression, pBAD-dam\xa0\textsubscript{SSU} plasmid was generated using dam\textsubscript{N}-NcoI and dam\textsubscript{C}-PmeI primers (Table 2) by replacing the NcoI-PmeI fragment of the pBAD/Thio-E vector (Invitrogen) under its araB araD promoter. To induce expression of the gene from the plasmid, arabinose (0.2%) was added to the medium (29). We referred to this culture as the Dam-overproducing A. hydrophila strain (Table 1). In some experiments, we also examined tight regulation of the dam gene in the pBAD system in A. hydrophila by either omitting arabinose or adding glucose and evaluating the effect of such a strain on bacterial virulence. To test the pBAD plasmid for use as a control, the pBAD/Thio-E vector was digested with Ndel and PmeI restriction endonucleases, treated with DNA polymerase I (Klenow fragment), and ligated. Both plasmids were then subjected to transformation/electroporation in E. coli JM109 and A. hydrophila SSU strains. The latter with pBAD vector alone was designated the A. hydrophila control strain (Table 1).

DNA methylation assays. The GATC-specific DNA methylation assay was conducted according to the published procedure (56). Briefly, the methylation mixtures were extracted by the transfer of 20 mM Tris-HCl, pH 8.0, 50 mM MgCl\textsubscript{2}, 7 μM Dam, and 1 mM dNTPs to 2-mercaptoethanol, 1 mM EDTA, 0.1 mg/ml bovine serum albumin (BSA), 1 μg N\textsubscript{6}-methyladenine-free lambda DNA, purified A. hydrophila Dam (3 μg), and 50 μM unlabeled AdoMet. After 1 h of incubation at 37°C, the reaction was stopped by heat inactivation at 65°C for 15 min. Then, methylated lambda DNA was examined by digestion with 5 U of restriction enzyme DpnI or DpnII in recom-
Cell membrane integrity and binding assays. The integrity of the cell envelope of both Aeromonas strains (with the pBAD vector alone or the pBAD-damSSU plasmid) was examined by analyzing sensitivity to TX-100 (1 to 2%) (13, 24, 70) and by evaluating the outer membrane permeability change with Torula yeast RNA (Sigma), which measured periplasmic RNase leakage from bacteria. For membrane permeability changes, bacterial cells (equal number) were spotted onto LB agar plates containing 1.5% Torula yeast RNA and incubated overnight at 37°C. Subsequently, 10% trichloroacetic acid was added onto the plates, and the RNA leakage from bacterial cells was examined by measuring the diameter of the clear zones around the bacterial colonies.

We infected human HT-29 colonic epithelial cells (American Type Culture Collection, Manassas, VA) with the above-mentioned bacteria at a multiplicity of infection (MOI) of 10 and incubated them at 37°C for 1 h. Unbound bacteria were aspirated, cells were washed four times with 1× Dulbecco’s PBS (DPBS) and lysed with 0.1% TX-100, and various dilutions of the cell lysates were plated onto 1.5% LB agar plates for determining CFU (24). The bacteria were grown in the presence of 0.2% arabinose.

To evaluate in vivo binding of A. hydrophila control and Dam-overproducing strains, 6- to 8-week-old female Swiss-Webster mice (Taconic Farms, California) were used. Briefly, mice were anesthetized under halothane and a ventral midline incision was made. A single 5-cm segment of small intestine was ligated with 00 suture and injected with 100 μl of the above-mentioned bacteria (1 × 10^8 CFU). After 2 h of infection, the animals were euthanized by cervical dislocation, and the intestinal loops were removed. The injected loops were measured, extensively washed in PBS, homogenized, and examined for colonization by determining CFU on LB agar plates containing antibiotics rifampicin and ampicillin (200 and 100 μg/ml, respectively) to select only for A. hydrophila.

Motility assay. LB medium with 0.35% agar was used to characterize the motility phenotype of A. hydrophila control and Dam-overproducing strains. The overnight cultures grown in the presence of 0.2% arabinose were adjusted to the same optical density, and equal numbers of CFU (10^8) were stabbed into 0.35% LB agar plates containing 0.2% arabinose. Plates were incubated at 37°C overnight, and the motility was assayed by examining migration of bacteria through the agar from the center towards the periphery of the plate (70).

