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Role of a Cytotoxic Enterotoxin in *Aeromonas*-Mediated Infections: Development of Transposon and Isogenic Mutants

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Transposon and marker exchange mutagenesis were used to evaluate the role of *Aeromonas* cytotoxic enterotoxin (Act) in the pathogenesis of diarrheal diseases and deep wound infections. The transposon mutants were generated by random insertion of Tn*5-751* in the chromosomal DNA of a diarrheal isolate SSU of *Aeromonas hydrophila*. Some of the transposon mutants had dramatically reduced hemolytic and cytotoxic activities, and such mutants exhibited reduced virulence in mice compared to wild-type *Aeromonas* when injected intraperitoneally (i.p.). Southern blot data indicated that transposition in these mutants did not occur within the cytotoxic enterotoxin gene (*act*). The transcription of the *act* gene was affected drastically in the transposon mutants, as revealed by Northern blot analysis. The altered virulence of these transposon mutants was confirmed by developing isogenic mutants of the wild-type *Aeromonas* by using a suicide vector. In these mutants, the truncated *act* gene was integrated in place of a functionally active *act* gene. The culture filtrates from isogenic mutants were devoid of hemolytic, cytotoxic, and enterotoxic activities associated with Act. These filtrates caused no damage to mouse small intestinal epithelium, as determined by electron microscopy, whereas culture filtrates from wild-type *Aeromonas* caused complete destruction of the microvilli. The 50% lethal dose of these mutants in mice was 1.0×10^8 when injected i.p., compared to 3.0×10^5 for the wild-type *Aeromonas*. Reintegration of the native *act* gene in place of the truncated toxin gene in isogenic mutants resulted in complete restoration of Act's biological activity and virulence in mice. The animals injected with a sublethal dose of wild-type *Aeromonas* or the revertant, but not the isogenic mutant, had circulating toxin-specific neutralizing antibodies. Taken together, these studies clearly established a role for Act in the pathogenesis of *Aeromonas*-mediated infections.

Aeromonas species, which have recently been placed in a new family, *Aeromonadaceae*, are responsible for causing a variety of human infections, including septicemia, wound infections, meningitis, pneumonia, and gastroenteritis (5). Among various virulence factors produced by *Aeromonas* species, enterotoxins are by far the most important in causing *Aeromonas*-mediated infections (1, 13, 28, 32). The cytotoxic enterotoxin gene (*act*) from the human diarrheal isolate SSU of *Aeromonas hydrophila* has been cloned, sequenced, and hyperexpressed in our laboratory (14). Four biological activities, namely, hemolytic, cytotoxic, and enterotoxic activities as well as lethality, have been shown in mice to be associated with cytotoxic enterotoxin (Act) (39). Act is a single-chain polypeptide with an estimated molecular mass of 52 kDa (40). The toxin protein is secreted as an inactive precursor (54 kDa), which is converted into the active form by proteolytic processing near the C terminus (14). Act is an aerolysin-related toxin which exhibited approximately 90% homology with an aerolysin from a fish isolate of *Aeromonas bestiarum* (previously designated *A. hydrophila*) (1, 23, 24). In contrast, comparison of Act with an aerolysin from a diarrheal isolate of *Aeromonas trota* revealed approximately 75% homology (1, 9, 26). Recently, an aerolysin-related toxin also was isolated from a gram-positive organism, *Clostridium septicum* (7).

We identified regions on Act involved in the biological functions of the toxin by deletion analysis, generation of antipeptide antibodies, and site-directed mutagenesis (16). Our data indicated that although Act had significant homology with aerolysin, there are enough differences that differential folding of these two protein molecules could occur (16, 17, 19). Further, our data suggested that there may be different loci coding for specific biological activities of Act.

Mechanism-of-action studies revealed that Act operated by creating pores, estimated to be 1.14 to 2.8 nm in diameter, in the erythrocyte membranes (17). The toxin appeared to undergo aggregation when preincubated with cholesterol, which resulted in a loss of Act's hemolytic activity (17), indicating cholesterol to be one of the receptors for Act (17). Recently, Nelson et al. (34) reported that Thy-1, a major surface glycoprotein of T lymphocytes, is a high-affinity receptor for aerolysin from *A. bestiarum*.

In the present study, we have constructed transposon and isogenic mutants that were defective in the production of Act from wild-type *A. hydrophila* SSU to determine Act's precise role in the overall virulence of *Aeromonas*. These mutants not only were devoid of Act-associated biological activities but were significantly less virulent in mice than wild-type *Aeromonas*, proving unequivocally the role of Act in *Aeromonas*-mediated infections.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. hydrophila* SSU, a diarrheal isolate, was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. The identity of this culture as *A. hydrophila* was confirmed by DNA-DNA hybridization and ribotyping (5). Isolate A52 of an *Aeromonas* species was provided by M. Kai, Tokai University, Kanagawa, Japan. A strain of *Escherichia coli* harboring plasmid pME9 with transposon Tn*5-751* was obtained from S. P. Howard, University of Regina, Regina, Saskatchewan, Canada. The transposon Tn*5-751* had

