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 Tn5-751 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 $1 \times 10^6$ when injected i.p., compared to $3.0 \times 10^8$ 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. Among various virulence factors produced by Aeromonas species, enterotoxins are by far the most important in causing diarrheal infections. The cytotoxic enterotoxin (Act) is one of the new family, Aeromonadaceae, which have recently been placed in a new family, Aeromonas, proving unequivocally the role of Act 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 pME29 with transposon Tn5-751 was obtained from S. P. Howard, University of Regina, Regina, Saskatchewan, Canada. The transposon Tn5-751 had been cloned, sequenced, and hyperexpressed in A. hydrophila.
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 pQ200KS, which contained a P15A origin of replication, a *sclA* 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 *sprr* (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 pM703.1, was provided to us by V. L. Miller, Washington University School of Medicine, St. Louis, Mo. Plasmid pXHC95 contained a 2.8-kb *BamHI* 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 *Km* resistance gene cassette, which represented a portion of the transposon Tn903 (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 −80°C. The concentrations of antibiotics used to grow cultures were as follows: 100 μg of ampicillin per ml, 40 μg of rifampin per ml for transposon mutants and 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 Tn5-751 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 × 10⁷ 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 10⁻⁶) 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 (Vitek, 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 (Strategene, 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-32P]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) (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 recombinant. Recombinant plasmid pXHC95 (14) was used to construct isogenic
Table 1. Hemolytic and cytotoxic activities of Act produced by wild-type and transposon mutants of A. hydrophila SSU

<table>
<thead>
<tr>
<th>Culture</th>
<th>Hemolytic activity in:</th>
<th>Cytotoxic activity in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture filtrates</td>
<td>Cell lysates</td>
</tr>
<tr>
<td>Wild-type SSU</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>Transposon mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>225</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>312</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>325</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>353</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

*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.*
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 Tn5-751 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 (SalI-BamH I) 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<sub>50</sub> of _Aeromonas_ strain A52 was calculated to be 3.9 × 10<sup>7</sup>. However, the LD<sub>50</sub> of wild-type _A. hydrophila_ SSU was 2.5 × 10<sup>7</sup>, indicating _Act_’s role in the organism’s virulence. The difference in the LD<sub>50</sub>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<sup>7</sup>). 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).
TABLE 2. Biological activities of Act produced in culture filtrates of A. hydrophila SSU and its isogenic mutant and revertant

<table>
<thead>
<tr>
<th>Culture</th>
<th>Hemolytic activitya</th>
<th>Cytotoxic activityb</th>
<th>Enterotoxic activity (µl/cm)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type A. hydrophila with native Act</td>
<td>1:512</td>
<td>1:2,048</td>
<td>118 ± 21</td>
</tr>
<tr>
<td>E. coli with suicide vector pJQ200</td>
<td>0</td>
<td>0</td>
<td>NDd</td>
</tr>
<tr>
<td>containing a truncated act gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double-crossover mutant of A. hydrophila with a truncated act gene</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Revertant strain of A. hydrophila in which the truncated act gene was replaced with the native act gene</td>
<td>1:512</td>
<td>1:2,048</td>
<td>112 ± 12</td>
</tr>
</tbody>
</table>

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 Cytotoxic activity was determined in mouse ligated intestinal loops. Results are means ± standard deviations.

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

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 levansucrase, 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 BamHI DNA fragment from plasmid pXHC97.3 was transferred 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 × 10^3, whereas the LD_{50} of the isogenic mutant was 1.0 × 10^6 (P = 0.01 by the Fisher exact test). The LD_{50} of wild-type Aeromonas was lower than that obtained earlier (2.5 × 10^3), 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.
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 cytolsin (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. taura* 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. taura* 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 entero-

pathogen 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 AS2 (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 AS2 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 $5 \times 10^7$ 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 (aerC) in both the 5’ (referred to as aerC) and 3’ (referred to as aerB) regions in *A. taura* 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 Tn5 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 BstXI 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-

![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 XhoI/SmaI 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).](http://iai.asm.org/DownloadedFrom)
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 LD₅₀ 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 is frequently isolated from patients with peritonitis and urinary tract infections, and there have been reports in which A. hydrophila has been shown to cause multilobar 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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