Cytotoxicity assay. RAW 264.7 murine macrophages (American Type Culture Collection) were seeded into 96-well plates (1 × 10^4 cells/well) and infected with the either-live A. hydrophila SSU control or the Dam-overproducing strain at an MOI of 10 for 3 to 4.5 h to examine the TSSS-associated cytotoxicity (70). Both strains were grown in the presence or absence of 0.2% arabinose. Host cells were also treated with 5 μl of filter-sterilized, overnight-grown bacterial culture supernatants (for determining TSSS Act-associated toxicity) (70). After incubation at 37°C for 2 h, the tissue culture medium was examined for the release of lactate dehydrogenase (LDH) enzyme using the CytoTox96 kit (Promega). The percentage of LDH released by RAW 264.7 macrophages infected with bacterial cells was determined following the manufacturer’s instructions. In macrophages treated with culture supernatants, cytotoxicity was reported per milliliter of the culture filtrate per 10^6 CFU and expressed as a fold change when compared between hydrophila control and Dam-overproducing strains.

Hemolytic activity. A. hydrophila SSU control and Dam-overproducing strains were grown overnight in LB medium with 0.2% arabinose and ampicillin (Ap; 100 μg/ml). The culture supernatants were collected and treated with trypsin to convert the precursor form of T2SS-associated Act to its mature form (13, 24, 70). For the hemolytic activity assay, 100 μl of 1× DPBS was added to each of the wells of a 96-well tissue culture plate flowed with sequential twofold dilution and the addition of 100 μl of 3% rabbit erythrocytes (Colorado Serum Co., Denver, CO). Our negative control included 1× DPBS and trypsin alone. The plate was incubated at 37°C for 1 h and observed for hemolytic activity associated with Act. The supernatants were taken from those wells that showed partial lysis of rabbit erythrocytes, and the hemoglobin release was recorded at 540 nm using a microplate ELISA reader. The hemolytic titers were calculated as the value of the hemoglobin release multiplied by the dilution of the culture supernatant. The hemolytic units were reported per milliliter of culture filtrate per 10^6 CFU. For neutralization studies, culture filtrates were mixed with either preimmune or hyperimmune rabbit sera (laboratory stock; 1:10 dilution) containing antibodies to Act. After incubation at 37°C for 1 h, the hemolytic and cytotoxic activities were measured.

Proteinase activity. An aliquot (200 μl) of overnight culture filtrates (in the presence of 0.2% arabinose) from A. hydrophila control and Dam-overproducing strains was added to disposable 6-ml snap-cap tubes which contained 800 μl of the DPBS and 5 μg of Hde azure powder substrate (Calbiochem, La Jolla, CA). The tubes were incubated in a shaker incubator at 37°C for 1 to 3 h. As the proteinase in the culture filtrates catalyzed the substrate, blue color was released from the substrate and was quantified at OD595. The proteinase activity was calculated per ml of culture filtrate per 10^6 CFU. The substrate incubated with the LB medium alone served as a negative control.

Animal experiments. Eight-week-old female Swiss-Webster mice were used to determine lethality induced by A. hydrophila SSU control and Dam-overproducing strains. Mice were inoculated intraperitoneally with a lethal dose (3 × 10^8 CFU, repressor 4×10^8 CFU) of both Aeromonas strains in a group of 10 mice each. One group of mice was inoculated with DPBS (n = 10) and served as a control. Mice were observed daily for signs of distress and mortality for up to 3 weeks. The animals were provided with 0.2% arabinose and ampicillin (40 mg/kg/day) in the drinking water for the first 2 to 3 days following challenge to retain the plasmid in bacteria. The treatment with arabinose and ampicillin started 1 day prior to the challenge.

Statistics. Wherever applicable, at least three independent experiments were performed; the data were analyzed by using Student’s t test, and P values of ≤0.05 were considered significant. The animal data were analyzed using Fisher’s exact test.

Nucleotide sequence accession number. The sequence of the A. hydrophila SSU dam gene was deposited in the GenBank database under accession number DQ067435.

RESULTS

Cloning and sequencing of the A. hydrophila SSU dam gene. To determine whether A. hydrophila strain SSU contains the DNA adenine MTase gene, we initially used restriction endonucleases which are sensitive to methylation of adenine residues in 5′-GATC-3′ sequences to digest gDNA. It is known that the DpnI enzyme cuts the methylated GATC sequence, but not unmethylated GATC. Conversely, DpnII does not cut methylated GATC, while it cuts the unmethylated GATC sequence (67). We noted that gDNA of A. hydrophila was sensitive to DpnI restriction endonuclease digestion and resistant to digestion with DpnII restriction enzyme (data not shown). These findings indicated that A. hydrophila might possess MTase activity with GATC specificity.