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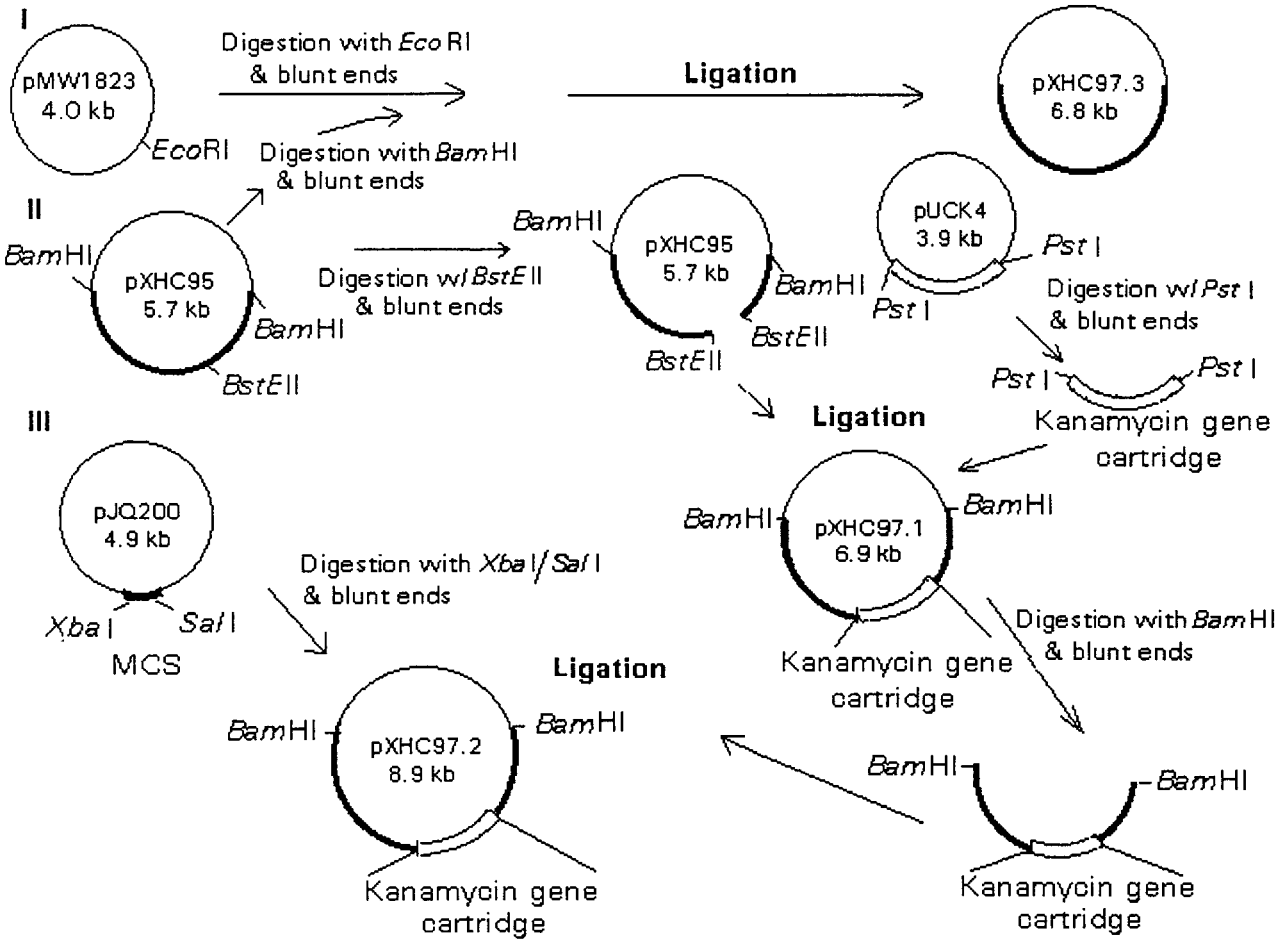

FIG. 1. Flow diagram showing construction of various recombinant plasmids. Recombinant plasmid pXHC95 contained a 2.8-kb *Bam*HI DNA fragment from chromosomal DNA of *A. hydrophila* SSU with the *act* gene (II). A kanamycin resistance gene cartridge from plasmid pUCK4 was introduced within the *act* gene to generate recombinant plasmid pXHC97.1 before ligation of the truncated *act* gene in the suicide vector pJQ200 to generate plasmid pXHC97.2 (III). A 2.8-kb *Bam*HI DNA fragment containing the *act* gene also was subcloned in another suicide vector, pMW1823, to generate plasmid pXHC97.3 (I) for the purpose of generating a revertant of *A. hydrophila* with parental biological activity of Act. MCS, multiple cloning site.

two antibiotic resistance genes coding for kanamycin and trimethoprim. Rifampin- and streptomycin-resistant spontaneous mutants of *A. hydrophila* were prepared during these studies. Suicide vector pJQ200KS, which contained a P15A origin of replication, a *sacB* gene from *Bacillus subtilis*, and a gentamicin resistance gene, was obtained from M. K. Hynes, The University of Calgary, Calgary, Alberta, Canada (36). *E. coli* S17-1, with streptomycin and trimethoprim resistance and lysogenized with λ*pir* (20, 36), was from S. J. Libby, North Carolina State University, Raleigh, N.C. Plasmid pMW1823, another suicide vector, with a chloramphenicol resistance gene from pACYC184, an origin of replication from plasmid pSC101, and the *mob* region from plasmid pJM703.1, was provided to us by V. L. Miller, Washington University School of Medicine, St. Louis, Mo. Plasmid pXHC95 contained a 2.8-kb *Bam*HI DNA fragment from *A. hydrophila* chromosomal DNA and harbored the *act* gene. This plasmid had an ampicillin resistance gene and was propagated in *E. coli* XL1-Blue cells. Plasmid pUC4K contained a 1.2-kb kanamycin resistance gene cassette, which represented a portion of the transposon Tn*903* (Pharmacia Biotech Inc., Piscataway, N.J.). The *E. coli* clones with recombinant plasmids, as well as *Aeromonas* cultures, were stored in Luria-Bertani (LB) medium containing 25% (vol/vol) glycerol at −70°C. The concentrations of antibiotics used to grow cultures were as follows: 50 μg of ampicillin per ml, 40 μg of rifampin per ml for transposon mutants and 300 μg of rifampin per ml for isogenic mutants, 25 μg of streptomycin per ml, 25 μg of trimethoprim per ml, 50 μg of kanamycin per ml, 15 μg of gentamicin per ml, and 20 μg of chloramphenicol per ml.

Transposon mutagenesis. The transposon Tn*5-751* from plasmid pME9 in *E. coli* was delivered to *Aeromonas* by conjugation as previously described (20, 38). Briefly, both *E. coli*(pME9) and streptomycin-resistant *A. hydrophila* SSU were grown under static conditions at 37°C overnight. The cultures were mixed (5 ml each) at a concentration of 8×10^6 cells/ml, centrifuged (4,000 × *g* for 10 min), resuspended in 200 μl of LB medium, and plated on LB plates without any antibiotic pressure. After 4 h of incubation at 37°C, the culture was removed from the plate and various dilutions (10^{-4} to 10^{-9}) of the sample were plated on LB plates with streptomycin, kanamycin, and trimethoprim. The cultures were identified as *Aeromonas* by a positive oxidase test to differentiate them from *E. coli* and by an automated identification system (Vitech, St. Louis, Mo.) in the Clinical Microbiology Laboratory, The University of Texas Medical Branch, Galveston. Further, dot blot hybridization (6) was utilized to differentiate toxin-bearing *Aeromonas* from nontoxigenic *E. coli* used in the conjugation experiment. The denatured total DNA samples were applied to a nitrocellulose membrane under vacuum in a dot blot apparatus (Bio-Rad, Hercules, Calif.). The filters were dried and baked at 80°C for 2 h. The blots were prehybridized and hybridized by using Quikhyb (Stratagene, La Jolla, Calif.) at 68°C as described by the manufacturer. The probes used included a 1.4-kb DNA fragment containing the full-length *act* gene and a 439-bp DNA fragment representing the 5′ end of the toxin gene (14) and were labeled with [α-^{32}P]dCTP (ICN, Irvine, Calif.) by using a random primer kit (GibcoBRL, Gaithersburg, Md.). The filters were washed at 68°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0) plus 0.1% sodium dodecyl sulfate (SDS) for 1 h and then in 1× SSC plus 0.1% SDS for 30 min at 68°C. The blots were exposed to the X-ray film at −70°C for 2 to 12 h.

