Molecular Characterization of a Glucose-Inhibited Division Gene, *gidA*, That Regulates Cytotoxic Enterotoxin of *Aeromonas hydrophila*

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By using a mini-transposon, we obtained two mutated strains of a diarrheal isolate, SSU, of *Aeromonas hydrophila* that exhibited a 50 to 53% reduction in the hemolytic activity and 83 to 87% less cytotoxic activity associated with the cytotoxic enterotoxin (Act). Act is a potent virulence factor of *A. hydrophila* and has been shown to contribute significantly to the development of both diarrhea and septicemia in animal models. Subsequent cloning and DNA sequence analysis revealed that transposon insertion occurred at different locations in these two mutants within the same 1,890-bp open reading frame for the glucose-inhibited division gene (*gidA*). A similar reduction in hemolytic (46%) and cytotoxic (81%) activity of Act was noted in the *gidA* isogenic mutant of *A. hydrophila* that was generated by marker exchange mutagenesis. Northern blot analysis revealed that the transcription of the cytotoxic enterotoxin gene (*act*) was not altered in the *gidA* transposon and isogenic mutants. However, by generating a chromosomal *act::alkaline phosphatase* gene (*phoA*) reporter construct, we demonstrated significantly reduced phosphatase activity in these mutants, indicating the effect of glucose-inhibited division (GidA) protein in modulating *act* gene expression at the translational level. The biological effects of Act in the *gidA* mutants were restored by complementation. The virulence of the *gidA* mutants in mice was dramatically reduced compared to the those of the wild-type (WT) and complemented strains of *A. hydrophila*. The histopathological examination of lungs, in particular, indicated severe congestion, alveolar hemorrhage, and acute inflammatory infiltrate in the interstitial compartment and the alveolar spaces when mice were infected with the WT and complemented strains. Minimal-to-mild changes were noted in the lungs with the *gidA* mutants. Taken together, our data indicate for the first time that GidA regulates the most-potent virulence factor of *A. hydrophila*, Act.

Aeromonas hydrophila is an emerging human pathogen that causes septicemia and gastroenteritis (13). The organism is increasingly becoming resistant to chlorination in water and to multiple antibiotics (3, 9). As a result, the Environmental Protection Agency has placed this organism on the "Candidate Contaminant List," and the monitoring of U.S. water supplies for this organism was started in 2002. Epidemiological studies implicated *Aeromonas* species in causing food-borne outbreaks and traveler's diarrhea (13). Pathogenesis of *A. hydrophila* infections is multifactorial and complex (1, 4). Two categories of enterotoxins, namely, cytotoxic and cytotoxic, were molecularly characterized in our laboratory (12, 14–16), and we demonstrated their role in causing gastroenteritis and/or septicemia by developing isogenic mutants (52, 57). In addition to these enterotoxins, Merino et al. (34, 35) reported that many strains of *Aeromonas* have an S-layer that resists complement-mediated killing by impeding complement activation. *Aeromonas* spp. produce a wide range of proteases which could cause tissue damage and aid in establishing an infection by overcoming host defenses and providing nutrients for cell proliferation (44). Other virulence factors, such as lectins, adhesins, type IV

pili, and flagella, are also associated with the pathogenesis of *Aeromonas* infections (13). Recently, we characterized *A. hydrophila* surface-expressed enolase, a glycolytic enzyme, which bound human plasminogen and could be involved in disseminating bacteria to different organs by dissolving fibrin clots (51). Interestingly, the enolase gene was differentially expressed in vivo (51).

Among the three enterotoxins of *A. hydrophila* characterized in our laboratory (52), the cytotoxic enterotoxin (Act) is the most potent and has hemolytic, cytotoxic, and enterotoxic activities (14, 21, 57). In addition, the toxin is lethal to mice when injected intravenously, with a 50% lethal dose of 27 ng (21). Act is a single-chain polypeptide, and in its mature form the toxin is 49 to 52 kDa. The culture filtrate from an *act* isogenic mutant from *A. hydrophila* SSU was devoid of hemolytic and cytotoxic activities and evoked a minimal enterotoxic response over an observation period of 4 to 6 h. Further, the mutant was significantly attenuated in causing infection in a mouse model (57). We demonstrated that Act activated proinflammatory cytokine and eicosanoid cascades in murine macrophages and in rat intestinal epithelial cells, leading to tissue damage and a fluid secretory response (17, 49). We recently published detailed studies in which we used microarrays to examine Act-induced cell signaling, leading to the activation of cytokine and eicosanoid cascades in macrophages (24, 49). The present

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study was undertaken to identify potential regulatory gene(s) that could control *act* gene expression.

Previously, we demonstrated that Act-associated hemolytic activity in *A. hydrophila* was affected by a number of environment stimuli, such as iron, calcium, pH, temperature, and glucose (53). Subsequently, we molecularly characterized a ferric uptake regulatory gene (*fur*) that repressed *act* gene expression in the presence of high amounts of iron (53). In searching for other regulatory genes, we used transposon mutagenesis and obtained *A. hydrophila* mutants with significantly lower hemolytic and cytotoxic activities. Our subsequent cloning and sequence analysis revealed that transposition in two studied mutants occurred within the glucose-inhibited division gene (*gidA*). A similar and significant reduction in the hemolytic and cytotoxic activity was also noted in the *gidA* isogenic mutant of *A. hydrophila*. Both *gidA* transposon and isogenic mutants were avirulent in mice, with minimal to mild pathology, compared to the wild-type (WT) and complemented strains of *A. hydrophila*, which were lethal to mice and caused severe pathology in the lungs, liver, and spleen.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources of *A. hydrophila* SSU and *Escherichia coli* strains, as well as the plasmids used in this study, are listed in Table 1. For transposon mutagenesis, the mini-transposon mini-Tn5 Sm/Sp (with resistance to streptomycin [STR] and spectinomycin [SPT]) (19) was delivered into *A. hydrophila* by the pUT plasmid, which is a derivative of the pGP704 suicide vector (38). The suicide vector pJQ200SK contained a P15A origin of replication (*ori*), a levanucrase gene (*sacB*) from *Bacillus subtilis*, and a gentamicin resistance (*Gm^r*) gene (46). The pJQact*phoA* plasmid contained an in-frame fusion of the *act* gene with the reporter alkaline phosphatase gene (*phoA*) in suicide vector pJQ200SK. This plasmid was used to generate reporter *A. hydrophila* SSU strains by single-crossover recombination in the chromosome for monitoring the *act* gene expression level by measuring phosphatase activity (Table 1) (53).

Enzymes, chemicals, and recombinant DNA techniques. The antibiotics ampicillin, gentamicin, tetracycline (TET), kanamycin (KAN), SPT, and STR were used at concentrations of 100, 15, 30, 50, 50, and 50 µg/ml, respectively. Unless otherwise stated, rifampin (RIF) was used at a concentration of 100 µg/ml for bacterial growth and 300 µg/ml during conjugation experiments. All of the antibiotics used were obtained from Sigma (St. Louis, Mo.). Restriction endonucleases and T4 DNA ligase were obtained from Promega (Madison, Wis.) and New England Biolabs (Beverly, Mass.). The Advantage cDNA PCR kit was purchased from BD Bioscience Clontech (Palo Alto, Calif.). Chromosomal DNA from various *A. hydrophila* mutants was isolated using a QIAamp DNA Mini kit (Qiagen, Inc., Valencia, Calif.). The digested plasmid DNA and the DNA fragments from the agarose gel were prepared and purified using a QIAprep Miniprep kit (Qiagen). All of the basic molecular biology techniques used in this study were previously described (5, 50, 57).