As a result, we designed dam1 and dam2 primers (Table 2) that corresponded to the regions of highest conservation among the dam genes from different gram-negative bacteria, such as the Vibrio species (e.g., V. cholerae, V. fischeri, and V. parahaemolyticus), E. coli, S. enterica serovar Typhimurium, and Yersinia species (e.g., Y. pseudotuberculosis and Y. pestis), to PCR amplify a portion of the dam gene (a 400-bp fragment) from the gDNA of A. hydrophila SSU. Upon Southern blot analysis under low-stringency conditions, this fragment reacted with an E. coli dam gene probe. Consequently, this 400-bp fragment was cloned into a PCR 2.1 vector and transformed in TOP10 chemically competent E. coli cells (TA cloning kit; Invitrogen). The DNA sequence analysis of this 400-bp fragment revealed 61% and 63% identities with the corresponding dam genes from different gram-negative species (e.g., Y. pseudotuberculosis and Y. pestis), to PCR amplify a portion of the dam gene (a 400-bp fragment) from the gDNA of A. hydrophila SSU. Upon Southern blot analysis under low-stringency conditions, this fragment reacted with an E. coli dam gene probe. Consequently, this 400-bp fragment was cloned into a PCR 2.1 vector and transformed in TOP10 chemically competent E. coli cells (TA cloning kit; Invitrogen). The DNA sequence analysis of this 400-bp fragment revealed 61% and 63% identities with the corresponding regions of the dam gene in E. coli and V. cholerae, respectively.

The coding regions of the dam gene in E. coli and V. cholerae contained 834 and 831 bp, respectively (7, 38).

We used two strategies to obtain a complete sequence of the A. hydrophila dam gene coding region as well as of the flanking DNA sequences to the dam gene. The nucleic acid sequence of the entire damSSU gene (Fig. 1) was determined by gDNA sequencing using the additional primers dam3 and dam4 (Table 2). The construction of a fosmid library of A. hydrophila SSU gDNA enabled us to confirm the DNA sequence of the dam gene and to obtain its flanking sequences for preparing isogenic mutants. The 400-bp dam gene fragment generated by PCR amplification that reacted with the E. coli dam gene
probe was used to screen the fosmid library of A. hydrophila. This probe did not react with the E. coli dam gene under high-stringency conditions and thus prevented identification of false-positive fosmid clones. Five positive fosmid clones that contained inserts of approximately 25 kb were obtained out of 800 clones that were screened. Further, this 400-bp dam gene fragment hybridized specifically with the gDNA digests (cut with various restriction enzymes) of A. hydrophila SSU. The synthesized cDNA was analyzed on a denaturing polyacrylamide gel. The length of the cDNA reflected the number of bases between the labeled primer and the 5’ end of the damSSU gene transcript. cDNA products with lengths of 54, 60, and 65 bp, were obtained with damP1 (Fig. 2, lane 4), damP2 (Fig. 2, lane 3), and damP3 (Fig. 2, lane 2) primers, respectively. Based on the DNA sequence, primer extension analysis identified a presumptive transcriptional start site (G) at a position 24 nucleotides upstream of the ATG start codon of the dam gene (Fig. 1). Putative –10 (GGGTAGAAT) and –35 (TAGCCA) elements of the promoter were also identified in this region using a software program found at www.softberry.com.

Analysis of the damSSU gene presumptive transcriptional start site and promoter region. Primer extension analysis was used to determine the presumptive transcriptional start site of the dam gene, and three primers were used. The damP1 primer was designed to nucleotide positions 1 to 30 (nucleotide A of the damSSU gene start codon [ATG] represented position 1). The damP2 primer represented nucleotide positions 7 to 36, while the damP3 primer spanned nucleotide positions 12 to 41 (Table 2 and Fig. 1). The primers were end labeled and hybridized to the RNA isolated from wild-type (WT) A. hydrophila SSU. The synthesized cDNA was analyzed on a denaturing polyacrylamide gel. The length of the cDNA reflected the number of bases between the labeled primer and the 5’ end of the damSSU gene transcript. cDNA products with lengths of 54, 60, and 65 bp, were obtained with damP1 (Fig. 2, lane 4), damP2 (Fig. 2, lane 3), and damP3 (Fig. 2, lane 2) primers, respectively. Based on the DNA sequence, primer extension analysis identified a presumptive transcriptional start site (G) at a position 24 nucleotides upstream of the ATG start codon of the dam gene (Fig. 1). Putative –10 (GGGTAGAAT) and –35 (TAGCCA) elements of the promoter were also identified in this region using a software program found at www.softberry.com.