The biological activity of Act in the culture filtrates, cell lysates, and cell debris of these transposon mutants was evaluated by hemolytic and cytotoxic assays (39). The cell lysates were prepared by resuspending the cells in phosphate-buffered saline (PBS) in the original culture volume, and the cells were sonicated (Sonifier cell disruptor 185; Branson Sonic Power Co., Danbury, Conn.). The mixture was centrifuged at 10,000 × *g* for 15 min at 4°C to separate cell lysate from cell debris, which then was resuspended in the original culture volume.

Construction of isogenic mutants of *Aeromonas* via double-crossover recombination. Recombinant plasmid pXHC95 (14) was used to construct isogenic

mutants (Fig. 1). The *Bst*EII restriction enzyme was used to digest this plasmid and to linearize it, since there was only one *Bst*EII site within the 2.8-kb *Bam*HI DNA insert (Fig. 1). There were no *Bst*EII sites in the pBluescript expression vector used to construct recombinant plasmid pXHC95. The ends of the 2.8-kb DNA fragment were made blunt by using a PCR polishing kit (Stratagene). Subsequently, a 1.2-kb kanamycin gene cartridge was isolated from plasmid pUCK4 by restriction with enzyme *Pst*I, which bordered the kanamycin gene cassette (Fig. 1). An appropriate-sized DNA fragment was excised from a 0.8% agarose gel, extracted with phenol-chloroform, ethanol precipitated (6), and finally purified with a GeneClean II kit (Bio 101, Vista, Calif.). The ends of the kanamycin gene cassette were made blunt and ligated to the blunt-ended 2.8-kb *Bst*EII DNA fragment by using T4 DNA ligase (Promega, Madison, Wis.).

The new recombinant plasmid was designated pXHC97.1 (Fig. 1) and transformed into *E. coli* XL1-Blue cells by electroporation (Cell-Porator Electroporation System I; GibcoBRL). The transformants were identified on LB agar plates containing ampicillin and kanamycin, and their identity was confirmed by miniplasmid isolation and digestion of the recombinant plasmid with *Bam*HI restriction enzyme (14). Fragments of 2.9 and 4.0 kb were visualized and represented, respectively, pBluescript vector DNA and a 2.8-kb *act* gene-containing DNA fragment truncated with a 1.2-kb kanamycin gene cassette (total fragment size, 4.0 kb). Further, PCR was performed to demonstrate the kanamycin gene cartridge and the *act*-specific gene sequences within the 4.0-kb DNA fragment. The two primers used to amplify the kanamycin gene cassette sequence were 5′-CGCTGAGGTCTGCTCGTGAAGAAGGTGTT-3′ (representing bp 434 to 464) and 5′-AAAGCCACGTTGTGTCTCAAAATCTCTGATGT-3′ (representing bp 1613 to 1645) from the pUCK4 plasmid. The primer sequences which amplified the *act* gene were 5′-ATAGAGTCTAGACTCCATGCAAAAACTA AAAAAACTGGCTTGT-3′ (bp 884 to 911) and 5′-CATCCTGTCGACTAAG CTTTTATTGATTGGCTGCTGGCGTCACG-3′ (bp 2341 to 2365) (14). The underlined bases represented *Xba*I and *Sal*I restriction sites, respectively. The deoxyoligonucleotides were synthesized by Biosynthesis, Inc., Lewisville, Tex. A Geneamp reagent kit with AmpliTaq DNA polymerase was used for PCR, as described by the manufacturer (Perkin-Elmer Cetus, Norwalk, Conn.). The sequence of the PCR product was verified by DNA sequence analysis with a Sequenase PCR sequencing kit (Amersham Life Sciences, Cleveland, Ohio).

This strategy to prepare isogenic mutants provided 1.9 and 0.9 kb of the flanking 5′ and 3′ DNA sequences, respectively, for double crossover. The biological activities (hemolytic and cytotoxic) of truncated (with the kanamycin resistance gene cassette) and native Act, with the *act* gene cloned in pBluescript vector under the control of a T7 promoter, were examined in *E. coli* (Fig. 1). The cultures were induced for 4 h at 37°C with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Subsequently, the recombinant plasmid pXHC97.1 was isolated from *E. coli* XL1-Blue cells by using a kit from Qiagen (Chatsworth, Calif.) and digested with the *Bam*HI restriction enzyme. A 4.0-kb DNA fragment, which encompassed the *act* gene truncated with a kanamycin resistance gene cassette, was isolated, purified, and ligated to the blunt-ended suicide vector pJQ200KS, which was digested originally with the *Xba*I and *Sal*I restriction enzymes. The newly constructed recombinant suicide vector was designated pXHC97.2 and transformed into *E. coli* S17-1 (Fig. 1). The transformants were identified on the LB agar plates supplemented with streptomycin, trimethoprim, ampicillin, kanamycin, and gentamicin, and the identity of cultures with the correct recombinant plasmid was verified by PCR with the specific primers described above.

Conjugation between *A. hydrophila* and *E. coli*. The recombinant *E. coli* S17-1(pXHC97.2) (Fig. 1) was conjugated with rifampin-resistant *Aeromonas*, as described previously for development of transposon mutants (20, 38), and the transconjugants were selected on LB agar plates with rifampin, kanamycin, and gentamicin to select for single-crossover transconjugants or with rifampin, kanamycin, and 5% sucrose to select for double-crossover transconjugants. The integration of the recombinant suicide vector into the chromosome of *Aeromonas* was confirmed by Southern blot analysis.

The probes used for Southern analysis included *act* gene-specific sequences (as described above), the 4.9-kb plasmid pJQ200KS, and a 1.2-kb kanamycin resistance gene cassette. The filters were hybridized and washed as described for dot blot hybridization.

Northern blot analysis. Total RNA was isolated from various transposon mutants and wild-type *A. hydrophila* by using the total RNA isolation kit from Qiagen. The RNA samples (6 μg) were subjected to electrophoresis on a 1.2% formaldehyde–agarose gel with 1× MOPS buffer (0.2 M MOPS [morpholinepropanesulfonic acid] [pH 7.0], 0.05 M sodium acetate, 0.01 M EDTA, pH 8.0) (6). The RNA was transferred to a nylon membrane (GibcoBRL), and after baking, the filters were prehybridized, hybridized, and washed as described for dot blot analysis. The amount of RNA in each lane was quantitated by densitometer scanning (Applied Imaging, Pittsburgh, Pa.) of 23S and 16S rRNA bands after ethidium bromide staining of the gel. All of the reagents used for Northern blot analysis were treated with diethylpyrocarbonate.

