APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2007, p. 7934–7946 Vol. 73, No. 24
0099-2240/07/$08.00+0 doi:10.1128/AEM.01115-07

Signature-Tagged Mutagenesis of *Edwardsiella ictaluri* Identifies Virulence-Related Genes, Including a *Salmonella* Pathogenicity Island 2 Class of Type III Secretion Systems▽

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Received 18 May 2007/Accepted 15 October 2007

***Edwardsiella ictaluri* is the leading cause of mortality in channel catfish culture, but little is known about its pathogenesis. The use of signature-tagged mutagenesis in a waterborne infection model resulted in the identification of 50 mutants that were unable to infect/survive in catfish. Nineteen had minitransposon insertions in miscellaneous genes in the chromosome, 10 were in genes that matched to hypothetical proteins, and 13 were in genes that had no significant matches in the NCBI databases. Eight insertions were in genes encoding proteins associated with virulence in other pathogens, including three in genes involved in lipopolysaccharide biosynthesis, three in genes involved in type III secretion systems (TTSS), and two in genes involved in urease activity. With the use of a sequence from a lambda clone carrying several TTSS genes, Blastn analysis of the partially completed *E. ictaluri* genome identified a 26,135-bp pathogenicity island containing 33 genes of a TTSS with similarity to the *Salmonella* pathogenicity island 2 class of TTSS. The characterization of a TTSS apparatus mutant indicated that it retained its ability to invade catfish cell lines and macrophages but was defective in intracellular replication. The mutant also invaded catfish tissues in numbers equal to those of invading wild-type *E. ictaluri* bacteria but replicated poorly and was slowly cleared from the tissues, while the wild type increased in number.**

The gram-negative enteric bacterium *Edwardsiella ictaluri* causes enteric septicemia of catfish (ESC), an economically significant disease of farm-raised channel catfish. Commercial catfish production accounts for 85 to 90% of the total finfish aquaculture production in the United States, with almost 300,000 tonnes produced annually (36), and significant losses due to ESC were reported on over 60% of all farms in operation (84). The clinical signs and pathogenesis of *E. ictaluri* infections have been reviewed previously (83). Briefly, ESC generally presents in two forms: a rapid-onset, acute septicemia with high mortality and a chronic form confined to the central nervous system and characterized by a distinct "hole-in-the-head" lesion (75). Subacute infections also occur, with lower mortality rates than in acute infections. Presentation in any given epizootic is dependent on fish condition and water quality, especially temperature. Generally, fish less than 1 year old are more susceptible than older fish, and disease is more acute within the optimal temperature range of 22°C to 28°C and more chronic outside of that range (29, 55). Chronic disease is also seen in populations that have survived epizootics and have developed some immunity, especially if environmental conditions are suboptimal.

Although there is substantial descriptive data relative to the invasion, spread, and persistence of *E. ictaluri* in channel catfish (6, 64, 65, 83), little is known about the virulence factors involved in the process. Chondroitinase activity has been correlated to virulence by several authors (19, 78, 85) and may mediate the cartilage degradation in the chronic "hole-in-the-head" lesion (85). Lipopolysaccharide (LPS) (49, 50) and fibrillar processes that are apparently involved in attachment (64, 79) have also been implicated in the pathogenesis of *E. ictaluri*. There is direct evidence that *E. ictaluri* can survive in catfish neutrophils (2, 64, 79), and several reports allude to intracellular replication and survival in catfish macrophages and neutrophils, based on microscopic observations (2, 6, 61, 64, 75, 79). Booth et al. (11) recently reported the entry, survival, and replication of *E. ictaluri* in vitro in head kidney-derived macrophages (HKDM), and there are also reports of invasion into fish cell lines (76, 77).

Signature-tagged mutagenesis (STM) is a mutagenesis system involving minitransposons that carry unique DNA tags, enabling the identification either by hybridization (39) or by PCR (51) of individual mutants in a mixture of mutants carrying different tags. The methodologies associated with STM were recently reviewed (15, 17). Briefly, a mixture of tagged mutants is used to establish an infection, and the mutants that are initially present are later compared to the mutants that remain at the death of the host. Those lost during the infection process are presumed to be attenuated as a result of the insertion of the transposon into a gene that is required for

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▽ Published ahead of print on 26 October 2007.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Escherichia coli		
CC118 λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA* λ*pir* lysogen	23
S17-1 λ*pir*	Tp Sm *thi pro recA* $HsdR^-$ $HsdM^+$; RP4-2-Tc::Mu::Km Tn*7* λ*pir*	23
XL1-Blue MRF′	(*mcrA*)*183* (*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proABlacIqZ.M15* Tn*5*(Kan)]	Stratagene, La Jolla, CA
Edwardsiella ictaluri		
93–146	Wild-type *E. ictaluri* isolated in 1993 from moribund channel catfish in a natural outbreak of ESC on a commercial farm	LSU aquatic animal diagnostic laboratory
Plasmids		
pBluescript SK-	Cloning vector	Stratagene, La Jolla, CA
pUT-miniTn*5*Km2	Delivery plasmid carrying mini-Tn*5*Km	23
pUT-miniTn*5*Km-MCS	pUT-miniTn*5*Km2 with multiple cloning site containing EcoRV, XbaI, and ApaI restriction enzyme sites	This study
pUT-Km-STM	pUT-miniTn*5*Km-MCS carrying a specific tag in the MCS	This study