Overproduction and purification of M.AhySSUDam. The damSSU gene was overexpressed, and M.AhySSUDam as a His tag fusion protein was purified using ProBond resin charged with nickel. Purified Dam was eluted from the column using 1 M imidazole. Based on SDS-polyacrylamide gel electrophoresis analysis and Coomassie blue staining, a single protein band with a molecular mass of 34 kDa was detected. The molecular mass of purified Dam was in agreement with the predicted size of 291 amino acid residues (32,662 Da) based on SDS-polyacrylamide gel electrophoresis analysis and Coomassie blue staining, a single protein band with a molecular mass of 34 kDa was detected. The molecular mass of purified Dam was in agreement with the predicted size of 291 amino acid residues (32,662 Da) based on SDS-polyacrylamide gel electrophoresis analysis and Coomassie blue staining, a single protein band with a molecular mass of 34 kDa was detected. The molecular mass of purified Dam was in agreement with the predicted size of 291 amino acid residues (32,662 Da) based on SDS-polyacrylamide gel electrophoresis analysis and Coomassie blue staining, a single protein band with a molecular mass of 34 kDa was detected.

GATC DNA methylation assay. It is known that the enzyme DpnI digests methylated GATC sequences but does not digest unmethylated GATC (Fig. 3, lane 1). Conversely, DpnII does
FIG. 4. MTase activity associated with the overproduced M.AhySSUDam of *A. hydrophila* SSU. The cell extracts from the *A. hydrophila* control strain (with the pBAD vector alone, designated as pBAD) and the Dam-overproducing strain (with pBAD-dam<sub>ASSU</sub>) were prepared, and the ability of M.AhySSUDam to transfer methyl-<sup>3</sup>H from AdoMet to N<sup>6</sup>-methyladenine-free lambda DNA was measured as described in Materials and Methods. Three independent experiments were performed, and the arithmetic mean ± standard deviation is plotted. An uninduced culture of *A. hydrophila* with pBAD-dam<sub>ASSU</sub> exhibited a basal level of MTase activity.

not cut methylated GATC, while it does cut unmethylated GATC sequences (Fig. 3, lane 2) (67). The N<sup>6</sup>-methyladenine-free lambda DNA was treated with purified M.AhySSUDam enzyme and then digested with DpnI and DpnII restriction endonucleases. Subsequently, the reaction products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. As illustrated in Fig. 3 (lanes 3 and 4), phage lambda DNA (treated with purified M.AhySSUDam) was cut by DpnI but was not cut by DpnII, indicating that Dam was indeed functional in the *A. hydrophila* strain. The cell extracts from the Dam-overproducing *A. hydrophila* strain showed a basal level of MTase activity.

The ability of M.AhySSUDam enzyme to transfer methyl groups from [methyl-<sup>3</sup>H]AdoMet to N<sup>6</sup>-methyladenine-free lambda DNA was also confirmed. As noted from Fig. 4, cell lysate from the *A. hydrophila* control strain had a basal expression level of MTase activity. However, cell lysate prepared from the Dam-overproducing *A. hydrophila* strain showed a considerable increase in MTase activity which was dose dependent. Overproduction of M.AhySSUDam from the <i>p<sub>BAD</sub></i> promoter, after induction with arabinose, resulted in an up-to-30-fold increase in Dam activity (Fig. 4), indicating the functional regulation of the <i>dam</i> gene from the pBAD-dam<sub>ASSU</sub> plasmid by the araBAD promoter in the *A. hydrophila* SSU strain. The levels of MTase activity in uninduced cultures were similar to that of the *A. hydrophila* control strain.

**The dam gene is essential for viability of *A. hydrophila* SSU.**

Different genetic strategies to obtain the <i>dam</i> knockout mutant of *A. hydrophila* SSU were futile, indicating that the <i>dam</i> gene is essential for the viability of the bacterium. However, it was possible to inactivate a chromosomal copy of the <i>dam</i> gene when the corresponding gene was first provided on the plasmid to the *A. hydrophila* strain in trans before the chromosomal copy was deleted or inactivated. Therefore, to successfully generate a chromosomal deletion of the <i>dam</i> gene, a pBAD-dam<sub>ASSU</sub> plasmid was first electroporated into *A. hydrophila*. For homologous recombination, we opted to use flanking upstream and downstream sequences of the <i>dam</i> gene in the suicide vector pDMS197 (this construct was designated as pDMS197-upkm1/km2down [Table 1]) instead of using the coding region of the <i>dam</i> gene (Fig. 5). This strategy ensured specific targeting of the <i>dam</i> gene on the chromosome rather than on the plasmid pBAD-dam<sub>ASSU</sub> (Fig. 5). To confirm insertion of the Km<sup>r</sup> cassettes and the chromosomal deletion of the <i>dam</i> gene in the mutants, we used PCR analyses with three combinations of primers (Table 2 and Fig. 6). As a control, we used gDNA of WT *A. hydrophila*.