Bacterial growth curves and Act production. The growth rate of WT and various *A. hydrophila* mutants over a period of the first 24 h was determined by plating (100 µl) of various dilutions (10^{-2} to 10^{-10}) of the cultures on Luria-Bertani (LB) agar plates containing appropriate antibiotics. An aliquot of the sample was taken every hour for 14 h and then every 2 h up to 24 h. After overnight incubation of the plates at 37°C, the number of CFU per milliliter of sample was calculated. The CFU count was also correlated with the optical density of the culture by measuring the A_{600} . The culture supernatants at various time points were used for examining the hemolytic activity as described later.

Transposon mutagenesis. The mini-transposon mini-Tn5 Sm/Sp, from plasmid pUT in *E. coli*, was delivered to *A. hydrophila* by conjugation as previously described (27, 48). Briefly, both *E. coli* and Rif^r *A. hydrophila* SSU were grown under static conditions at 37°C overnight. The cultures were mixed in different ratios and centrifuged at 5,000 rpm (Sorvall RC 5B plus centrifuge; Kendo Laboratory Products, Newtown, Conn.) for 10 min. The cells were resuspended in 200 µl of LB medium and transferred onto LB plates without any antibiotic pressure. After 4 h of incubation at 37°C, the cultures were removed from the plates and various dilutions (10^{-4} to 10^{-9}) of the sample were transferred to LB agar plates with RIF, SPT, and STR antibiotics. Subsequently, the transconju-

gants were spotted onto 5% sheep blood agar plates (Fisher Scientific, Pittsburgh, Pa.) for examination of hemolytic activity. The cultures were identified as *Aeromonas* by using oxidase test reagent (Difco, Sparks, Md.) to differentiate them from *E. coli* (52).

Northern blot analysis to depict *act* gene transcription in the *gidA* transposon mutants. By using an RNaseq kit (Ambion, Inc., Austin, Tex.), we isolated RNA from WT *A. hydrophila* and its *gidA* transposon and isogenic mutants. The isolated RNA samples (10 µg) were then subjected to Northern blot analysis as described by us previously (52). A 1.4-kb [α - 32 P]dCTP-labeled *act* gene that was PCR amplified from the plasmid pXHC95 (57) was used as a probe to detect the *act* transcript. The amount of RNA in each lane was quantitated by scanning 23S or 16S rRNA bands after ethidium bromide staining of the gel, using the Gel Doc 2000 System (Bio-Rad Laboratories, Hercules, Calif.).

Southern blot analysis on the chromosomal DNA of transposon mutants of *A. hydrophila* SSU with *act* gene and Sp/Sm^r gene cassette probes. An aliquot (10 µg) of the chromosomal DNA from the transposon mutants M1 and M19 (Table 1), as well as WT *A. hydrophila*, was digested with suitable enzymes and subjected to 0.8% agarose gel electrophoresis (52). Southern blot analysis was performed with the *act* and Sp/Sm^r gene probes as described in our previous studies (52, 53, 57).

Localization of the transposon insertion sites in the mutants of *A. hydrophila* SSU. Based on Southern blot analysis data obtained with the Sp/Sm^r gene probe, the *Pst*I-digested chromosomal DNA fragments in the size range of 6.4 to 6.7 kb from the transposon mutants were recovered and ligated to the pBluescript vector (Table 1). The correct transformants were screened on LB agar plates containing SPT and STR, and their identities were confirmed again by Southern blot analysis using an Sp/Sm^r gene probe. The cloned DNA fragments were further analyzed by automated DNA sequencing. The automated DNA sequencing was performed in the Protein Chemistry Core Facility at The University of Texas Medical Branch, Galveston. Based on the Blast search with the GenBank database, the interrupted open reading frames (ORFs) on the chromosome of transposon mutants M1 and M19 were located. The precise transposon insertion sites within the ORFs were determined by DNA sequence comparison between the transposon mutants and WT *A. hydrophila*.

Generation of a mutated *gidA* gene (*MgidA*) of *A. hydrophila* SSU by marker exchange mutagenesis. As shown in Fig. 1, based on the DNA sequence obtained from M1 and M19 transposon mutants, two primers (*gidA*-5 with an *Eco*RI restriction site [underlined], 5' GTGAATTCATGCAATACCATGAACAAT TTG 3' [forward], and *gidA*-3 with a *Pst*I restriction site, [underlined] 5' GTCTGCAGGTAGGATCCTCAGGCGTT 3' [reverse]) were synthesized to depict the coding region of the *gidA* gene. The *gidA* gene (1,890 bp) was PCR amplified from the chromosomal DNA of WT *A. hydrophila* and subsequently cloned into pBluescript vector at the *Eco*RI/*Pst*I sites, forming a recombinant plasmid, pBlg*idA* (Fig. 1). In the *gidA* gene coding region, there was a unique *Bgl*II restriction site; the plasmid pBlg*idA* was thus linearized with *Bgl*II digestion (Fig. 1). A 1.2-kb Km^r gene cartridge was isolated from plasmid pUC4K (Amersham) by using restriction enzyme *Bam*HI, which bordered the Km^r gene cassette. This Km^r gene cassette was ligated to plasmid pBlg*idA* at the *Bam*HI-compatible *Bgl*II site to interrupt the *gidA* gene, which generated a new recombinant plasmid, pBlg*idA*-Km (Fig. 1). Subsequently, the DNA fragment, which was now 3.2 kb, was removed from the plasmid pBlg*idA*-Km by *Xba*I/*Xho*I digestion and ligated to a suicide vector, pJQ200SK, at the *Xba*I/*Xho*I sites, forming a new recombinant plasmid, pJQ*gidA*-Km, in *E. coli* strain S17-1 (Fig. 1; Table 1). This strategy to prepare an isogenic mutant provided 358 bp and 1.5 kb of the flanking 5' and 3' DNA sequences to the mutated *gidA* gene, respectively, to permit double-crossover homologous recombination.

Complementation of the transposon mutant (M19) and *gidA* isogenic mutant (*MgidA*) of *A. hydrophila* SSU. By using specific primers (*gidA*C₅ with an *Eco*RI site [underlined], 5' GAGAATTCGTGCTTATCAACAGAAGCGG 3' [forward], and *gidA*-3 with a *Pst*I site [underlined], 5' GTCTGCAGGTAGGATCCTCAGGCGTT 3' [reverse]), a 2.1-kb DNA fragment containing the *gidA* gene and its putative promoter region was amplified from the chromosomal DNA of *A. hydrophila*. It was then ligated to vector pBR322 at the *Eco*RI/*Pst*I restriction sites to generate a recombinant plasmid pBR*gidA* (Table 1), which was first transformed into *E. coli* HB101 that carried a helper plasmid, pRK2013 (with Km^r gene) (52, 53). Subsequently via conjugation, the recombinant pBR*gidA* plasmid was transferred into the *gidA* mutants of *A. hydrophila* (M19 and *MgidA*) (Table 1). The transconjugants were screened on LB agar plates containing TET, RIF, and SPT-STR (for M19) or TET, RIF, and KAN (for *MgidA*). The presence of pBR*gidA* recombinant plasmid in M19 and *MgidA* mutants was confirmed by plasmid isolation and restriction enzyme analysis. By using the same method, pBR322 vector alone was introduced into M19 and *MgidA* mutants, along with WT *A. hydrophila* to be used as negative controls (Table 1).