survival in the host. Because the tagged transposons carry an antibiotic resistance marker, the region flanking the transposon can be subcloned from a restriction digest using antibiotic selection. The subcloned gene carrying the insertion can be identified by using the tag as a primer to sequence the DNA flanking the minitransposon and comparing the sequence to known bacterial DNA databases. Numerous colonization/virulence factors from a variety of bacterial pathogens have been identified by using STM, and summaries of those studies are available (58, 73). Based on the hypothesis that a basic knowledge of ESC pathogenesis and virulence factors is important to the ultimate prevention and control of the disease, the main objective of this study was to identify virulence-associated genes of *E. ictaluri* by using the STM procedures of Lehoux (51) in our channel catfish waterborne-infection model (82). This is the first report of virulence factors identified for *E. ictaluri* using modern molecular screening methods.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, *Escherichia coli* was grown in Luria-Bertani broth (LB) at 37°C and *E. ictaluri* strains were grown in brain heart infusion (BHI) broth at 28°C. Strain CC118 λ*pir* of *E. coli* was used to maintain the STM delivery plasmids and to isolate plasmid DNA prior to introduction into the conjugation strain, S17-1 λ*pir*. Antibiotics were used in the following concentrations: kanamycin (Km) at 50 μg ml^{-1}, colistin (Col) at 10 μg ml^{-1}, and ampicillin (Amp) at 200 μg ml^{-1}. *Edwardsiella ictaluri* transconjugants reisolated from fish were grown on Trypticase soy agar plates supplemented with 5% sheep blood (BA).

SPF channel catfish. Channel catfish egg masses were obtained from a commercial producer with no history of ESC outbreaks. The eggs were disinfected with 100 ppm free iodine and hatched in closed recirculating systems in the specific-pathogen-free (SPF) laboratory at the LSU School of Veterinary Medicine. The fish were reared on commercial catfish diets fed at 2 to 3% of their body weight per day until used for the infection and coinfection experiments. The 1.5- to 2-year-old fish used to harvest HKDM were reared entirely within the SPF laboratory and weighed 1,000 to 1,500 g.

Production of an STM plasmid and validation of the STM procedures. When using both a wild-type *E. ictaluri* and an attenuated, tagged *aroA* mutant (82), our attempts to validate the use of the tagged pUTmini-Tn*5*Km2 plasmid and PCR conditions reported by Lehoux et al. (51) proved to be inconsistent for both mutant production and tag identification in *E. ictaluri*. We therefore modified pUT-miniTn*5*Km2 (22) for STM by inserting a new multiple cloning site containing EcoRV, XbaI, and ApaI sites (5′GATATCTCTAGAGGGCCC3′). The new plasmid, pUT-miniTn*5*Km-MCS, was digested with EcoRV and treated with calf intestinal alkaline phosphatase. The 12-tag sequences reported by Lehoux et al. (51) were synthesized and designated STM-A/B to STM-Y/Z, excluding the letters I and Q. Annealed tags were phosphorylated using T4 polynucleotide kinase (New England Biolabs) and ligated to the plasmid using T4 DNA ligase (New England Biolabs). The new plasmids were designated pUT-Km-STM-A/B to pUT-Km-STM-Y/Z, depending on which tag was inserted, and the orientation and single-copy insertion of each tag were verified by PCR. A minitransposon carrying tag STM-G/H was also ligated into the *aroA* gene (82) of wild-type *E. ictaluri* and used to validate the STM procedure.

Tagged *E. ictaluri* transconjugant libraries were made by conjugation using procedures previously described (57). Briefly, *E. ictaluri* 93-146 was mated with *E. coli* S17-1 λ*pir* containing the tagged pUT-Km-STM, after which the transconjugants were suspended in 10 mM $MgSO_4$. The specificities of the tags in the *E. ictaluri* genomic background were confirmed by PCR on a mixture containing DNA from individual mutants carrying each of the 12 tags, as well as a mixture containing all of the tags except the one being used as the primer. The presence of individual tags was confirmed by the amplification of a predicted 643-bp product. Three of the original tags (51), E/F, J/K, and W/X, were not used further because they amplified nonspecific products from the tagged *E. ictaluri* chromosomal DNA, even when DNA carrying the specific tags was not included in the screening (data not shown).