PCR confirmed the chromosomal deletion of the <i>dam</i> gene with a set of primers, F1 and R1 (Fig. 5 and 6), that represented external sequences relative to the flanking up- and downstream sequences of this gene. Specifically, these primers were designed for regions located outside of 144-bp upstream and 369-bp downstream sequences from the dam structural gene that were used to construct the pDMS197-upkm1/km2down plasmid (Table 1). We observed PCR products with gDNA of both the mutant and WT bacteria. The mutant showed the expected size of the product (3 kb) when compared to that of the WT bacterium (1.8 kb) (Fig. 6, lanes 4 and 1). A pair of F1/dam2 primers (the dam2 primer was located within the <i>dam</i> gene coding region) verified the deletion of the <i>dam</i> gene in the mutant. A correctly sized band of 0.75 kb was amplified from the WT gDNA, and no PCR product was amplified from the gDNA of the mutant (Fig. 6, lanes 2 and 5). PCR amplification with R1/KF primers (the KF primer was located in the Km<sup><i>1</sup></i> gene cassette) confirmed the insertion of Km<sup><i>r</sup></i> gene cassettes instead of the <i>dam</i> gene in the mutant. No PCR product was amplified from the gDNA of the WT bacterium; however, a band of an expected size of 2.6 kb was amplified from the gDNA of the mutant (Fig. 6, lanes 3 and 6).

To further provide evidence that the <i>dam</i> gene was necessary for the viability of *A. hydrophila*, we demonstrated that the chromosomal <i>dam</i> gene-deleted mutant was not viable, based on determining colony counts, when plated on LB agar plates with glucose, which shuts off the expression of the <i>dam</i> gene from the pBAD-dam<sub>ASSU</sub> plasmid (data not shown).

**Integrity of the cell membrane of the *A. hydrophila* SSU Dam-overproducing strain.**

We determined growth rates of the *A. hydrophila* control strain and its Dam-overproducing strain in LB medium with 0.2% arabinose. No significant difference in growth curves was noted in these two strains. The presence or absence of arabinose did not affect the growth phenotype. Also, both of these strains equally tolerated bile salts present in MacConkey’s agar plates as determined by colony counts. Further, the ability of control and Dam-overproducing strains to bind to HT-29 colonic epithelial cells was not affected, indicating the intactness of the bacterial cell envelope. Both the control and Dam-overproducing strains were resistant to the effect of TX-100 up to a concentration of 2%, and the release of periplasmic RNase I from the Dam-overproducing strain was not statistically different than that of the control *A. hydrophila* strain. These data indicated that increased MTase activity in the Dam-overproducing strain did not alter cell membrane integrity (data not shown).
Effect of M. AySSU Dam overproduction on *A. hydrophila* SSU virulence. It was recently shown that alterations in the level of Dam attenuated the virulence of a number of pathogens, including *S. enterica* serovar Typhimurium, *Y. pseudotuberculosis*, *H. influenzae* (4, 76, 86), and others. Although Dam in *E. coli* and *S. enterica* serovar Typhimurium is not essential for bacterial growth, it is required for the viability of such pathogens as *V. cholerae*, *Y. pseudotuberculosis* (38), and *Y. enterocolitica* (25) as we also demonstrated in this study in *A. hydrophila*.

To determine whether altered Dam production affected the
virulence potential of *A. hydrophila* SSU, we evaluated various biological activities associated with *A. hydrophila* control and Dam-overproducing strains. Motility is an important pathogenic factor for bacteria to reach the host target tissue, to colonize, and then to cause disease. We noted that overproduction of Dam significantly reduced (58%) the motility of the bacterium (Fig. 7A). To determine if overproduction of Dam would have an effect on the cytotoxicity associated with the T3SS of *A. hydrophila*, RAW 264.7 murine macrophage cells were infected with the dam knockdown mutant with F1/R1 primers (1.8 kb); 2, WT gDNA with F1/dam2 primers (0.75 kb); 3, WT gDNA with R1/KF primers (no product); 4, gDNA from the dam gene mutant with F1/R1 primers (3.0 kb); 5, gDNA from the dam knockdown mutant with F1/dam2 primers (no product); 6, gDNA from the dam knockdown mutant with R1/KF primers (2.6 kb).

Interestingly, when the cultures were grown in the absence of arabinose, no difference in Act production was noted between the control and the Dam-overproducing strain. Expression of the genes (e.g., *aopB*, *aopD*, *ascV*, and *acrV*) that constitute the T3SS apparatus was not altered (data not shown).