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>A. hydrophila</i>		
SSU		CDC ^a
SSU-R	Rif ^r strain of <i>A. hydrophila</i> SSU	Laboratory stock
M1	<i>gidA</i> transposon mutant of <i>A. hydrophila</i> SSU-R; Rif ^r Sp/Sm ^r	This study
M19	<i>gidA</i> transposon mutant of <i>A. hydrophila</i> SSU-R; Rif ^r Sp/Sm ^r	This study
MgidA	<i>gidA</i> isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r , sucrose resistance	This study
SSU-R/pBR322	SSU-R containing pBR322 vector; Rif ^r Tc ^r	This study
M19/pBR322	<i>gidA</i> transposon mutant M19 containing pBR322 vector; Rif ^r Sp/Sm ^r Tc ^r	This study
MgidA/pBR322	<i>gidA</i> isogenic mutant containing pBR322 vector; Rif ^r Km ^r Tc ^r	This study
M19/pBRgidA	<i>gidA</i> transposon mutant M19 complemented with the <i>gidA</i> gene via pBR322 vector; Rif ^r Sp/Sm ^r Tc ^r	This study
MgidA/pBRgidA	<i>gidA</i> isogenic mutant complemented with the <i>gidA</i> gene via pBR322 vector; Rif ^r Km ^r Tc ^r	This study
SSU66	<i>A. hydrophila</i> SSU-R with chromosomally integrated <i>act::phoA</i> fusion; Rif ^r Gm ^r sucrose sensitive	This study
M19phoA	Transposon mutant M19 with chromosomally integrated <i>act::phoA</i> fusion; Rif ^r Gm ^r Sp/Sm ^r , sucrose sensitive	This study
MgidAphoA	<i>gidA</i> isogenic mutant with chromosomally integrated <i>act::phoA</i> fusion; Rif ^r Gm ^r Km ^r , sucrose sensitive	This study
SSU66/pBR322	SSU66 containing pBR322 vector; Tc ^r Rif ^r Gm ^r , sucrose sensitive	This study
M19phoA/pBR322	M19phoA containing pBR322 vector; Tc ^r Rif ^r Gm ^r Sp/Sm ^r , sucrose sensitive	This study
MgidAphoA/pBR322	MgidAphoA containing pBR322 vector; Tc ^r Rif ^r Gm ^r Km ^r , sucrose sensitive	This study
M19phoA/pBRgidA	M19phoA complemented with the <i>gidA</i> gene via pBR322; Tc ^r Rif ^r Gm ^r Sp/Sm ^r , sucrose sensitive	This study
MgidAphoA/pBRgidA	MgidAphoA complemented with the <i>gidA</i> gene via pBR322; Tc ^r Rif ^r Gm ^r Km ^r , sucrose sensitive	This study
<i>E. coli</i>		
DH5α	<i>recA gyrA</i>	Laboratory stock
S17-1	Streptomycin and trimethoprim resistance, λpir	Laboratory stock
Plasmids		
pRK2013	Helper plasmid, Km ^r	ATCC ^b
pBR322	Ap ^r Tc ^r	Amersham
pBluescript SK	Ap ^r	Stratagene
pUC-4K	Contains a 1.2-kb kanamycin ^r gene cassette	Amersham
pHP45Ω	Contains a 2.0-kb Sm/Sp ^r gene cassette	45
pJQ200SK	Suicide vector; P15A, <i>sacB</i> Gm ^r	46
Mini-Tn5 Sm/Sp	Minitransposon containing Sp/Sm ^r gene cassette delivered by the pUT plasmid	19
pJQ200actphoA	Vector pJQ200SK containing <i>act::phoA</i> fusion; Gm ^r	53
pBlueM1	pBluescript vector containing a 6.6-kb <i>Pst</i> I DNA fragment from M1, which harbored the interrupted <i>gidA</i> gene generated by mini-Tn5 transposition	This study
pBlueM19	pBluescript vector containing a 6.6-kb <i>Pst</i> I DNA fragment from M19, which harbored the interrupted <i>gidA</i> gene generated by mini-Tn5 transposition	This study
pBgldA	pBluescript vector containing coding region of <i>gidA</i> gene of <i>A. hydrophila</i>	This study
pBgldA-Km	<i>gidA</i> gene in plasmid pBluegidA interrupted at the <i>Bgl</i> II site with a Km ^r gene cassette	This study
pJQgidA-Km	Vector pJQ200SK containing a Km ^r gene cassette interrupting the <i>gidA</i> gene for generating a <i>gidA</i> isogenic mutant, MgidA	This study
pBRgidA	<i>A. hydrophila</i> <i>gidA</i> gene with its putative promoter region cloned in pBR322 at the <i>Eco</i> RI/ <i>Pst</i> I sites	This study

^a CDC, Centers for Disease Control and Prevention, Atlanta, Ga.^b ATCC, American Type Culture Collection.

Reverse transcriptase PCR (RT-PCR). Total RNA from WT *A. hydrophila* SSU, different *gidA* mutants, and their complemented strains was isolated, and an aliquot (1 µg) of the RNA was subjected to reverse transcription by using the Advantage RT for PCR kit. A negative control was set for each reverse transcription reaction by omitting RT from the reaction. A 5-µl aliquot from each of the reverse transcription reaction mixtures was used as a template for further PCRs by using the primers that were within the *gidA* gene: 5' GCCTGATCCA CATCGCATG 3' (forward) and 5'TGCCGATGCACTTGCTCTCC3' (reverse). The PCR program used was described by us previously (52).

Integration of *act::phoA* reporter gene in the chromosome of *A. hydrophila* SSU

mutants. The strategy used to integrate an *act::phoA* reporter gene into the chromosome of *A. hydrophila* was described previously (Table 1) (53). The *A. hydrophila* mutants with integration of *act::phoA* in the chromosome exhibited diffused blue color around the colonies as a result of the secretion of PhoA, which reacted with the substrate BCIP (5-bromo-4-chloro-3-indolylphosphate) (53). The identity of the genuine single-crossover mutants (e.g., MgidAphoA and M19phoA) (Table 1) was confirmed by Southern blot analysis using the *act* gene probe (53, 57). A strain that was designated SSU66 represented WT *A. hydrophila* containing the fusion reporter gene cassette in the chromosome and was generated in our previous study (53). The complementation of these reporter

FIG. 1. Flow diagram showing the strategy used for preparation of the *gidA* isogenic mutant of *A. hydrophila* SSU. By using PCR, the coding region of the *gidA* gene was amplified from the chromosomal DNA of WT *A. hydrophila* and was subsequently cloned into pBluescript vector at the *EcoRI*/*PstI* sites to generate a recombinant plasmid, pBlgidA. A Km^r gene cassette from plasmid pUC4K interrupted the *gidA* gene at the *BglII* restriction site and generated the recombinant plasmid pBlgidA-Km. The mutated *gidA* gene was removed from the pBlgidA-Km plasmid by *XhoI*/*XbaI* digestion and cloned into a suicide vector, pJQ200SK, at the compatible sites, forming a recombinant plasmid, pJQgidA-Km, for the generation of the *gidA* mutant of *A. hydrophila*. The solid bar represents the *gidA* gene, while the open bar represents the Km^r gene cassette. These plasmids are not drawn to scale. The sequences of the primers (*gidA5* and *gidA3*) used to amplify the *gidA* gene are described in Materials and Methods.

strains was performed by delivering the *gidA* gene in *trans* using a pBR322 vector (52, 53).

Alkaline phosphatase assay to quantitate the *act:phoA* fusion gene expression. The PhoA activity in the culture supernatants of the *act:phoA* reporter gene strains of *A. hydrophila* was measured as previously described (53). The density of the blue color was measured at 630 nm. The PhoA activity was calculated per milliliter of the culture supernatant per 10^8 CFU.

Measurement of biological activities. The biological activities associated with Act in the culture filtrates of various mutants, WT, and complemented strains of *A. hydrophila* were determined by hemolytic and cytotoxic assays.