Generation and identification of *E. ictaluri* attenuated mutants by STM. The procedures for STM used in this study are depicted in Fig. 1. Briefly, an aliquot of each tagged library was removed from the freezer, thawed, and spread on BHI plates with Km to select for transconjugants and Col to eliminate the *E. coli* donor (BHI-KmCol). Nine colonies, one from each plate of individual tags, were inoculated into individual tubes containing 6 ml of BHI-KmCol broth and incubated at 28°C for 16 h. After the incubation, 1-ml samples of individual transconjugants were frozen and stored in microfuge tubes at −80°C. The remaining 5 ml of culture for each transconjugant from each of the nine tagged libraries was combined to create 45-ml pools of transconjugants that were used in the immersion challenges (In pools). In addition, DNA was isolated from 500 μl of each In pool prior to challenge and frozen at −20°C to be used in PCR to verify that all nine mutants, each carrying an individual tag, were present.

For the immersion challenge, experimental fish were placed into 20-liter tanks supplied with a continuous flow of dechlorinated municipal water maintained at 25°C ± 1°C at a flow rate of 500 to 600 ml per minute. Fish were stocked at a density of 25 per tank and fed commercial catfish feed ad libitum every other day during a 4-week acclimation period. One tank of 20 fish was infected with each In pool by adding enough bacterial culture to achieve a final concentration of approximately 1×10^8 CFU per ml of tank water. When fish began dying, generally 6 to 7 days postinfection, bacteria were isolated from three dead or moribund fish by spreading 200 μl of a liver tissue homogenate on BA plates. The resulting solid lawn of bacteria was washed off with 5 ml sterile saline and collected in sterile test tubes (Out pool). Each of these Out pools was processed by PCR, and transconjugants that were missing from the Out pools potentially carried mutations in virulence-related genes.

Cloning of flanking DNA and sequence analysis. Each putative virulence-associated mutant was further evaluated to determine the gene of insertion.

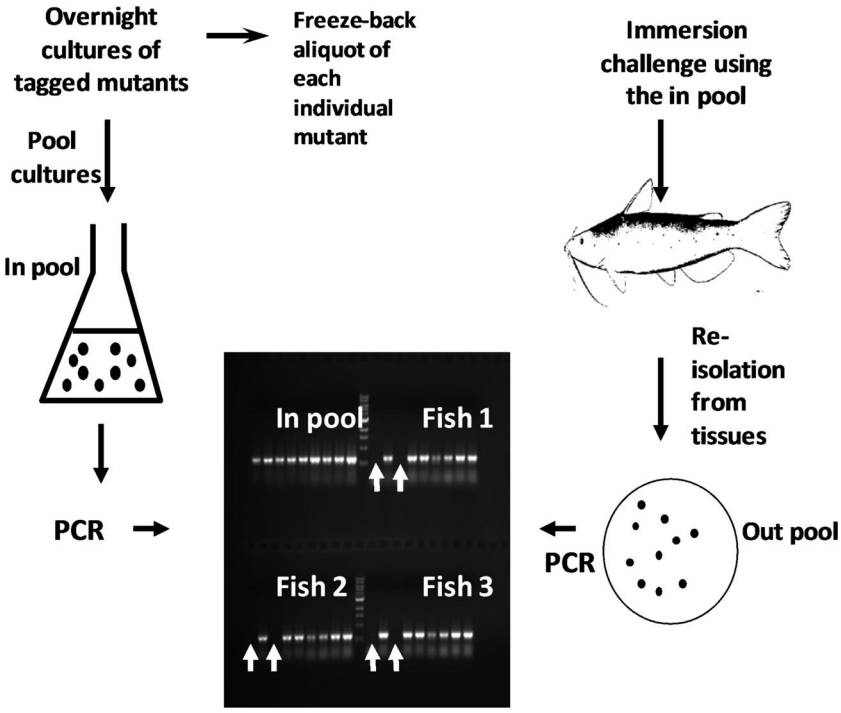

FIG. 1. Overview of the signature-tagged mutagenesis procedures. The two PCR products missing for all three fish (white arrows) represent putative attenuated mutants for further characterization.

Briefly, chromosomal DNA was digested with ClaI, which does not cut the Km-STM minitransposon, ligated into the ClaI site of pBluescript SK$^-$ (Stratagene, La Jolla, CA), and electroporated into *E. coli* XL1-Blue MRF′. Km/Amp-resistant colonies containing plasmids carrying the mini-Tn*5*-Km-STM transposon and associated chromosomal DNA flanking the site of insertion were selected on LB-Km/Amp, and plasmid DNA was purified by using a QIAprep spin mini-prep kit (QIAGEN, Valencia, California). The region adjacent to the tagged end of the transposon was sequenced by using the tag-specific primer on an ABI prism 377 automated sequencer (PE-Applied Biosystems, Foster City, CA), and the resulting sequence was analyzed against the NCBI databases using Blastx to ascertain homologous sequence information.