The pathogenic and virulence characteristics of *A. hydrophila* are also associated with the production of T2SS-associated exoenzymes (e.g., proteases and lipases) (15, 36). We noted increased production of proteinase (2.4-fold) with the *A. hydrophila* Dam-overproducing strain compared to its appropriate control in the culture supernatant (Fig. 8D). These results indicated that overproduction of Dam enzyme did, indeed, alter the virulence potential of *A. hydrophila* based on in vitro assays.

To finally confirm the effect of Dam overproduction on bacterial virulence, we injected mice intraperitoneally with either the *A. hydrophila* control strain or the Dam-overproducing strain at a lethal dose of 3 × 10⁷ CFU. All of the animals infected with the *A. hydrophila* control strain died within 2 days. However, animals infected with the Dam-overproducing strain did not die over a tested period of 3 weeks, indicating bacterial attenuation as a result of Dam overproduction. We also noted that the ability of the Dam-overproducing strain to colonize the small intestine of mice remained unaltered compared to that of the control strain. After 2 h of infection, approximately 2 × 10⁶ to 7 × 10⁶ CFU of control and Dam-overproducing strains bound the intestinal epithelial cells compared to 1 × 10⁷ CFU that were injected into the ligated ileal loops (data not shown).

**DISCUSSION**

The role that Dam plays in altering bacterial virulence is only beginning to be understood (45), and studies have shown that adenine methylation can either directly or indirectly alter the interaction of regulatory proteins with DNA (18). Dam can act as a de novo methylase by methylating both nonmethylated and hemimethylated GATC sites (79) and plays a pivotal role in controlling gene expression by the formation of DMPs (80). The role that Dam plays in altering bacterial virulence is only beginning to be understood (45), and studies have shown that adenine methylation can either directly or indirectly alter the interaction of regulatory proteins with DNA (18). Dam can act as a de novo methylase by methylating both nonmethylated and hemimethylated GATC sites (79) and plays a pivotal role in controlling gene expression by the formation of DMPs (80). In this study, we identified, cloned, and sequenced the *dam* gene from a diarrheal isolate, SSU of *A. hydrophila*. Further, the role of *A. hydrophila* Dam in altering bacterial virulence was evaluated using both in vitro and in vivo systems.
Previous studies indicated that Dam methylation played a role in *S. enterica* serovar Typhimurium cell envelope integrity (62). These investigators showed that Dam mutants enhanced the release of extracellular proteins to the medium, with no obvious alteration in the T3SS-associated secretion of proteins. We therefore examined the effect of Dam on cell membrane integrity of *A. hydrophila* SSU. We noted that the growth phenotypes of the control and Dam-overproducing *A. hydrophila* strains as well their abilities to adhere to HT-29 colonic or mouse small intestinal epithelial cells were similar. Likewise, membrane permeability remained unaltered in the Dam-overproducing strain of *A. hydrophila* compared to the control strain. These data indicated that the virulence defect in the Dam-overproducing strain of *A. hydrophila* was directly the result of alterations in gene expression and not the pleiotropic effects of Dam on cell physiology (30, 38, 45).

Our data indicated that Dam was functional (Fig. 3 and 4) and essential for the viability of *A. hydrophila* (Fig. 5 and 6). Similarly, in *V. cholerae*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, deletion of the *dam* gene was lethal to the corresponding bacterium (25, 38). However, a recent study indicated that the *dam* gene could be deleted from another strain of *Y. pseudotuberculosis* without loss in viability (78). The reason for the growth requirement of Dam is not clear, but for *V. cholerae*, which has two chromosomes, Dam is required for initiation and replication of both (23). Studies with the *E. coli dam* mutants revealed that Dam MTase was not required for the viability of the bacterium; however, the viability was dependent on the increased expression of the SOS regulon, and more specifically of the genes *recA* and *nuv* within this regulon (61). It was noted that RecA facilitated cleavage of the LexA repressor, a negative regulator of approximately 20 unlinked...
operons involved in DNA repair and mutagenesis (84), indicating that methyl-directed mismatch repair played a crucial role in the cell viability of *E. coli dam* mutants (51, 60). In *Y. enterocolitica*, Falker et al. (25) speculated that the potential role of Dam lay in the expression of essential genes required for bacterial viability rather than in Dam’s role in DNA mismatch repair when compared to the *dam* gene of *E. coli*. These results are in contrast to those in *E. coli* and *Salmonella* in which, for example, Dam is one of the three dispensable methyltransferases in addition to Dcm (methylates the internal cytosine residues in the sequences CCwGG) and EcoK (modifies adenine residues in the sequences AAC[N6]GTGC and GCA C[N6]GTT) (44).