(i) **Hemolytic assay.** The culture filtrates from various *Aeromonas* cultures (grown for 18 h in LB medium at 37°C with shaking [180 rpm]) were first treated with trypsin (final concentration, 0.05%) at 37°C for 1 h and then subjected to hemolytic assay as follows. A 100- μ l aliquot of phosphate-buffered saline (PBS) was added to each of the wells of a 96-well microtiter plate. Next, 100- μ l aliquots of culture filtrates were added, followed by a serial twofold dilution, with subsequent addition of 100 μ l of 2.5% rabbit erythrocytes (Colorado Serum Company, Denver, Colo.). The plate was incubated at 37°C for 1 h and observed for the lysis of red blood cells (RBCs). The supernatant was taken from those wells that showed partial lysis of rabbit erythrocytes, and the hemoglobin release was recorded at 540 nm (21). 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 the culture filtrate per 10^8 CFU. As RBCs do not produce any protease, the culture filtrates were treated with trypsin to convert all of the precursor form of Act to a mature form of the toxin. RBCs treated with trypsin alone served as a negative control, and it had no effect on the lysis of RBCs.

(ii) **Cytotoxicity assay.** The RAW264.7 murine macrophage cell line (American Type Culture Collection, Manassas, Va.) was used for the cytotoxicity assay. The cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum supplemented with penicillin-STR (Invitrogen) and were incubated at 37°C with 5% CO_2 . As stated above, the culture filtrates from various *Aeromonas* cultures were serially diluted and added to the macrophages. After 1 h of incubation, the lactate dehydrogenase release was measured at 490 nm by using the CytoTox96 nonradioactive cytotoxicity assay kit from Promega. The cytotoxic activity was calculated as the value of the lactate dehydrogenase release multiplied by the dilution of the culture supernatant. The cytotoxicity units were measured per milliliter of the culture filtrate per 10^8 CFU. For cytotoxicity assay, culture filtrates were not treated with trypsin, since furin-like protease produced by host cells could activate Act (2).

Lethality associated with *gidA* mutants of *A. hydrophila* SSU in mice and histopathology. WT *A. hydrophila*, its *gidA* transposon and isogenic mutants, and the complemented strains were grown in LB medium overnight. The cells were harvested by centrifugation and washed twice with PBS. The bacterial cells were resuspended in PBS and injected intraperitoneally into female Swiss-Webster mice weighing 20 to 25 g (Taconic Farms, Inc., Germantown, N.Y.) (five mice per group in triplicate) at a dose that was 3 logs higher (5.0×10^8 CFU) than the 50% lethal dose, and the mouse deaths were recorded for 2 weeks after challenge (57). A portion of the tissues, from, for example, the lungs, liver, and spleen was excised from mice, and the tissues were fixed in 10% buffered formalin and stained with hematoxylin-eosin in the Histology Core Facility, The University of Texas Medical Branch. The tissues were taken from mice infected with WT and complemented strains of *A. hydrophila* 24 h postchallenge, a time at which they died. For mice infected with the mutants, tissues were excised randomly from

three mice at days 1, 3, and 7 postchallenge. The remaining six mice were kept under observation for 2 weeks. Histology data at 3 days after challenge were shown; however, the pathology was very similar, irrespective of the day the mice were sacrificed.

A three-tiered scoring system (0, 1+, and 2+) was developed to evaluate the histopathology observed in the lungs, spleen, and liver. Observed differences between the variously treated groups of animals were very clear based on histologic analysis. In addition, no variability within animals in the same group was observed. Measurements of capillary septa were obtained by using an Olympus 10× ocular equipped with a 1-cm linear reticule containing 1- and 0.1-mm subdivisions. Measurements were obtained perpendicularly to the alveolar septa. Cell densities were obtained by using the same ocular equipped with square chambers for cell counting. A total of 10 noncontiguous fields were evaluated with either 25×10^{-4} -mm² (spleen and lungs) or 318×10^{-4} -mm² square chambers (for liver).

Lung scoring was as follows: 0, normal histology; 1+, nondiscernible or barely discernible capillaries in alveolar septa and/or interstitium with one to three polymorphonuclear neutrophils (PMNs) per square chamber in alveolar spaces and/or interstitium and alveolar septal thickness ranging from 2.5 to 8.0 μ m; 2+, prominent dilatation of capillaries in alveolar septa and/or interstitium with five to eight PMNs per square chamber in alveolar spaces and/or interstitium and alveolar septal thickness ranging from 7 to 13 μ m.

Spleen scoring was as follows: 0, normal histology; 1+, small germinal centers in splenic follicles (10 to 40% of total diameter) with >50% immunoblasts per total number of mononuclear cells in splenic follicles and 0 to 2 apoptotic bodies per square chamber in splenic follicles; 2+, prominent germinal center formation (>40% of total diameter) with <20% immunoblasts per total number of mononuclear cells in splenic follicles and 3 to 10 apoptotic bodies per square chamber in splenic follicles.

Liver scoring was as follows: 0, normal histology; 1+, 0 to 3 inflammatory cells per square chamber in hepatic sinusoids and no sinusoidal congestion; 2+, 10 to 30 inflammatory cells per square chamber in hepatic sinusoids.

The liver and spleen tissue specimens were also homogenized and transferred to LB agar plates containing RIF (300 μ g/ml) to monitor bacterial load in the tissues at days 1, 3, and 7 postinfection. With the WT and complemented strains of *A. hydrophila*, tissues were excised 1 day after infection (time of their death). Data for mutants were obtained 3 days postinfection; however, no bacteria were found in these tissues at days 1, 3, and 7 postinfection. The data were expressed as the number of bacteria recovered per 100 mg of liver and spleen tissues.

Statistical analysis. Wherever appropriate, the data were analyzed using Student's *t* test, and *P* values of ≤ 0.05 were considered significant.

Nucleotide sequence accession number. The complete DNA sequence of the cloned chromosomal DNA fragment (4,574 bp) without the mini-Tn5 was submitted to GenBank under accession number of AY333759.

RESULTS

Transposon mutants of *A. hydrophila* SSU. Approximately 5,000 transposon mutants of *A. hydrophila* were initially screened on 5% sheep blood agar plates for hemolytic activity, and a total of 24 colonies which exhibited reduced or no Act production were picked up. These 24 isolates were further tested for hemolytic activity in the culture filtrates, and a total of 20 mutants out of the 24 were confirmed to have no hemolytic activity. Southern blot analysis on the chromosomal DNA of these 20 transposon mutants with the *act* gene probe revealed that transposition occurred within the *act* structure gene. In the remaining four mutants, one contained free plasmid, while in another the transposon insertion occurred at more than one site on the chromosome, based on Southern blot analysis with the Sp/Sm^r gene cassette probe. Thus, these mutants were not the ideal candidates for further study.