CI assays. For the competitive index (CI) assays, mutant strains and wild-type *E. ictaluri* bacteria were grown separately in BHI at 28°C for 18 h. All mutants tested grew well in broth culture, with final plate counts averaging 9.2×10^9 CFU/ml with a range of 5.8×10^9 to 1.1×10^{10} CFU/ml. Based on the values for optical density at 600 nm, cultures were diluted so that equal numbers of wild-type and mutant bacteria were mixed together and used in the immersion challenge at a final concentration of 1×10^8 CFU/ml of tank water. Liver samples were removed from at least three dead or moribund fish in each tank, homogenized, serially diluted, and drop plated on BHI to determine the total CFU recovered and on BHI-Km to determine the number of mutant CFU recovered. The CI was determined by dividing the recovery ratio of mutant CFU/wild-type CFU by the input ratio of mutant CFU/wild-type CFU. The values, ranging from 0 to 1, indicate the level of attenuation, with values closer to 0 indicating greater attenuation and values closer to 1 greater virulence. A CI of 0.0 indicates that no mutants were recovered.

LPS immunoblotting. Putative LPS mutant and wild-type *E. ictaluri* cells were grown in BHI broth, harvested by centrifugation, and washed three times in phosphate-buffered saline, pH 7.3. The cells were resuspended in sterile, distilled, deionized water to a concentration of 0.1 ml of cell pellet/ml of water and sonicated until the cell suspensions cleared. The sonicated cells were centrifuged to remove particulate cell debris, and the supernatants were subjected to electrophoresis on a 12% polyacrylamide gel under denaturing conditions. Purified *E. ictaluri* LPS was run as a positive control. Lysates were transferred to 0.45 μM nitrocellulose membranes at a constant 100 V for 1 h in 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol, pH 8.3. The blots were air dried and stored in the dark until the LPS was immunodetected by using mouse anti-LPS primary monoclonal antibody Ed9 (1) and goat anti-mouse immunoglobulin G heavy-plus-light chains–horseradish peroxidase-labeled secondary antibodies with an enhanced chemiluminescence (ECL) Western blotting analysis system (GE Healthcare, Piscataway, NJ).

Further sequencing of the *E. ictaluri* type III secretion system (TTSS). A 259-bp PCR product was amplified from the pBluescript plasmid carrying the subcloned 65ST insertion by using primers identified from the *E. ictaluri esaU* sequence. The fragment was labeled by using the ECL DNA-labeling system (GE Healthcare) and was used to screen a previously described λ Zap Express *E. ictaluri* genomic library (82) according to the manufacturer's instructions (Stratagene, Inc., La Jolla, CA). The pBK-CMV phagemid was excised from hybridization-positive plaques according to the Zap Express protocol and was sequenced as described above. The sequence derived was subsequently used to search the partially completed *E. ictaluri* genome database (www.microgen.ouhsc.edu/cgi-bin/blast_form.cgi) to identify a 41,205-bp fragment carrying 33 genes (26,135 bp) in a pathogenicity island encoding a TTSS. Because of the similarity between the *E. ictaluri* and the *Salmonella* and *E. tarda* TTSS sequences, gene names were assigned based on homology. In order to evaluate similarity to putative *Salmonella* homologues when Blastx alignments were not identified, the L-ALIGN program at www.ch.embnet.org was used to directly compare sequences, and various proteomics programs at us.expasy.org were used to compare primary and secondary protein structures. *Salmonella* gene names were assigned to previously unnamed *E. tarda* open reading frames (ORFs) when L-ALIGN alignments and protein structure analysis indicated similarity (see Table 4).

Confirmation of a single transpositional event in the TTSS mutant 65ST. Genomic DNA from 65ST was isolated using standard methods (5), and 10 μg was cut to completion with ClaI, which does not cut the mini-Tn*5* Km-STM transposon. Cut genomic DNA was separated on a 1% agarose gel and transferred to an ECL Hybond N$^+$ nylon membrane (GE Healthcare). A 373-bp Km-STM PCR product was amplified using primers Tn*5*kan+ (5′ACACGTAG AAAGCCAGTCCG3′) and Tn*5*kan− (5′CCCAGTCATAGCCGAATAG3′), labeled using the ECL nucleic acid-labeling system, hybridized to the 65ST genomic DNA on the membrane, and detected using the ECL detection reagents.

Intracellular replication of the TTSS mutant 65ST. A standard gentamicin survival assay (26) was used to evaluate the abilities of the wild-type and the TTSS mutant *E. ictaluri* bacteria to enter and to survive and replicate in channel catfish HKDM and the channel catfish ovary (CCO) cell line. Briefly, HKDM

were isolated by the method of Booth et al. (11) and viable counts were determined by using trypan blue dye exclusion (70). Dissociated cells were suspended to a final concentration of 1×10^7 cells/ml in channel catfish macrophage medium (CCMM) consisting of RPMI 1640 medium (GIBCO, Invitrogen Corporation, Carlsbad, CA) diluted to a catfish tonicity of 243 mOsm/kg by adding 1 part sterile deionized/distilled water (RPMI 9:1) and containing 15 mM HEPES buffer solution (GIBCO), 0.18% sodium bicarbonate solution (GIBCO), 0.05 mM beta-mercaptoethanol (Sigma Chemicals Co., St. Louis, MO), and 5% heat-inactivated pooled channel catfish serum (27, 60). One ml of the cell suspension was added to each well of a 24-well plate and allowed to adhere for 16 h at 28°C with 5% CO_2, after which the wells were washed three times with RPMI 9:1 to remove nonadherent cells and 1 ml of fresh CCMM was added per well. Following adherence and washing, the attached cells averaged 91.4% macrophages, 5.4% neutrophils, and 3.1% unidentified cells, as determined by nonspecific esterase and Sudan black B staining (25), and averaged 2.5×10^5 cells per well.