Regardless of the role of Dam in bacterial viability, both deletion and overproduction of the *dam* gene have been shown to attenuate bacterial virulence (4, 31, 39). We explored the role of Dam in the pathogenesis of *A. hydrophila* and showed decreased motility and cytotoxicity associated with the T3SS of the Dam-overproducing strain (Fig. 7A and B). Motility is an important virulence factor of many gram-negative pathogens and a significant invasion-related factor for bacteria such as *S. enterica* serovar Typhi (42). Motility was shown to be decreased in a *dam* mutant of *E. coli* (58). Similarly, a decrease in motility might have contributed to the lack of invasiveness of *S. enterica* serovar Typhi *dam* mutants (42). Since invasiveness of *S. enterica* serovar Typhimurium to the host cells is also dependent upon the T3SS (26), it was noted that the *dam* mutants were indeed defective in the secretion of Salmonella pathogenicity island 1-encoded effector proteins, including those proteins essential for the invasion of the bacterium (28).
A possible mechanism by which overproduction of Dam leads to decreased T3SS-associated cytotoxicity in *A. hydrophila* could be explained by findings observed in *Y. pseudotuberculosis*. The overproduction of Dam in *Y. pseudotuberculosis* altered the expression and secretion of a T3SS-associated effector protein YopE (yersinia outer membrane protein E), which is secreted by the WT bacterium under low calcium and high temperature (37°C) conditions and is also known to be antigenic (39). Yops translocated into the host cell via the T3SS act to inhibit phagocytosis of the bacterium and to induce proinflammatory cytokine release (5). The overproduction of Dam in *Y. pseudotuberculosis* disrupted both the thermal and calcium regulation of YopE synthesis and relaxed the thermal but not the calcium dependence of YopE secretion (39). Currently, the effector proteins secreted by the *A. hydrophila* T3SS are not known. However, the phenomenon we observed of reduced T3SS cytotoxicity associated with Dam overproduction could be related to the altered secretion and/or synthesis of T3SS effectors. Our Northern blot analysis data indicated no alteration in the expression of T3SS apparatus genes *aopB, aopD, ascV*, and *acrV* in Dam-overproducing versus control *A. hydrophila* strains (data not shown). These data suggested that the T3SS machinery itself remained unaltered in the Dam-overproducing strain.

It is also possible that Dam overproduction in *A. hydrophila* may play a role in the increased expression of negative regulators of the T3SS or, conversely, the decreased expression of positive T3SS regulatory genes. An example of a T3SS regulator was recently elucidated in *Pseudomonas aeruginosa*. A specific locus was defined (sad4RS) which was comprised of genes for a putative sensor histidine kinase and two response regulators (41). Among the genes regulated by this three-component SadARS system are those required for the T3SS. This report showed that SadS and SadA were important for controlling expression of T3SS genes. SadA contains a helix-turn-helix motif and may regulate T3SS gene expression at the transcription level. In *Bordetella pertussis*, the BvgA response regulator also contains a helix-turn-helix motif, and under activating conditions (in the Bvg’ phase), this protein binds to virulence gene promoters and activates transcription (54). Recent data suggest that the SadARS regulatory system may function to promote biofilm formation, possibly, in part, by repressing expression of the T3SS (41). Similarly, it was reported recently that ExsE is a negative regulator of the T3SS in *P. aeruginosa* (65) and is secreted via the T3SS under conditions of low calcium. Therefore, it is intriguing to determine whether a homolog of ExsE exists in *A. hydrophila* and whether overproduction of Dam might prevent release of ExsE via the T3SS and hence reduced expression of the T3SS-secreted effectors.

In addition to T3SS-associated cytotoxicity, the biological activities associated with the T2SS-associated Act, a potent virulence factor of *A. hydrophila*, were also affected by M.AhySSUDam. Interestingly, overproduction of Dam augmented the virulence potential of Act. Both the cytotoxic and hemolytic activities associated with Act were markedly increased in the culture filtrate of the *A. hydrophila* Dam-overproducing strain compared to the control strain (Fig. 8A and B), indicating a positive effect on bacterial virulence by Dam. Indeed, *act* gene expression was increased in the Dam-overproducing strain of *A. hydrophila*, based on Northern blot analysis (data not shown) and ELISA (Fig. 8C), for the *act* transcript and Act protein, respectively. A similar pattern was noticed for proteinase production (Fig. 8D), which was upregulated in the Dam-overproducing strain compared to the control strain of *A. hydrophila*.