Hemolytic activity was measured in culture filtrates, cell lysates, and membrane fractions of the remaining two transposon mutants (designated M1 and M19). Minimal or no hemolytic activity was detected in the cell lysates and membrane fractions (data not shown), and a 50 to 53% reduction in hemolytic activity and an 83 to 87% reduction in cytotoxic

TABLE 2. Hemolytic and cytotoxic activities of WT *A. hydrophila* SSU, *gidA* mutants, and their complemented strains

<i>Aeromonas</i> strain tested	Mean activity $\pm$ SD ^a (U/ml/10 ⁸ CFU)	
	Hemolytic	Cytotoxic
WT <i>A. hydrophila</i>	83.8 $\pm$ 3.2	1226.7 $\pm$ 36.8
M1	39.2 $\pm$ 4.0 ^b	214.4 $\pm$ 18.3 ^b
M19	42.2 $\pm$ 3.1 ^b	154.9 $\pm$ 9.9 ^b
<i>MgidA</i>	45.6 $\pm$ 0.3 ^b	229.4 $\pm$ 33.8 ^b
WT <i>A. hydrophila</i> /pBR322	82.3 $\pm$ 5.2	1015.4 $\pm$ 68.9
M19/pBR322	42.1 $\pm$ 2.1 ^b	241.4 $\pm$ 12.2 ^b
M19/pBR <i>gidA</i>	81.8 $\pm$ 2.7	867.4 $\pm$ 35.4
<i>MgidA</i> /pBR322	46.9 $\pm$ 2.3 ^b	326.5 $\pm$ 31.2 ^b
<i>MgidA</i> /pBR <i>gidA</i>	85.0 $\pm$ 3.5	1129.5 $\pm$ 49.1

^a Three independent experiments were performed.

^b Statistically significant value (*P* $\leq$ 0.05) compared to WT *A. hydrophila* SSU using Student's *t* test.

activity were observed in the culture filtrates of these mutants compared to those of the WT *A. hydrophila* (Table 2). These results indicated that transposition did not alter the export machinery in these two mutants. To rule out the possibility that transposition caused delayed toxin production, WT *A. hydrophila* and its transposon mutants M1 and M19 were grown for 96 h. At 4-h intervals, a sample was removed, and the CFU count (for measuring bacterial growth) and the hemolytic activity (by measuring hemoglobin release at 540 nm) in the culture filtrates were monitored. The hemolytic and cytotoxic activities were first detected at 6 to 8 h and reached a maximum at 18 h in both of the mutants, which was similar to those recorded for the parental *A. hydrophila* strain. It was noted that the growth of transposon mutants was 10% slower than that of WT *A. hydrophila* during the first 3 to 4 h; however, no difference in the CFU count was noted at the subsequent time points.

Cloning of the *gidA* gene from *A. hydrophila* SSU. Based on Southern blot analysis, a 6.6-kb DNA fragment was detected in the *Pst*I-digested chromosomal DNA of transposon mutant M19 when an Sp/Sm^r gene cassette was used as a probe (data not shown). This DNA fragment was cloned into pBluescript vector to generate a recombinant plasmid, pBlueM19 (Table 1). DNA sequence analysis revealed that the 6.6-kb DNA fragment contained a complete sequence of the *gidA* gene, which was interrupted by Tn5. Based on the entire *gidA* gene sequence, two primers, *gidA*-5 and *gidA*-3 (described in Materials and Methods), were used to amplify the native *gidA* gene from the chromosomal DNA of WT *A. hydrophila*. The native *gidA* gene was subsequently cloned into pBluescript vector at *Eco*RI/*Pst*I sites.

The DNA sequence of the *gidA* gene revealed that it contained 1,890 bp and had 68 and 74% homology at the DNA and amino acid levels, respectively, to the *gidA* gene recently isolated from the plant pathogen *Pseudomonas syringae* (29), while our studies were in progress. The homology of the *gidA* gene between *A. hydrophila* and *E. coli* was 68% at the DNA level and 71% at the amino acid level. By DNA sequence comparison between the native *gidA* gene with that of the interrupted version present on the 6.6-kb DNA fragment in pBlueM19 plasmid, the exact insertion site for transposition

FIG. 2. Schematic diagram showing the *gidA* region of *A. hydrophila* SSU and insertion sites in the *gidA* mutants. The *gidA* gene (long arrow) of *A. hydrophila* was located near the origin of the replication site (*oriC*), and three ORFs downstream of *gidA* included *gidB*, *soj*, and part of the *spoOJ* gene (short arrows). Based on the 4,574-bp DNA fragment that was sequenced, the transposition in mutants M1 and M19 occurred in the *gidA* gene at bp 2552 and 1954, respectively. The *gidA* isogenic mutant had an insertion, with the *Km^r* gene cassette at bp 1113 of the *gidA* gene. The figure is not drawn to scale.

was determined to be at bp 1954 within the *gidA* gene ORF (bp 759 to 2648) (Fig. 2).

By using a similar strategy, the transposon insertion in the M1 mutant was determined to be within the *gidA* gene. In the M1 mutant, transposition occurred at bp 2552 (Fig. 2). Other genes detected on the 6.6-kb *Pst*I fragment of transposon mutants included *gidB*, *soj*, and *spoOJ*, based on sequence comparison with other bacterial genome sequences (Fig. 2). The function of these genes includes chromosome replication and partition and cell division (25, 40). Since transposition in both the mutants (M1 and M19) occurred within the *gidA* gene, subsequent detailed studies were performed only with the M19 mutant.

Characterization of *gidA* isogenic mutants (MgidA) of *A. hydrophila* SSU. The strategy used to develop MgidA is depicted in Fig. 1. Conjugation of WT *A. hydrophila* with *E. coli* S17-1 harboring pJQgidA-Km plasmid (Table 1) with the interrupted *gidA* gene should result in transconjugants that were resistant to RIF, KAN, and 5% sucrose but sensitive to gentamicin. Such mutants should have undergone genuine, double-crossover homologous recombination, resulting in the replacement of the native *gidA* gene with an insertionally inactivated version of *gidA*, and with concomitant loss of the suicide vector harboring *Gm^r* and *sacB* genes.

To confirm the correct genotype of the isogenic mutants, the chromosomal DNA from WT *A. hydrophila* and MgidA mutants was isolated and subjected to PCR and Southern blot analysis. For PCR analysis, the *gidA* gene primers *gidA*-5 and *gidA*-3 were used. These primers detected a 1.9-kb DNA fragment from the chromosome of WT *A. hydrophila*, which represented the correct size of the *gidA* gene. However, the size of the *gidA* gene was larger by 1.2 kb in the isogenic mutants, due to the insertion of the *Km^r* gene cassette. The PCR data were confirmed by performing Southern blot analysis.

As depicted in Fig. 3, the size of the chromosomal DNA fragment from WT *A. hydrophila* digested with *Pst*I restriction enzyme was 4.6 kb when a *gidA*-specific gene probe was used for Southern blot analysis (panel I, lane 1). However, in the MgidA mutants, the *Pst*I restriction enzyme digestion divided this 4.6-kb DNA into two fragments of 1.1 and 3.5 kb (Fig. 3, panel I, lanes 2 and 3), due to the insertion of a *Km^r* gene

FIG. 3. Confirmation of the identity of the *gidA* isogenic mutants of *A. hydrophila* SSU based on Southern blot analysis. Chromosomal DNA from WT *A. hydrophila* (lane 1) and isogenic mutants (MgidA) (lanes 2 and 3) was digested with *Pst*I restriction enzyme. As a positive control, a purified *Km^r* gene cassette was loaded in lane 4 of panel II, while an *Xba*I/*Xho*I-digested suicide vector, pJQ200SK, was used in lane 4 of panel III. Three different probes were used. In panel I, the probe was a *gidA* gene from *A. hydrophila*. In panel II, a 1.2-kb *Km^r* gene cassette was employed as the probe, while in panel III, plasmid pJQ200SK was used for hybridization. The two bands in panel I, lanes 2 and 3, reflect fragments containing the *gidA* gene. These fragments were generated by the *Pst*I restriction enzyme digestion as a result of the insertion of a *Km^r* gene cassette within the *gidA* gene that introduced two additional *Pst*I sites (Fig. 1).