To evaluate the efficiency of entry and replication, 1×10^4 of either wild-type or TTSS mutant *E. ictaluri* cells that had been opsonized for 30 min in normal autologous serum were added to triplicate wells of the 16-h HKDM cultures, giving a multiplicity of infection (MOI) of 1 bacterium:10 HKDM. After infection, the plates were centrifuged at 200× gravity to synchronize contact of the bacteria with the adhered cell layer and allowed to incubate for 30 min. The medium was then removed from each well, and CCMM with 100 μg/ml gentamicin was added for 1 h to kill residual extracellular bacteria. The cells were then washed three times with RPMI 9:1, and CCMM containing a 0.35-μg/ml bacteriostatic dose of gentamicin was added to control the extracellular growth of bacteria released from the cells. At 0 (90 min postinfection), 5, and 10 h, the HKDM were lysed by the addition of 100 μl of a 1% solution of Triton X-100 (Fisher Scientific, Fair Lawn, NJ), and the numbers of surviving *E. ictaluri* cells were determined by spreading serial dilutions on BA. A similar assay was used for the CCO cells, except that L-15 medium (GIBCO) was used, the cells were split and passed using standard cell culture methods, a 100-μg/ml dose of gentamicin was used throughout the assay, and the MOI was 1:1, bacteria to cells. In addition to the enumeration of intracellular bacteria, coverslips with both uninfected and infected HKDM and CCO cells were removed from wells without being lysed, stained using a Hema 3 stain kit (Fisher Diagnostics, Middletown, VA), mounted on glass slides with Permount (Fisher Chemical, Fair Lawn, NJ), and observed by light microscopy.

Invasion and persistence of the TTSS mutant, 65ST, in vivo. The comparative invasion and persistence of the wild-type *E. ictaluri* and the TTSS mutant, 65ST, were evaluated in vivo using SPF channel catfish fingerlings with an average weight of 5 g. Sixty fish were stocked in each of two tanks, and one of the groups was experimentally infected by immersion exposure with wild-type *E. ictaluri* while the other was experimentally infected with strain 65ST. Briefly, after the water levels were dropped to 4 liters per tank, overnight broth cultures of *E. ictaluri* 93-146 or strain 65ST were each added directly to the tanks at doses of 2.2×10^8 CFU/ml of tank water. The numbers of CFU/ml were estimated by using a standard curve for optical density at 600 nm and confirmed by drop plating serial dilutions onto BA. The water flow was stopped for 15 min following initial exposure and then resumed.

To assess the initial invasion, five fish were removed from each tank at 0.5, 2, 4, and 8 h postexposure and euthanized by being transferred to water containing 1 g/liter MS-222. In order to assess the persistence in the tissues, five fish were also collected at 24, 48, 72, 96, 120, 144, and 168 h. Five fish were also collected from each tank prior to the experimental infections for a preinfection sample. Using aseptic technique, samples of the head kidney were suspended in 0.5 ml sterile 0.9% saline solution, weighed, and homogenized. The resulting suspension was serially diluted in 0.9% saline solution in triplicate using 96-well plates, and 20-μl aliquots of each triplicate well were dropped onto BHI plates for quantification. Colonies were counted after incubation for 48 h, and the CFU/gm of tissue was calculated.

Statistical methods. The percentages of uptake in the CCO and HKDM gentamicin survival assays were calculated by dividing the mean number of CFU/well at time zero by the number of CFU in the initial bacterial inoculum. Differences in percent uptake and growth between strains were analyzed by using the general linear model from Statistical Analysis Systems (SAS), version 9.1 (SAS Institute, Incorporated, Cary, North Carolina). In the in vivo invasion/persistence study, differences in CFU/g tissue for the mutant and the wild-type *E. ictaluri* bacteria were analyzed by using two-way analysis of variance following a log transformation of the CFU count data. When the overall model indicated significance at a P value of <0.05, Scheffe's test was used for pairwise comparison of the main effects, and a least square means procedure was used for pairwise comparison of the interaction effects.

RESULTS

Screening of the *E. ictaluri* transconjugant library. A total of 119 pools, containing nine mutants per pool, were each used to infect one tank of 25 fish. Of the 1,071 mutants screened, 50, or about 4.7% of the total transconjugants, were missing from the Out pools, indicating that they carried an insertion in virulence-related genes. This corresponds to the upper end of the 1.5 to 6.5% range identified by using STM in other gram-negative pathogens (58, 73).