Although the mechanism(s) by which Dam overproduction alters gene expression is far from clear, a recent study based on the microarray analysis of different mutants has implicated SeqA protein as playing an important role in the alteration of gene expression in the Dam-overproducing *E. coli* strain (43). These investigators noted that the absence of SeqA protein (*seqA* mutant) and high DNA methyltransferase levels (Dam-overproducing strain) affected global gene expression in an almost identical manner. However, a different pattern of gene expression was noted in the dam mutant of *E. coli* (43). In addition to DNA initiation and replication, SeqA has been shown to exert its function in nucleoid organization through interaction with hemimethylated DNA (43). Similar to other chromosome structure-maintaining proteins, such as H-NS, Fis, IHF, HU, etc. (53), a global regulatory role has been proposed for SeqA (43). It has been speculated that Dam and SeqA compete for binding to hemimethylated DNA behind the replication fork. Either deletion of the *seqA* gene or overproduction of Dam increases the negative superhelicity of the chromosome (43, 87), thus facilitating open complex formation by RNA polymerase on promoters in general, which leads to redistribution of the RNA polymerases in bacteria, resulting in up- and downregulation of certain genes (37, 43).

Dam overproduction leads to attenuation of *V. cholerae* in animals (38). We observed a similar pattern of attenuation of *A. hydrophila* when animals injected with the Dam-overproducing strain did not die at a dose (2 LD50) that killed 100% of the animals infected with the *A. hydrophila* control strain.

Previous studies also indicated that Dam overproduction in *Y. pseudotuberculosis* caused the ectopic secretion of LcrV (low calcium response protein V) under conditions that are non-permissive for synthesis and secretion in the WT strain (i.e., under high-calcium and low-temperature conditions). LcrV is a *Yersinia* T3SS virulence protein involved in the expression and translocation of Yop proteins, as well as in the suppression of host inflammatory activities via interleukin-10 by activation of toll-like receptor 2 (4, 8, 39, 81). It was demonstrated in *Y. pseudotuberculosis* that the protection conferred by the Dam-overproducing strain against the WT bacterium is highly dependent on the presence of LcrV (4). Such dependence on LcrV may be due to its role as a principal immunogen and/or its role in the synthesis and localization of Yops, which may also contribute to the immunity observed in Dam-overproducing, *Yersinia*-vaccinated hosts (4). A recent study indicated that oral immunization of mice with a dam mutant of *Y. pseudotuberculosis* protected them against infection with *Y. pestis* (78).

Taken together, the overproduction of Dam in *A. hydrophila* SSU altered the expression of two key virulence factors of this bacterium, namely T3SS- and T2SS-associated Act, in addition to motility and proteinase production. Overexpression of the *dam* gene might alter the expression of virulence genes in a positive or a negative way. Perhaps this dual nature of Dam in *A. hydrophila* may be responsible for causing diseases via aberrant virulence gene expression. Although expression of the *act* gene was increased in the Dam-overproducing strain compared to the WT *A. hydrophila*, overall virulence of the bacterium appears to depend upon the interplay between the T3SS- and/or T2SS-associated Act, and possibly other factors. Finally, in our studies, we pro-
vided evidence that the Dam-overproducing strain was avirulent in mice compared to the WT bacterium. Our in vivo binding studies also indicated that bacterial attenuation due to Dam overproduction was not related to a defect in colonization.

At present it is not clear whether the decreased virulence of Dam-overproducing *A. hydrophila* is attributable to the direct increase in MTase activity or whether it occurs indirectly through other *A. hydrophila* proteins that could have been affected by increased MTase activity. Our future studies will be focused on obtaining the mutated form of Dam with no MTase activity and comparing the effects of overproduction of inactive and active Dam on *A. hydrophila* virulence. Our studies also will be aimed at delineating whether animals immunized with the Dam-overproducing strain are protected against challenge with the WT *A. hydrophila*.

ACKNOWLEDGMENTS

This work was supported by a grant from the NIH NIAID (AI41611) and from the American Water Works Association Research Foundation. L. Pillai, a predoctoral fellow, obtained funding from the NIH T32 training grant in Emerging and Tropical Infectious Diseases. A. A. Fadl was supported by the McLaughlin Postdoctoral Fellowship.

We thank M. J. Mahan (University of California, Santa Barbara) for providing the pT76 plasmid for the *E. coli dam* gene probe and M. J. Susman for editing the manuscript. All of the DNA sequencing was performed at the Protein Chemistry Core Facility, UTMB, Galveston, Tex.

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