cassette, which contained a *Pst*I site at both ends (Fig. 1). A 1.2-kb DNA fragment was detected in the digested chromosomal DNA of the isogenic mutants when the *Km^r* gene cassette was used as a probe (Fig. 3, panel II, lanes 2 and 3). As expected, this probe did not react with the digested chromosomal DNA from the WT *A. hydrophila* (panel II, lane 1). Lane 4 in panel II contained a purified 1.2-kb *Km^r* gene cassette that was used as a probe and served as a positive control. No band was detected in the digested chromosomal DNA of both mutants (Fig. 3, panel III, lanes 2 and 3) and WT *A. hydrophila* (Fig. 3, panel III, lane 1) when suicide vector pJQ200SK was used as a probe. The probe reacted with the pJQ200SK plasmid digested with *Xba*I/*Xho*I restriction enzymes and served as a positive control (Fig. 3, panel III, lane 4). These data indicated that the MgidA mutants had completely lost the suicide vector sequences as a result of double-crossover homologous recombination. Compared with the WT *A. hydrophila*, the selected MgidA mutant exhibited 46 and 81% reduction in hemolytic and cytotoxic activities, respectively (Table 2). The growth rate of the MgidA was similar to that of transposon mutants, with no difference in the CFU count at 18 h, the time point that was used for measuring the biological activities. The growth of the mutant was slower in the first 3 to 4 h by 10% compared to the WT *A. hydrophila*; however, no toxin was synthesized during this time by both the mutant and the WT *A. hydrophila*. No difference in the CFU count was noted after the initial 3 to 4 h of growth.

As the *gidA* gene was shown to be involved in the cell division of *E. coli* (55), morphological examination of the MgidA mutant by Gram staining showed that the bacterial cells were filamentous (Fig. 4B) compared to the cocco-bacillus nature of parental *A. hydrophila* when grown in a medium containing 0.2% glucose (Fig. 4A). A similar pattern was noticed in the *gidA* transposon mutants (data not shown).

Restoration of biological activities of transposon mutant M19 and MgidA isogenic mutant by complementation. The

FIG. 4. Morphology of *A. hydrophila* mutants as determined by Gram staining. (A) WT *A. hydrophila*. A typical cocco-bacillus nature of the cells was noted. (B) *MgidA* mutant; the cells were longer. (C) Complemented *MgidA* strain; a typical morphology of the cells was restored. The bacterial cells were grown in NY medium (N-Z amine [8 g], yeast extract [4 g], NaCl [5 g], distilled H₂O to 1 liter) containing 0.2% glucose.

native *gidA* gene was supplied to the M19 and *MgidA* mutant via plasmid vector pBR322. As shown in Table 2, the hemolytic activity in transposon mutant M19 and isogenic mutant *MgidA* with pBR322 vector was 49 and 43% lower compared to that of WT *A. hydrophila* containing vector pBR322. However, the hemolytic activity was fully restored in the complemented strain M19, with a hemolytic titer of 81.8 ± 2.7 U/ml/ 10^8 CFU (results here and elsewhere are presented as arithmetic means $\pm$ standard deviations). Likewise, the complemented *MgidA* mutant restored the hemolytic titer to 85.0 ± 3.5 U/ml/ 10^8 CFU (Table 2). WT *A. hydrophila* with the pBR322 vector alone had a hemolytic titer of 82.3 ± 5.2 U/ml/ 10^8 CFU. A similar pattern was seen with the cytotoxic activity as well, with cytotoxicity titers of $1,015.4 \pm 68.9$, 241.4 ± 12.2 , and 867.4 ± 35.4 U/ml/ 10^8 CFU in WT *A. hydrophila*, M19, and the complemented strain of the M19 transposon mutant. The cytotoxicity titers for WT *A. hydrophila*, *MgidA*, and its complemented strain were $1,015.4 \pm 68.9$, 326.5 ± 31.2 , and $1,129.5 \pm 49.1$ U/ml/ 10^8 CFU (Table 2). Further, the cocco-bacillus nature of *A. hydrophila* was restored after complementation (Fig. 4C).

Detection of the *gidA* gene transcription in WT *A. hydrophila* SSU and its various mutants. Total RNA from WT *A. hydrophila*, *gidA* mutants and their complemented strains was subjected to cDNA synthesis, and the synthesized cDNA was used for PCR using two specific primers (5' GCCTGATCCA CATCGGCATG 3' [forward] and 5' TGCCGATGCACTTG CTCTCC 3' [reverse]) generated within the *gidA* gene of *A. hydrophila*. As shown in Fig. 5, a 609-bp DNA fragment, which represented a portion of the *gidA* gene transcript, was amplified from WT *A. hydrophila* (lanes 2 and 5) and the complemented mutants M19 and *MgidA* (lanes 7 and 9). The fragment was missing from the *gidA* mutants M19 (lanes 3 and 6) and *MgidA* (lanes 4 and 8). The same-size band was amplified from the chromosomal DNA of WT *A. hydrophila* and served as a positive control (lane 10). No band was amplified in any of these strains when RT was omitted from the reverse transcription reaction mixture. This control ensured that the PCR amplification was occurring only from the *gidA* gene mRNA, and not from the chromosomal DNA (data not shown).

Evaluation of *act* gene expression level in *gidA* mutants of *A. hydrophila* SSU. The isolated RNA from WT *A. hydrophila*, the

M1, M19, and *MgidA* mutants, and their proper control strains was subjected to Northern blot analysis with the *act* gene-specific probe. We noticed no significant difference in the *act* gene transcription level between the *gidA* mutants and the WT of *A. hydrophila* (Fig. 6, panel I). To evaluate whether the effect of the *gidA* gene was at the translational level of Act, several reporter gene constructs with proper control strains were constructed and tested (Table 1). The amount of PhoA secreted from these strains reflected the level of Act produced (56). As shown in Fig. 6, panel II, PhoA activity was decreased in the *gidA* mutants compared to that of WT *A. hydrophila*. PhoA activity was restored after complementation. These data indicated that translation of the *act* gene was affected in the *gidA* mutants.

The *gidA* gene mutants of *A. hydrophila* SSU were avirulent in a mouse model. WT *A. hydrophila* and the M19 and *MgidA*

FIG. 5. RT-PCR depicting transcript of the *gidA* gene in various *A. hydrophila* mutants. Total RNA was isolated from different cultures of *A. hydrophila*. A hexamer primer was used for the cDNA synthesis. Subsequently, PCR was performed by using a pair of primers designed within the *gidA* gene, as stated in Materials and Methods. An aliquot (5 μ l) of the PCR product from each of the cDNA reactions was loaded onto a 0.8% agarose gel for electrophoresis. Lane 1, λ DNA digested with *Hind*III marker; lanes 2 and 5, WT *A. hydrophila*; lanes 3 and 6, transposon mutant M19; lanes 4 and 8, *MgidA* mutant; lane 7, complemented M19 mutant; lane 9, complemented *MgidA* mutant. Chromosomal DNA from the WT of *A. hydrophila* was used as a template for the PCR and served as a positive control (lane 10). Abbreviations: compl, complementation; c, control.