Identification of the gene of insertion. Cloning and sequencing of the DNA flanking the inserted transposon of all 50 attenuated mutants indicated that none of the mutants contained vector plasmid integrations. This is consistent with our earlier results using a similar pGP704-based suicide vector, pLOF, in *E. ictaluri*, in which plasmid integrations occurred in only 0.6% of the mutants generated (57). Details of the matches found by analysis of the NCBI bacterial databases are presented in Table 2. In summary, 19 mutants had insertions in miscellaneous genes in the chromosome, 10 matched to various hypothetical proteins, and 8 had insertions that were in genes matching to virulence genes identified in other pathogens. Thirteen mutants had insertions in genes with no significant amino acid or nucleotide matches in the NCBI databases (Table 3).

Miscellaneous chromosomal insertions. Although most of the transconjugants with an insertion in miscellaneous genes in the chromosome have obvious housekeeping roles (Table 2), several may have direct roles in virulence. Mutant 65NO has an insertion in a gene with high similarity to *insA*, the transposase of the insertion element, IS*1*, a common insertion sequence in the *Enterobacteriaceae* that is often present in multiple copies in a genome. The capacity of insertion sequences to modify gene expression, sequester genes, and promote genome rearrangements (31) makes 65NO an interesting mutant, and the severe level of attenuation, with a CI of 0.0, indicates that an important gene has been affected. Mutant 69ST carries an insertion in the *miaA* gene, which is involved in the posttranscriptional modification of tRNA. Transconjugants 197GH and 234AB both carry the transposon insertion in the arginine decarboxylase (*adiA*) gene.

Insertions in hypothetical genes in other pathogens. Of the 10 mutants with insertions in hypothetical genes, 2 have insertions in genes encoding proteins that have similarity to putative adhesion-related proteins. The insertion in 193UV is in a gene possibly encoding a fimbrial assembly/usher protein that has a CI of 0.00000044, indicating an important role in virulence. Further sequence analysis revealed homologues to proteins identified as a putative fimbrial chaperone and putative outer membrane proteins. These putative genes are flanked by transposon insertions, and the region has a 43.8% G+C content, compared to the 53% G+C content of the *E. ictaluri* chromosome (37), suggesting the horizontal transfer of a pathogenicity island (34).

The insertion in 233PR is in a gene with similarity to hypothetical adhesion-related genes but is more moderately attenuated, with a CI of 0.00089. Analysis of surrounding sequences revealed several genes with similarity to putative hemolysin/adhesion genes associated with a genomic island in some of the verotoxin-producing strains of *E. coli* CL3 (74) and *Yersinia*

TABLE 2. *E. ictaluri* virulence-related genes identified by STM[a]

Type and name of mutant	Homologous gene product (organism)[b]	Function of matching protein in NCBI databases (accession no.)	Length in amino acids	% Amino acid identity	% Amino acid similarity	% Nucleotide identity	CI
Miscellaneous chromosomal mutants							
50ST	NagA (YPK)	Glucosamine catabolism (NP_406152)	134	71	85	71	ND
65NO	InsA (SHD)	Insertion element (AAA25031)	50	80	82	86	0.0
69ST	MiaA (YPS)	Amino acyl tRNA synthesis (NP_404020)	140	80	90	71	0.014
69YZ	GidA (SAT)	Glucose-inhibited division protein (NP_462773)	212	91	96	78	ND
86LM	PyrD (SHF)	Pyrimidine biosynthesis (NP_706868)	118	81	91	73	ND
95GH	LipA (SET)	Lipoic acid synthesis (NP_459625)	71	95	97	81	ND
155LM	GuaB (SET)	Purine biosynthesis (NP_457045)	205	84	88	81	ND
157AB	Ppc (SHF)	Anaerobic respiration (NP_709756)	30	90	93	73	ND
172UV	FolD (SET)	Folic acid biosynthesis (NP_455132)	16	87	93	66	ND
196GH	Fom1 (STW)	Glycolysis (BAA32495)	223	53	69	51	ND
197GH	AdiA (SAT)	Biodegradation of arginine (NP_463161)	91	66	82	64	0.16
199UV	NuoH (YPS)	Aerobic respiration, electron transport (NP_406080)	193	90	96	79	ND
203AB	Hpt (SAT)	Purine metabolism (NP_459175)	69	78	92	75	ND
207AB	0304 (VCH)	Purine metabolism (NP_229959)	211	51	73	59	ND
223UV	Ssb (SHF)	Single-stranded DNA binding protein (NP_709860)	119	84	90	77	ND
225UV	HupA (YPS)	DNA binding protein HU-alpha (NP_407181)	15	100	100	86	ND
231ST	Cls (PSS)	Phospholipid biosynthesis (ZP_00125405)	113	32	56	46	ND
234AB	AdiA (SHF)	Biodegradation of arginine (NP_838857)	192	61	74	67	0.00013
236UV	ECA	Universal stress protein (CAG74674.1)	142	63	80	35	0.00000022
Hypothetical proteins							
60AB	SET	Protein STY3950 (AL513382)	162	46	57	62	0.000012
71AB	BRJ	Protein bll4065 (overlaps protein with no database match in 147AB) (NP_770705)	194	45	65	44	0.00049
72NO	PST	Protein with pentapeptide repeats (Q52118)	131	45	58	29	0.00000062
168AB	SAT	Methyl-accepting chemotaxis protein (NP_462053)	162	33	55	44	0.0
180GH	YojN (SAT)	Putative signal transduction histidine kinase (NP_461211)	157	41	53	62	0.00051
193UV	YPS	Putative fimbrial usher protein (YPO0302)	73	29	52	40	0.00000044
194AB	SET	Putative exported protein (NP_457745)	218	57	72	61	0.000011
196ST	OBI	Sigma-L-dependent transcriptional regulator (NP_691931)	105	27	43	36	0.0000011
233PR	PHL	Putative adhesin (NP_927889)	123	34	55	36	0.00089
245YZ	DSD	Putative Fic protein family; COG3177 (ZP_00129414)	181	55	67	66	0.0
Virulence-related mutants							
58PR	LsgA (HPD)	LPS biosynthesis protein (NP_873379)	140	23	49	43	0.002
142YZ	WaaL (EDT)	LPS biosynthesis (AAL01247)	22	86	95	95	0.0
173ST	WaaL (EDT)	LPS biosynthesis (AF326578)	108	80	86	84	0.0000064
65ST	EsaU (CHV)	Type III secretion apparatus protein (NP_902280)	266	43	57	59	0.0
84LM	UreG (YEN)	Urease accessory protein (P42871)	35	86	100	79	0.000013
166ST	EIC	Repeat region, *E. ictaluri* plasmid pEI2, upstream from type III secretion chaperone/effector (AF244084)	51	100	100	100	0.0
217UV	SspH1 (SAT)	Type III secretion effector protein (AAD40326)	680	43	56	66	0.0000012
243NO	UreF (YPS)	Accessory for urease assembly (NP_406188)	80	62	82	61	0.00000045