Measurement of biological activities. The biological activity of Act was determined by cytotoxic, hemolytic, and enterotoxic assays.

(i) Cytotoxic assay. The culture filtrates, cell lysates, and cell membranes from various *Aeromonas* cultures (grown for 18 h in LB medium at 37°C) were diluted twofold with PBS and added to Chinese hamster ovary (CHO) cells. After 18 to 20 h of incubation at 37°C in the presence of 5% CO_2, the cytotoxic activity was recorded. The cytotoxic unit was defined as the reciprocal of the highest dilution of the toxin demonstrating 50% destruction of CHO cells (39).

TABLE 1. Hemolytic and cytotoxic activities of Act produced by wild-type and transposon mutants of *A. hydrophila* SSU[a]

Culture	Hemolytic activity in:			Cytotoxic activity in:		
	Culture filtrates	Cell lysates	Membranes	Culture filtrates	Cell lysates	Membranes
Wild-type SSU	128	16	4	4,096	16	4
Transposon mutants						
42	16	0	0	4	0	0
225	32	0	0	32	0	0
312	32	0	0	64	0	0
325	32	0	0	8	0	0
353	16	0	0	32	0	0

[a] Hemolytic and cytotoxic activities were defined as the reciprocal of the highest dilution of the toxin that caused 50% lysis of erythrocytes or 50% destruction of CHO cells, respectively. All cultures were grown overnight with shaking (150 rpm) at 37°C in LB medium. The cells were harvested by centrifugation, and the culture filtrates, cell lysates, and membranes were saved for measuring the toxin activity.

(ii) Hemolytic assay. A volume of 100 μl of PBS was added to each of the wells of a 96-well microtiter plate. Next, 100 μl of twofold-diluted toxin preparation was added, followed by 100 μl of 2% rabbit erythrocytes (39). The plate was incubated at 37°C for 1 h and observed for hemolytic activity. The hemolytic unit was defined as the reciprocal dilution of Act demonstrating 50% lysis of rabbit erythrocytes. To demonstrate that the residual cytotoxic and hemolytic activities in various transposon mutants were indeed due to Act, antibodies specific for Act were used to neutralize Act's biological effects. The toxin preparations were mixed with antibodies and incubated at 37°C for 1 h before being placed on the erythrocytes and CHO cells. The ability of these antibodies to neutralize the biological activity of Act then was evaluated. Preimmune serum was used as a negative control in neutralization experiments.

(iii) Enterotoxic assay. Outbred Swiss-Webster mice (25 to 30 g; Taconic Farms, Inc., Germantown, N.Y.) were anesthetized with halothane (River Edge, N.J.). An abdominal incision was made, and a single 5-cm loop was constructed, as previously described with 00 silk suture (35). A 100-μl test sample was injected into the loop. After 6 h of observation, the animals were euthanized by cervical dislocation, and the intestinal loops were removed. The amount of luminal fluid was measured and expressed as microliters per centimeter.

LD_{50} determination. The wild-type *A. hydrophila*, its transposon and isogenic mutants, and the revertant were grown in LB medium and centrifuged, and the cells were washed twice with PBS. The bacterial cells were resuspended in PBS and injected intraperitoneally (i.p.) into Swiss-Webster mice (6 to 10 mice per group) at various doses (10^4 to 10^{10} CFU), and the mice were observed for death. The 50% lethal dose (LD_{50}) was determined by the method of Reed and Muench (37).

Electron microscopy. The mouse intestinal loops injected with culture filtrates from wild-type *A. hydrophila* and its isogenic mutants were removed after 6 h for electron microscopic studies. Small pieces (1 mm) of small intestine were fixed (17), and ultrathin sections were cut with a Sorvall MT-6000 ultramicrotome (RMC, Tucson, Ariz.) and stained with uranyl acetate and lead citrate. The sections were examined and photographed in a model 201 electron microscope (Phillips Electron Optics, Eindhoven, The Netherlands) at 60 kV.

SDS-polyacrylamide gel electrophoresis and Western blot analysis. Culture filtrates from *A. hydrophila* and its mutants were analyzed by SDS–10 to 12% polyacrylamide gel electrophoresis (30) and Western blot analysis (Bio-Rad) with toxin-specific antibodies developed in mice or rabbits. Goat anti-mouse or goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase or horseradish peroxidase was used as the secondary antibody (diluted 1:3,000). The blots were developed with either an alkaline phosphatase substrate kit (Bio-Rad) or an enhanced chemiluminescence substrate (Pierce, Rockford, Ill.).

RESULTS

Transposon mutagenesis of chromosomal DNA of *A. hydrophila* SSU. Approximately 4,000 transposon mutants of *A. hydrophila* were screened for biological activity. Culture filtrates from five mutants displayed drastically reduced biological activities, as measured by hemolytic and cytotoxic assays (Table 1). All of these mutants were confirmed to be *Aeromonas*. We also performed dot blot hybridization of the total

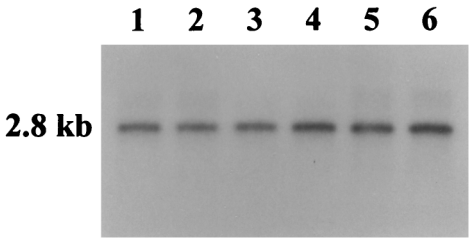

FIG. 2. Southern blot analysis of the genomic DNAs from wild-type and transposon mutants of *A. hydrophila*. The genomic DNA (15 μg) was digested with the *Sal*I and *Bam*HI restriction enzymes and subjected to Southern blot analysis. The probe used was a 1.4-kb *Xba*I/*Sal*I DNA fragment (^{32}P labeled), which depicts the coding region of the *act* gene. The blot was prehybridized, hybridized (5×10^6 cpm/ml), and washed as described in Materials and Methods. The lanes contained digested DNAs from the transposon mutants 353 (lane 1), 325 (lane 2), 312 (lane 3), 225 (lane 4), and 42 (lane 5) and from wild-type *A. hydrophila* SSU (lane 6) as a positive control.

DNAs from wild-type *A. hydrophila* and its transposon mutants by using a 439-bp *act*-specific gene probe. The DNAs from these cultures exhibited a positive signal, while total DNA from *E. coli* did not hybridize with this probe. It was crucial to differentiate *Aeromonas* from *E. coli* used in the conjugation before proceeding further, because *Aeromonas* and *E. coli* exhibited very similar biochemical profiles. We indeed obtained streptomycin-, kanamycin-, and trimethoprim-resistant colonies with no hemolytic activity, but these were identified as *E. coli*. The frequency at which these colonies appeared was relatively high (25 to 30%). We also confirmed that the streptomycin-resistant spontaneous mutants of *Aeromonas* used in the conjugation experiment exhibited biological activity similar to that of wild-type *Aeromonas*.