FIG. 6. Translation of the *act* gene was affected in *gidA*-deficient mutants of *A. hydrophila* SSU based on Northern blot analysis and production of PhoA from the *act::phoA* reporter gene constructs. In panel I, total RNA from different cultures of *A. hydrophila* was isolated and subjected to Northern blot analysis. Lanes 1, RNA from WT *A. hydrophila*; lanes 2 and 3, RNA from *gidA* transposon mutants M1 and M19; lanes 4, RNA from the *gidA* isogenic mutant (*MgidA*). (A) The probe used was a 1.4-kb *act* gene. (B) The RNA loaded in each lane was quantitated by scanning 16S and 23S rRNA bands after ethidium bromide staining of the gel. In panel II, the PhoA activity was measured to represent the translation of the *act* mRNA in various *A. hydrophila* strains containing the *act::phoA* reporter gene construct. The arithmetic mean values and standard deviations (error bars) were plotted. The unit of measurement for the y axis is PhoA activity per milliliter per 10^8 CFU. Column 1, WT *A. hydrophila*; column 2, M19 transposon mutant; column 3, isogenic mutant *MgidA*; column 4, complemented (comp) M19 transposon mutant; column 5, complemented *MgidA* mutant. An asterisk denotes statistically significant difference ($P \leq 0.05$) compared to WT *A. hydrophila* using Student's *t* test.

mutants with their complemented strains were tested for the ability to induce death in mice. In the WT *A. hydrophila* group, all of the animals died within 24 h. In the transposon mutant M19 and *MgidA* isogenic mutant group of mice, all of the mice survived up to a tested period of 2 weeks. Complementation restored the virulence, and all mice died within 24 h, as noted for WT *A. hydrophila* (data not shown). The lungs, liver, and spleen were excised from mice in each group for further analysis. Figure 7 shows histopathological findings in the lungs 3 days postinfection with the *gidA* mutants and 1 day after infection with the WT and complemented strains of *A. hydrophila*. However, the pathology was very similar on days 1 and 7 postinfection with the *gidA* mutants as seen on day 3.

Severe vascular congestion and acute inflammatory infiltrates (score, 2+) composed of mostly PMNs in the alveolar spaces and interstitium were seen in the animals infected with WT and complemented strains (Fig. 7B, D, and F). The ani-

mals infected with the *MgidA* mutant exhibited mild histopathology that consisted of mostly mild inflammatory infiltrates (score, 1+) in the interstitium of the lungs (Fig. 7E). Lung histologic sections from mice infected with the M19 mutant (Fig. 7C) were virtually identical to those of the uninfected animals (score, 0) (Fig. 7A).

The histopathologic findings in the liver and spleen were also evaluated. In animals infected with the mutant strains, the liver did not show any histologic abnormalities except for the presence of scattered mononuclear inflammatory cells in the hepatic sinusoids (score, 1+). Spleen sections from these animals revealed follicular hyperplasia with germinal center formation and >50% immunoblasts; occasional apoptotic lymphocytes (tingible body macrophages) were also observed (score, 1+). In contrast, animals infected with the WT and complemented strains revealed prominent inflammatory infiltrates composed of mononuclear cells and neutrophils in the portal triads and sinusoidal spaces in the liver (score, 2+). Sections of spleen revealed marked follicular hyperplasia with large germinal centers formation, <20% of immunoblasts in the germinal centers, and abundant apoptotic bodies within the splenic follicles (score, 2+). Scattered neutrophils were seen in the splenic sinusoids. Overall, the differences were clearly evident between the animals infected with either the WT strain or the complemented strains versus the animals infected with the mutant strains.

The number of bacteria recovered from the livers and spleens of the mice infected with WT *A. hydrophila* and the complemented strains at the time of death (24 h) was in the range of 10^7 to 10^8 CFU (Fig. 8). No bacteria were recovered from the tissues of animals infected with *gidA* mutants on day 1, 3, and 7 postinfection. These data indicated that systemic infection occurred in mice infected with WT *A. hydrophila* and the complemented strains. However, the *gidA* mutants failed in infected mice to cause systemic infection because of their rapid clearance from the host system.

DISCUSSION

In this study, a Tn5-derived mini-transposon was used to generate insertion mutants in *A. hydrophila* (19). Among the 24 genuine transposon mutants obtained, 2 mutants (M1 and M19) had reduced hemolytic (50 to 53%) and cytotoxic (83 to 87%) activities with an intact *act* gene in their genomes (Table 2). Transposition in both of these mutants occurred in the same ORF that encoded the GidA protein consisting of 630 amino acid residues (Fig. 2).

Since transposon mutants could lead to polar effects, a *gidA* isogenic mutant was prepared, which similarly exhibited significantly reduced hemolytic (46%) and cytotoxic (81%) activities, and these effects could be fully restored in both the isogenic and transposon mutants after complementation (Table 2). It was noticed that reduction in cytotoxic activity in the mutants was much more pronounced than the effect on hemolytic activity compared to WT *A. hydrophila*. This possibly could be due to the sensitivity of the assays used.

As shown in Fig. 4, the *gidA* isogenic mutant of *A. hydrophila* showed filamentous morphology (Fig. 4B) compared to WT *A. hydrophila* (Fig. 4A). We noticed that this observed phenotype caused the optical density of the mutant culture at 600 nm to

FIG. 7. Lung sections of mice infected with various mutants of *A. hydrophila*. All sections were stained with hematoxylin and eosin. (A) Uninfected mouse showing normal lung histology. (B) Mouse infected with WT *A. hydrophila* (24 h postinfection). Note marked infiltration of alveolar spaces and interstitium by PMNs (inset). Severe vascular congestion was also observed. (C) Mouse infected with the M19 transposon mutant (3 days postinfection). The histology was virtually identical to that of the uninfected mouse. (D) Mouse infected with the complemented M19 mutant. Note marked infiltration of interstitium and alveolar spaces by PMNs (inset) as shown in panel B. (E) Mouse infected with the *MgidA* mutant (3 days postinfection). Minimal to mild inflammatory infiltrates composed mostly of PMNs in the interstitial areas (inset) were noted. Alveolar spaces were free of infiltrates. (F) Mouse infected with the *MgidA*-complemented strain. Note marked infiltration of alveolar spaces and interstitium as depicted in panel B. All panels and insets are shown at magnifications of $\times 100$ and $\times 400$, respectively.

appear less compared to that of WT *A. hydrophila* due to differences in the light-scattering properties of normal and filamentous cells.

We therefore determined the growth rates of the WT and the mutant cultures by counting CFU rather than measuring the absorbance of the culture. We noted no difference in the CFU count between the *gidA* mutants and WT *A. hydrophila*

after 3 to 4 h of growth. During this early growth period, the mutants grew 10% more slowly and no toxin production occurred. Toxin in both the WT and the mutants was first detected at around 6 to 8 h, and became maximal at 18 h, with a parallel increase in the bacterial number for both the mutants and WT *A. hydrophila*. Taken together, these data indicated a direct role of *GidA* in regulating Act production and not a

FIG. 8. Systemic infection by various strains of *A. hydrophila* in mice. As stated in Materials and Methods, the bacteria recovered from the liver and spleen tissue specimens (1 day for the WT and complemented strains and 3 days postinfection for the mutants) were plated and expressed as the number of recovered bacteria per 100 mg of tissue. Column 1, WT *A. hydrophila*; column 2, *gidA* transposon mutant M19; column 3, isogenic mutant *MgidA*; column 4, complemented (compl) M19 mutant; column 5, complemented *MgidA* mutant. The gray bar represents the number of bacteria recovered from the spleen, while the black bar represents bacteria recovered from the liver. The arithmetic mean $\pm$ standard deviation (error bar) was plotted.

pleiotropic effect of reduced bacterial growth leading to low toxin production.

The function of GidA was initially thought to be involved in chromosome replication and cell division. This was based on several pieces of evidence. First, disruption of the *gidA* gene in *E. coli* affected cell division when cells were grown in a medium containing glucose (55). Second, the *gidA* gene is located in close proximity to the *oriC* (26, 30, 41, 42, 58), and initiation of chromosomal replication is still inhibited by RIF after a point in the replication process that requires protein synthesis, indicating that a transcriptional event might be involved (29, 31, 36). Third, in the synchronous cell studies, the activity of the *gidA* promoter was turned off after the chromosomal replication initiation events were accomplished (8, 43, 54). However, recent studies implicated GidA in a number of biological and pathogenic processes, as it is a widely distributed and conserved protein with multiple functions (56).