[a] The length in amino acids measures the region sequenced from the tag. In some cases the insert was immediately upstream of the gene indicated but is predicted to affect expression. Amino acid identity and similarity values show the comparison of the amino acids adjacent to the tag with those in the most-similar homologue. In instances where nucleotide matches were not identified by Blast, nucleotide identity was determined by alignment in L-ALIGN at www.ch.embnet.org/software/LALIGN_form.html. CI is calculated relative to the wild-type strain 93-146 as described in Materials and Methods. CI values are the averages of the results for a minimum of three fish. In fish where no mutant bacteria were recovered, the CI is recorded as 0.0.

[b] Most-similar homologue of gene product, from organism indicated in parentheses. BRJ, *Bradyrhizobium japonicum*; CHV, *Chromobacterium violaceum*; DSD, *Desulfovibrio desulfuricans*; ECA, *Erwinia carotovora*; EPEC, enteropathogenic *Escherichia coli*; EIC, *Edwardsiella ictaluri*; EDT, *Edwardsiella tarda*; HPD, *Haemophilus ducreyi*; OBI, *Oceanobacillus iheyensis*; PHL, *Photorhabdus luminescens*; PSS, *Pseudomonas syringae*; PST, *Pantoea stewartii* subsp. *stewartii*; SAT, *Salmonella enterica* serovar Typhimurium; SET, *Salmonella enterica* subsp. *enterica* serovar Typhi; SHD, *Shigella dysenteriae*; SHF, *Shigella flexneri*; STW, *Streptomyces wedmorensis*; VCH, *Vibrio cholerae*; YEN, *Yersinia enterocolitica*; YPK, *Yersinia pestis* KIM; YPS, *Yersinia pestis*.

TABLE 3. CIs for *E. ictaluri* mutants with transposon insertions in genes with no significant matches in the genetic databases at either the amino acid or nucleotide level

Mutant	% G+C content[a]	CI
61ST	40.7	0.000020
147AB[b]	48.7	0.000001
184GH	54.3	0.0000041
185GH	48.8	0.0000022
195CD	51.3	0.0
203UV	46.0	0.000045
205NO	46.0	0.0
219YZ	48.2	0.00016
220PR	43.4	0.000000053
222 CD	59.8	0.079
242 PR	58.8	0.00000070
242 YZ	43.9	0.0
254 CD	56.2	0.0000011

[a] The *E. ictaluri* genome has a reported 53% G+C content (37). The percentages of G+C were calculated using 500 bp flanking both sides of the STM transposon insertion site.
[b] Overlaps with hypothetical protein in 71AB.

pestis (68) and a region that encodes sequences homologous to CdiA and CdiI, which are associated with contact-dependent inhibition of growth in *E. coli* (3). This region, containing about 10 ORFs, is also bordered by TnpA transposon sequences, again indicating possible horizontal transfer of this region, but has a percent G+C content similar to that of the overall *E. ictaluri* genome.