Southern blot analysis was performed by using plasmid pME9 with Tn*5-751* as a probe to demonstrate that transposition indeed occurred in the chromosomal DNA of *A. hydrophila*. Our data indicated the presence of a single copy of the transposon in the digested (*Sal*I-*Bam*HI) chromosomal DNAs of all five mutants of *A. hydrophila* tested. Digested genomic DNAs from *E. coli* and wild-type *A. hydrophila* did not react with this probe (data not shown). Similar genomic digests also were probed with the *act*-specific gene probe in Southern blots (Fig. 2). Interestingly, a 2.8-kb DNA fragment hybridized with this probe, irrespective of whether the chromosomal DNA was isolated from wild-type *A. hydrophila* or its transposon mutants (Fig. 2). These data implied that transposition might have occurred in some other region (e.g., a regulatory element) and not within the structural gene coding for the toxin.

The biological activity of Act in culture filtrates, cell lysates, and membranes of these transposon mutants was measured. Minimal or no toxin activity was detected in the cell lysates and membrane fractions, and reduced biological activity was observed in culture filtrates of these mutants (Table 1). These results indicated that transposition did not alter the export machinery of *Aeromonas*. To rule out the possibility that transposition caused delayed toxin production, both wild-type *A. hydrophila* SSU and its transposon mutants were grown for 96 h. Every 4 h, a culture sample was removed and total viable counts were determined. The supernatants, cell lysates, and cell membranes were examined for hemolytic and cytotoxic activities. Wild-type *Aeromonas* demonstrated the highest hemolytic and cytotoxic activities at 18 h. The transposon mutants similarly exhibited the highest, albeit significantly reduced, biological activity at 18 h, although the mutants had viable counts similar to those of wild-type *Aeromonas*. After this time point, no further increase in hemolytic and cytotoxic activities was noted for the mutant cultures. Coincident with these data was the reduced amount of Act antigen on Western blots in transposon mutants compared to wild-type *Aeromonas* (data not shown). By using specific polyclonal antibodies to Act, it was demonstrated that the residual biological activity in these transposon mutants was contributed by Act, since both the remaining hemolytic and cytotoxic activities of Act were abolished by Act-specific antibodies.

Based on the Southern blot data in Fig. 2, we performed Northern blot analysis on the total RNA isolated from wild-type *A. hydrophila* SSU and its transposon mutants to examine the expression of the toxin gene. A weak transcript or no transcript was detected in the transposon mutants (Fig. 3, lanes 1 to 5), whereas a transcript of approximately 1.4 kb was detected in wild-type *A. hydrophila* (Fig. 3, lane 6). The data in Fig. 3 and Table 1 demonstrated that the transposon mutants synthesized Act, albeit at low levels.

Lethality studies with the Act-deficient *Aeromonas* strain A52 and transposon mutants of *A. hydrophila* SSU. Isolate A52 of *Aeromonas* is naturally deficient in the *act* gene, as confirmed by Southern analysis and biological activity measurements. Both *A. hydrophila* SSU (Act positive) and *Aeromonas* strain A52 (Act negative) were injected i.p. into mice to demonstrate the role of Act in *Aeromonas*-mediated infections. The LD_{50} of *Aeromonas* strain A52 was calculated to be 3.9×10^9. However, the LD_{50} of wild-type *A. hydrophila* SSU was 2.5×10^7, indicating Act's role in the organism's virulence. The difference in the LD_{50}s between these two cultures was statistically significant ($P = 0.01$) by the Fisher exact test. The sera from animals that survived the challenge with *A. hydrophila* SSU contained toxin-specific antibodies; however, the sera from animals challenged with *Aeromonas* strain A52 were devoid of toxin-specific antibodies as determined by Western blot analysis (data not shown).

Wild-type *Aeromonas* isolate SSU and all of the five transposon mutants with reduced hemolytic and cytotoxic activities (Table 1) were injected i.p. into mice to validate the data obtained with a toxin-deficient strain of *Aeromonas*. For these studies, we selected only one dose of bacteria (5×10^7). All mice injected with wild-type *Aeromonas* died within 6 to 24 h. Pure cultures of *A. hydrophila* could be isolated from the spleens and livers of the dead animals. However, none of the mice injected with the transposon mutants at this dose died over a 2-week observation period (data not shown).

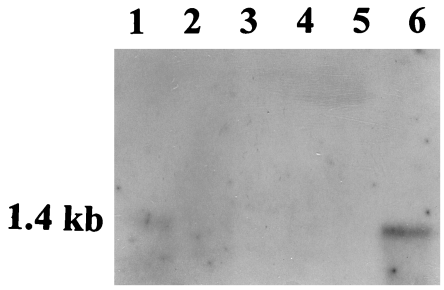

FIG. 3. Northern blot analysis of the total RNAs isolated from the wild-type and transposon mutants of *A. hydrophila*. The total RNA was isolated by using a total RNA isolation kit (Qiagen). A 439-bp ^{32}P-labeled DNA fragment, which represented the 5′ end of the toxin structural gene, was used as a probe in these blots. The blot was prehybridized, hybridized, and washed as described in Materials and Methods. The blots were exposed to X-ray film at −70°C for 12 h. The lanes contained RNAs from mutant cultures 353 (lane 1), 325 (lane 2), 312 (lane 3), 225 (lane 4), and 42 (lane 5) and from wild-type *A. hydrophila* SSU (lane 6).

TABLE 2. Biological activities of Act produced in culture filtrates of *A. hydrophila* SSU and its isogenic mutant and revertant

Culture	Hemolytic activity[a]	Cytotoxic activity[a]	Enterotoxic activity (μl/cm)[b]
Wild-type *A. hydrophila* with native Act	1:512	1:2,048	118 ± 21
E. coli with suicide vector pJQ200[c] containing a truncated *act* gene	0	0	ND[d]
Double-crossover mutant of *A. hydrophila* with a truncated *act* gene	0	0	0
Revertant strain of *A. hydrophila* in which the truncated *act* gene was replaced with the native *act* gene	1:512	1:2,048	112 ± 12

[a] The hemolytic and cytotoxic activities were defined as the reciprocal of the highest dilution of the toxin causing 50% lysis of erythrocytes or 50% destruction of CHO cells, respectively.

[b] Enterotoxic activity was determined in mouse ligated intestinal loops. Results are means ± standard deviations.