We previously showed that addition of glucose in the medium repressed Act production by *A. hydrophila* (53). Since in the *gidA* mutants of *Aeromonas* Act production was reduced, we performed an experiment in which Act production by the *gidA* mutants was evaluated in the presence and absence of glucose. The premise of our study was whether there is a link between regulation of Act production by glucose and GidA. Our initial study indicated that the addition of glucose (0.1 to 0.2%) in the medium still suppressed Act production, as measured by the hemolytic activity assay, in the GidA mutants of *A. hydrophila*. These data suggested that a mechanism(s) in ad-

dition to GidA was responsible for reduced Act production in the presence of glucose. These studies will be pursued in the future.

GidA-like protein is widely distributed in nature (29, 56). It is conserved among eubacteria and has been classified into two groups, based on the size of the protein. The larger protein (GidA_L) has 611 to 679 amino acid residues, and the smaller protein (GidA_S), which is truncated at the carboxyl-terminal end compared to GidA_L, contains 435 to 482 amino acid residues (56). The GidA of *A. hydrophila* belongs to the longer version (GidA_L) of the GidA family of proteins, which is usually located near the replication origin (*oriC*) of the chromosome (Fig. 2), whereas the chromosomal positioning of the gene encoding GidA_S is variable (56). Some eubacteria, such as *Myxococcus xanthus*, *B. subtilis*, *Aquifex aeolicus*, and *Thermotoga maritima* carry both versions of GidA (56). The GidA_S shares 24% identity with the GidA_L in *M. xanthus* (56).

White et al. (56) reported that GidA is a flavin adenine dinucleotide (FAD)-binding protein involved in development of *M. xanthus*. The *gidA* knockout mutant of *M. xanthus* was unstable, and after a few generations, was converted into a strain that was no longer able to form fruiting bodies (56). Although these investigators focused on the smaller version of GidA in *M. xanthus*, both larger and smaller forms of GidA from different bacteria revealed a conserved dinucleotide-binding motif at the N terminus. The distribution of GidA_S in *M. xanthus* varied, based on different growth conditions (56). GidA_S was found in both cytoplasm and periplasm when *M. xanthus* was vegetatively grown, while starved cells contained only the cytoplasmic GidA_S. Further studies revealed that the cytoplasmic GidA_S was associated with the inner membrane and was transported to the periplasm (56). FAD proteins involved in oxidation-reduction reactions required for energy production are associated with the membrane or located in the periplasm. The FAD moiety of GidA and conservation of the FAD-binding site among all GidA proteins suggest that GidA might act as a sensor for the redox state of cells similar to the flavoproteins, e.g., aerotaxis signal transducer in *E. coli* (56).

In another study, Karita et al. (28) noted that the *gidA* gene was closely located near the *dapE* gene (which encodes succinyl-diaminopimelate desuccinylase) and that the *gidA* gene was essential for the cell viability of *Helicobacter pylori*. These investigators were unable to obtain a *gidA*-deficient mutant while providing evidence that insertion mutagenesis indeed occurred within the target gene (28). By using transposon mutagenesis, Duwat et al. (20) generated transposon mutants of *Lactococcus lactis*, which were sensitive to mitomycin and UV light. In one of these mutants, the transposition occurred in the *gidA* gene, and the capacity of this mutant to mediate homologous recombination was affected (20). Lidholm and Gustafsson (32) reported that the presence of the *gidA* gene on the chloroplast genomes of conifers was important for the ability of these plants to synthesize chlorophyll in the dark (23, 32). GidA was also found as a mitochondrial protein in *Saccharomyces cerevisiae*. Mutations in an *MT01* gene (which codes for a GidA homolog) were pleiotropic on the expression of several mitochondrial genes in yeast (18). Most recently, while our studies were in progress, Kinscherf and Willis (29) reported GidA as a global regulator in the plant pathogen *P. syringae*. Mutation in the *gidA* gene affected diverse phenotypic traits, such as li-

podepsipeptide antibiotic production, swarming, presence of fluorescent pigment, and virulence. In *A. hydrophila*, we have now shown that disruption in the *gidA* gene reduced the hemolytic and cytotoxic activities associated with Act. Further, the *gidA* mutants were avirulent in mice (Table 2; Fig. 8). However, unlike in *H. pylori*, mutation in the *gidA* gene was not lethal to *P. syringae* and *A. hydrophila*.

The mechanism of how GidA regulates gene expression is not clear. One study demonstrated that the *gidA* gene has a function similar to that of *miaA* (which encodes aminoacyl tRNA synthetase), a gene involved in tRNA modification that stabilizes codon-anticodon interactions (39). In another study, inactivation of either the *gidA* or the *mmE* gene (allele of *trmE* gene that encodes GTP binding protein) in *E. coli* greatly increased the occurrence of a two-base frameshift during the translation of particular sites in mRNA (10). Since the *mmE* gene product is known to be involved in the modification of some tRNAs (11, 29), a similar role was proposed for the *gidA* gene. Detailed studies showed that GidA up regulated the expression of *salA* (which codes for a putative DNA-binding protein) and that of *sydB* (which encodes syringomycin) genes at the translational level in *P. syringae* (29). In our study, we also noted that GidA affected the translation of Act, as the transcription of the *act* gene remained unchanged in the *gidA* mutants (Fig. 6). A role has been suggested for *gidA* in moderating translation fidelity, and this may explain the striking absence of *gidA* analogs in *Archaea*, since it had different ribosomes and tRNAs (7, 29).

Despite the conservation of the *gidA* gene among different bacteria, the phenotypes of different *gidA* mutants do not point to a common mechanism for GidA function in these organisms. Moreover, a detailed understanding of GidA is complicated by the fact that homologs of GidA exist in two sizes and are transcribed from genes that are not linked genetically (56). In addition to size, other differences have also been noticed between these two versions of GidA, such as distribution in the cells and the export machinery. GidA_L was found only in the periplasm fraction of *M. xanthus* cells, while GidA_S was found in cytoplasm as well as in the periplasm. An SRR motif was found in the N terminus of the small GidA protein of *M. xanthus*, a twin-arginine translocation-like (Tat) system (6) that was suggested for GidA_S export (56). While the GidA_L lacks the (S/T)RR motif, it may be exported through a different pathway. These differences might reflect various roles of different versions of GidA.

Microbial infection represents interactions between the host and pathogen, in which the microbe uses its strategies for survival and multiplication by combating defenses of the immune system of the host (33). One such microbial strategy is to use the regulatory system by which bacteria modulate their genes, as well as genes of the host, during infection. There are reports of such regulators in the literature, e.g., the *phoP-phoQ* regulatory system in *Salmonella enterica* serovar Typhimurium. This system controls expression of several bacterial genes, such as genes involved in macrophage survival (22, 37). Likewise, the accessory gene regulator (*agr*) in *Staphylococcus aureus* controls expression of alpha-hemolysin (*hla*) and staphylococcal enterotoxin C (*sec*) genes (47).

In this study, we have identified for the first time a regulatory gene (*gidA*) from an enteric pathogen, *A. hydrophila*, that reg-

ulates expression of a crucial virulence gene *act*. The biological activity of Act was decreased in both the *gidA* transposon and isogenic mutants and was restored after complementation. Most importantly, these *gidA* mutants were avirulent in the mice. Our future studies will be targeted at defining whether *gidA* regulates other virulence factors produced by *A. hydrophila* and the specific interaction that occurs between Act and GidA. GidA could be an important regulatory molecule, controlling virulence-associated factors of other enteric pathogens.

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