[c] Suicide vector pJQ200 gives a gentamicin-resistant and sucrose-sensitive phenotype to the cultures.

[d] ND, not done.

Characterization of double-crossover mutants of *Aeromonas* with an altered *act* gene. The strategy used to develop an isogenic mutant of *Aeromonas* is depicted in Fig. 1. The single-crossover transconjugants obtained after conjugation of wild-type, rifampin-resistant *A. hydrophila* SSU with *E. coli* S17-1 harboring plasmid pXHC97.2 did not grow in the presence of 5% sucrose, as it induces the *sacB* gene, coding for levan sucrase, which is lethal to cells when produced in large amounts. The hemolytic activity of these single-crossover mutants was similar to that of wild-type *Aeromonas*.

The colonies which grew in the presence of sucrose should represent genuine double-crossover mutants, since the suicide vector sequences containing *sacB* and gentamicin resistance genes should be lost as a result of the second crossover. Those colonies which grew in the presence of sucrose but were sensitive to gentamicin were chosen for further studies. The genuine double-crossover mutants with no hemolytic activity on 5% sheep blood agar plates were obtained at a frequency of 0.01%. These mutants were grown in LB medium for 18 h, and the culture filtrates, cell lysates, and membranes were examined for hemolytic and cytotoxic activities. Table 2 shows that the double-crossover mutants had no biological activity (e.g., hemolytic, cytotoxic, and enterotoxic activities) compared to wild-type *A. hydrophila*. Western blot analysis also did not demonstrate any protein band corresponding to Act in the culture filtrates of double-crossover mutants. A band corresponding to 52 kDa was detected in the culture filtrates of wild-type *A. hydrophila* and single-crossover mutants (data not shown).

By electron microscopy, extensive tissue damage was found in the ligated small intestine injected with the culture filtrates from wild-type *Aeromonas* (Fig. 4A). The fluid accumulation response in a group of 10 mice was 118 ± 21 μl/cm (mean ± standard deviation) (Table 2). No tissue damage or fluid secretion was observed when the loops were challenged with culture filtrates from double-crossover mutants (Fig. 4B). The enterocytes had intact microvilli and a normal appearance.

Southern blot analysis of the chromosomal DNAs of *A. hydrophila* SSU and its isogenic mutants. Southern blot analysis of the genomic DNAs of wild-type *Aeromonas* and its mutants was performed to confirm the identity of isogenic mutants. It is evident from Fig. 5A, lane 6, that a 2.8-kb chromosomal DNA fragment reacted with the *act* gene probe in wild-type *Aeromonas*. Total DNA from a single-crossover transconjugant showed bands at 9.3 and at 2.8 kb (Fig. 5A, lane 5). The band at 2.8 kb represented the native *act* gene of *Aeromonas*, whereas the band at 9.3 kb contained a truncated *act* gene and the suicide vector. A fragment of 4.0 kb reacted with the *act* gene probe in the digested DNAs of the double-crossover mutants (Fig. 5A, lanes 3 and 4), instead of a 2.8-kb fragment observed with the wild-type *A. hydrophila* (Fig. 5A, lane 6). The size shift was due to insertion of a kanamycin resistance gene cassette in the *act* gene. When similar DNA samples were probed with a kanamycin gene cassette (Fig. 5B), only the double-crossover and single-crossover mutants showed signals at 4.0 and 9.3 kb, respectively (Fig. 5B, lanes 1 to 4). Genomic DNA from wild-type *Aeromonas* did not react with this probe (Fig. 5B, lanes 5 and 6). When plasmid pJQ200KS was used as a gene probe, only the digested DNAs from single-crossover mutants reacted (Fig. 5C, lanes 3 and 4). As predicted, no band was detected in the digested DNAs from double-crossover mutants, indicating loss of the suicide vector sequences (Fig. 5C, lanes 1 and 2). Likewise, genomic DNA from wild-type *A. hydrophila* did not react with this probe (Fig. 5C, lanes 5 and 6).

Reintegration of the native *act* gene in the double-crossover mutants with an inactive *act* gene. The truncated *act* gene in the double-crossover mutant was replaced with the functionally active *act* gene by homologous recombination. To perform this experiment, the 2.8-kb *Bam*HI DNA fragment from plasmid pXHC95 was made blunt ended and ligated to the blunt-ended suicide vector pMW1823 at the *Eco*RI restriction site (Fig. 1). The recombinant plasmid pXHC97.3 was transformed into *E. coli* S17-1. After the identity of chloramphenicol-resistant recombinant clones was confirmed, the plasmid pXHC97.3 was transferred from *E. coli* into an isogenic mutant of *Aeromonas* (rifampin resistant) which contained the truncated *act* gene. Colonies resistant to rifampin were inoculated on a blood agar plate and observed for a surrounding zone of hemolysis after overnight incubation at 37°C. *Aeromonas* revertants exhibiting hemolytic activity were obtained at a frequency of 0.05%. The hemolytic activities in the culture filtrates of wild-type and revertant strains of *Aeromonas* were identical, indicating that the revertant had regained the biological activity of Act (Table 2). All of the *Aeromonas* mutants exhibited similar growth rates in synthetic M-9 medium (data not shown).

Different doses of the wild-type *A. hydrophila* SSU, its isogenic mutant, and the revertant were injected i.p. into mice, which were observed for death over a 1-week period. The LD_{50} of wild-type *Aeromonas* and the revertant was 3.0×10^5, whereas the LD_{50} of the isogenic mutant was 1.0×10^8 ($P = 0.01$ by the Fisher exact test). The LD_{50} of wild-type *Aeromonas* was lower than that obtained earlier (2.5×10^7), because all of the cultures were passed through animals twice before lethality studies were performed. The cultures were injected into mouse ligated small intestine, and after 6 h, blood was drawn from the heart and spread on blood agar plates. Organisms recovered from the blood after the second passage were used in the lethality studies and were found to be more virulent than those subcultured on the synthetic medium.

The animals which survived the bacterial challenge (with wild-type *Aeromonas* or its mutants) were bled after 14 days, and the toxin-specific antibodies in the sera were examined by Western blot analysis. In an immunoblot in which pure Act was probed with sera from animals injected with either wild-type *Aeromonas* or its revertant, a band of 52 kDa was visualized,

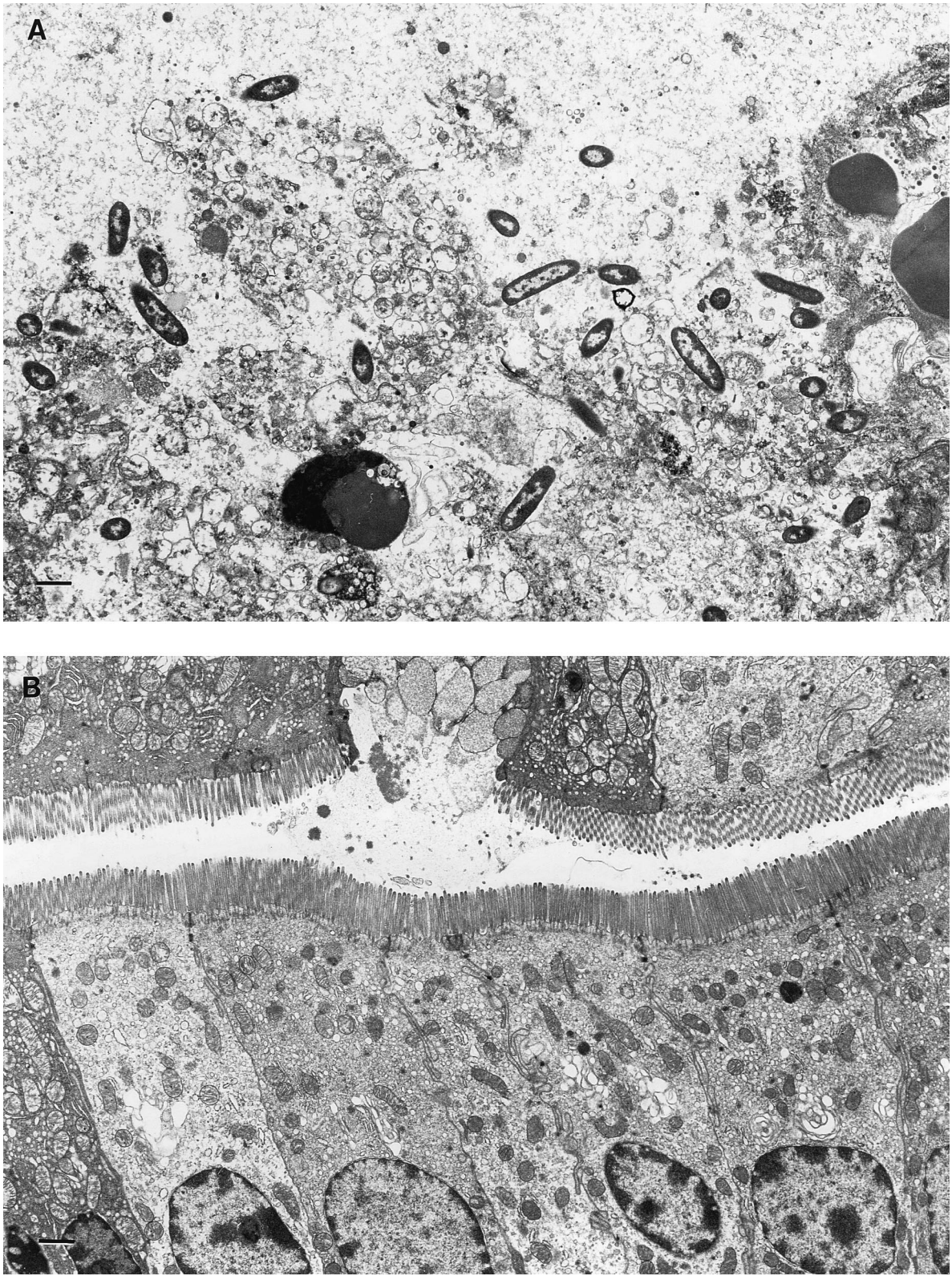

FIG. 4. Electron microscopy of intestinal tissues of mice injected with culture filtrates from wild-type *Aeromonas* and its isogenic mutant. Mouse ligated loops were placed in fixative and cut into 1-mm pieces. Ultrathin sections were stained and photographed in a Philips 201 electron microscope. (A) After administration of Act (contained in culture filtrate) from wild-type *Aeromonas*, enterocytes were completely destroyed. (B) Mouse loops challenged with culture filtrate from a double-crossover mutant. Normal enterocytes with intact brush borders surround the lumen. Mucus is being emptied from a goblet cell into the lumen in the upper layer of cells. Bars, 1 μm.

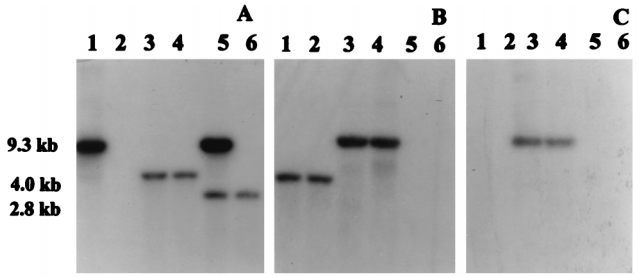

FIG. 5. Southern blot analysis of the chromosomal DNAs from *A. hydrophila* SSU and its isogenic mutants. Total DNAs (15 μg) from *A. hydrophila* and its mutants were digested with the *Bam*HI restriction enzyme and subjected to Southern blot analysis. The probes used were a 439-bp *Xba*I/*Sal*I DNA fragment, which depicts part of the coding region of the *act* gene (A), a 1.2-kb kanamycin resistance gene cassette (B), and a 4.9-kb pJQ200 suicide vector (C). The blots were probed and washed as described in Materials and Methods. (A) Digested DNAs from *E. coli* with suicide vector and truncated *act* gene (lane 1), double-crossover mutants of *A. hydrophila* (lanes 3 and 4), a single-crossover mutant of *A. hydrophila* (lane 5), and wild-type *A. hydrophila* (lane 6). (B and C) Digested DNAs from double-crossover mutants of *A. hydrophila* (lanes 1 and 2), single-crossover mutants of *A. hydrophila* (lanes 3 and 4), and wild-type *A. hydrophila* (lanes 5 and 6).

and these antibodies could effectively neutralize the tested hemolytic activity of Act. In contrast, sera from animals injected with the *Aeromonas* isogenic mutant, as well as the preimmune serum, did not react with Act in Western blots (data not shown).

DISCUSSION

Aeromonas species, like many other bacterial pathogens, secrete a number of extracellular proteins which play important roles in the pathogenesis of disease (3). Hemolysins have been shown to be produced by many gram-negative bacteria, and Welch and Falkow (41) were able to establish a correlation between the hemolytic titer in *E. coli* and lethality in rats. In contrast, Wright and Morris (42) noted that the cytolysin (with hemolytic and cytotoxic activities) produced by *Vibrio vulnificus* had a minimal effect on the pathogenesis of *V. vulnificus* infections.

Asao et al. (4) noted that the hemolysin produced by *Aeromonas* had multiple biological activities, including hemolytic, cytotoxic, and enterotoxic activities and lethality in mice, similar to the case for Act (14). Chakraborty et al. (9) reported that their aerolysin from *A. trota* reacted with antibodies to hemolysin isolated by Asao et al. (4). Hirono and Aoki (21) reported another hemolysin from *Aeromonas* which exhibited minimal homology with aerolysin. By marker exchange mutagenesis, Chakraborty et al. (10) showed that aerolysin-deficient mutants were less virulent in mice than wild-type *Aeromonas*. Molecular cloning and DNA sequence analysis of our *act* gene from *A. hydrophila* (14) revealed that it differed significantly from the aerolysin gene of *A. trota* and from the hemolysin purified by Asao et al. (4). The differences included the inability of one of the neutralizing Act monoclonal antibodies to react with the two other proteins in Western blot analysis and its failure to neutralize the hemolytic activity of these toxins. Site-directed mutagenesis within the *act* gene revealed many other differences between Act and aerolysins (16). We also have demonstrated that Act stimulated the chemotactic activity of human leukocytes and inhibited the phagocytic function of mouse phagocytes (27), clearly indicating a role for Act in *Aeromonas*-mediated infections. Further, in a clinical study, *A. hydrophila* was isolated as the sole enteropathogen from patients' diarrheal stools and from the ready-to-eat shrimp cocktail that those patients had ingested (2), indicating a definitive epidemiological link between diarrhea and direct exposure to *Aeromonas*.

The LD_{50} of *Aeromonas* strain A52 (Act negative) was almost two logarithmic doses greater than the LD_{50} of *A. hydrophila* SSU when injected i.p. However, since we were unaware of the various virulence factors produced by A52 compared to SSU, we opted to generate transposon and isogenic mutants of wild-type *A. hydrophila* SSU. At present, no oral-challenge models are available for *Aeromonas*, and therefore, the current model has limitations in mimicking the true disease process in humans. Regardless, this is the first report of a study in which an *act* gene-deficient mutant was prepared from an authentic strain of *A. hydrophila* to unequivocally establish the role of Act in *Aeromonas*-mediated infections in mice after i.p. challenge.

The transposon mutants of *A. hydrophila* SSU with dramatically reduced biological activity were not lethal to mice at a dose of 5×10^7 compared to the wild type. Southern blot data suggested that transposition might not have occurred within the structural gene for Act in these mutants. Our Northern blot data demonstrated that transcription of the *act* gene in the transposon mutants was affected (Fig. 3). The exact location of the transposition in these mutants has not been determined and is under investigation. It is plausible that the transposition might have occurred in some regulatory element whose product was essential for the transcription of the *act* gene. Earlier, Chakraborty et al. (9) used transposon insertions to demonstrate that the DNA sequences flanking the aerolysin structural gene (*aerA*) in both the 5′ (referred to as *aerC*) and 3′ (referred to as *aerB*) regions in *A. trota* were important for the expression of the aerolysin gene. However, Howard et al. (24) noted that the expression of their aerolysin gene from *A. bestiarum* was not affected when Tn*5* insertions were introduced immediately downstream of the stop codon for the aerolysin structural gene. Although regulation of the *act* gene is a subject of intense investigation in our laboratory, at present nothing is known about the *act* operon in *A. hydrophila*. It is therefore plausible that transposition in these mutants, although not within the structural gene, may be in the *act* operon.

Transposition may lead to polar mutations, and the possibility that some other virulence genes might have been affected in the transposon mutants due to a polar effect cannot be ruled out. Therefore, we generated an isogenic mutant of *Aeromonas*. The frequency of double-crossover events was very low (0.01%). We obtained at a high frequency colonies which acquired sucrose resistance but still were gentamicin resistant. This could have occurred as a result of various types of mutations within the *sacB* gene (36). Further, the DNA sequences flanking the *act* gene had to be increased significantly (1.9 kb at the 5′ end and 0.9 kb at the 3′ end) to obtain double-crossover mutants.

Originally, we removed 63% of the coding region of the toxin by using the *Bst*XI restriction enzyme, which resulted in a flanking 422 bp of the DNA sequence at the 5′ end and 179 bp at the 3′ end of the *act* gene (14). Donnenberg and Kaper (15) similarly removed 66% of the *eae* gene of *E. coli* and had 519 and 120 bp of flanking DNA sequences in order for the double-crossover event to occur. They reported successful isolation of double-crossover mutants. However, this strategy did not provide us any genuine double-crossover mutants, although single-crossover mutants were obtained. These single-crossover transconjugants were grown without antibiotic selection to the late logarithmic phase, allowing second recombination events to accumulate. Although we obtained the de-

sired phenotype (gentamicin sensitivity and sucrose resistance), we noticed that the suicide vector was indeed not lost and that the biological activity of the toxin remained intact. Thousands of colonies were screened on the blood agar plates for the loss of hemolytic activity, without any success. These data indicated mutations in the gentamicin resistance and *sacB* genes.

A dramatic difference between the LD_{50}s of the isogenic mutant and wild-type *Aeromonas* when injected into animals was noted. Even with the construction of an isogenic mutant, it is possible that unlinked mutations might influence the biological effects of Act. We therefore reintroduced the native *act* gene in the isogenic mutant to restore the biological activity of Act. Indeed, full hemolytic, cytotoxic, and enterotoxic activities were regained by this *Aeromonas* revertant. Further, the revertant was as virulent in mice as wild-type *A. hydrophila* SSU, indicating that there was no polar effect in the isogenic mutants. Finally, we have demonstrated that Act was produced during the infection process, since antisera obtained from mice surviving infection with wild-type *A. hydrophila* and the revertant had Act-specific antibodies. However, the sera from animals injected with the isogenic mutant did not show an Act-specific band in Western blots.

In conclusion, we have demonstrated that elimination of the biological effects of Act by either transposon or marker exchange mutagenesis significantly affected the pathogenicity of *Aeromonas* in mice. These data were substantiated by using an isolate of *Aeromonas* that naturally did not produce Act. These observations are very provocative, since *Aeromonas* increasingly has been isolated from patients with peritonitis and urinary tract infections, and there have been reports in which *A. hydrophila* has been shown to cause multilobular lung abscesses and a fatal bacteremia with myonecrosis and gas gangrene in a hemodialysis patient treated with deferoxamine (25, 29, 31, 33). The reported isolation of *Aeromonas* worldwide from 5 to 7% of individuals suffering from gastroenteritis, the presence of this organism in a variety of foods, and the prevalence of Act-related molecules in most *Aeromonas* isolates examined have resulted in an increased awareness of their association with human infections (8, 11, 12, 18, 22). Overall, our data indicate that Act has an impact on virulence in mice, but further studies will be necessary to clearly correlate this observation with human illness.

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