The mutation in 72NO, with a CI of 0.00000062, is in a gene whose product is related to a variety of hypothetical proteins with similarity to a family of gene products that carry pentapeptide repeats. These repeats are described by the motif A(D/N)*XX, where the asterisk denotes a polar amino acid and the X is any amino acid (7). The first 75 amino acids following the transposon insertion in 72NO comprise a pentapeptide-repeat domain with a very similar repeat motif, except that asparagine is not as common at position two.

Two other mutants had insertions in genes with hypothetical roles in signal recognition. The insertion in 180GH is in a gene encoding a putative signal transduction histidine kinase (HK). Further sequence analysis immediately downstream from the HK revealed a positive response regulator containing a CheY-like receiver domain and a helix-turn-helix domain. There is a 7-bp overlap of the two ORFS, suggesting that they comprise an operon encoding a two-component signal transduction system (54). Two-component systems respond to environmental cues by transferring a phosphate group from a conserved histidine residue on the HK to a conserved aspartate residue on the response regulator, causing a transformational change in the protein structure, resulting in either activation or repression of transcription. Because several of these two-component systems were identified using in vivo expression technologies (44, 86), a role in host environment recognition is postulated, although the relatively high CI of 0.0005 would indicate a more minor role. The role in virulence of the putative methyl-accepting chemotaxis protein (MCP) mutated in 168AB was confirmed by the fact that no 168AB mutants were recovered from any of the three fish evaluated in the competitive assay.

Insertions in known virulence-related genes in other pathogens. In total, eight insertions were in genes encoding *E. ictaluri* proteins with similarity to proteins known to be directly involved in the virulence of other bacterial pathogens (Table 2). Three of those, 58PR, 142YZ, and 173ST, encode proteins involved in the synthesis of LPS. Immunodetection of Western-blotted whole-cell lysates with monoclonal antibody to *E. ictaluri* O-antigen (monoclonal antibody Ed9) (1) determined that all three are negative for the production of O-antigen side chains (data not shown). The severe level of attenuation of the LPS mutants is consistent with the high level of attenuation previously reported for *E. ictaluri* LPS mutants (50).

Two of the mutants, 84LM and 243NO, carried insertions in genes involved in urease activity, and both were confirmed in the competitive assay to be severely attenuated, with CI values of 0.000013 and 0.00000045, respectively. This is interesting in light of the fact that *E. ictaluri* is urease negative in standardized biochemical tests. The insert in 84LM is in a gene similar to *ureG*, which encodes a GTP-binding protein that is thought to function in energy-dependent urease assembly (63). The insert in 243NO is in a gene similar to *ureF*, encoding a protein thought to function in the generation or delivery of carbon dioxide to the metallocenter of the enzyme to facilitate interaction between the urease apoprotein and the UreE holoprotein (63). Further analysis of the *E. ictaluri* genome has identified a urease operon containing seven genes with a gene arrangement similar to those in *Escherichia coli* O157:H7, *Morganella morganii*, and *Yersinia enterocolitica*, as well as putative urea and ammonia transporters (10).

The three remaining virulence-related mutants, 65ST, 166ST, and 217UV, had insertions in genes encoding proteins with similarity to proteins from TTSS, and all were confirmed to be severely attenuated in the competitive assays (Table 2). The insertion in 65ST is in a gene encoding a protein with homology to EsaU/SsaU and to the homologous proteins in a number of other gram-negative bacteria carrying a TTSS. In other gram-negative pathogens, EsaU/SsaU homologues are associated with the inner-membrane structural component of the type III secretion apparatus, which is essential for type III secretion and virulence (20, 41). The CI value of 0.0 for 65ST suggests an essential role in virulence for EsaU and the TTSS in *E. ictaluri*. Consequently, the EsaU mutant is characterized further below.

Interestingly, two of the TTSS-related insertions, in transconjugants 166ST and 217UV, are located either in or immediately upstream of putative TTSS effector proteins previously reported on the two native plasmids of *E. ictaluri*, pEI1 and pEI2 (28). The transposon insertion in 166ST is in repeat 1 of pEI2, 120 bp upstream from the start codon for *orf1* (28). This region does not encode any recognizable ORFs, but the mutant has a CI of 0.0 and the insertion may be in a region with a regulatory role in the expression of *orf1*. The similarity in size, pI, and sequence to the *spa15*-encoded chaperone of *Shigella flexneri* (67) suggests that *orf1* of pEI2 is a TTSS chaperone. *orf1* is in an apparent operon with a putative TTSS effector that has similarity to OspB, a TTSS effector in several gram-negative pathogens. The insertion in 217UV is in *orf1* of pEI1 (28), which has similarity to leucine-rich-repeat (LRR) effector proteins in a variety of pathogens, including several *Salmonella*-translocated effectors (STE), several IpaH proteins in *Shigella*, YPO1007 and

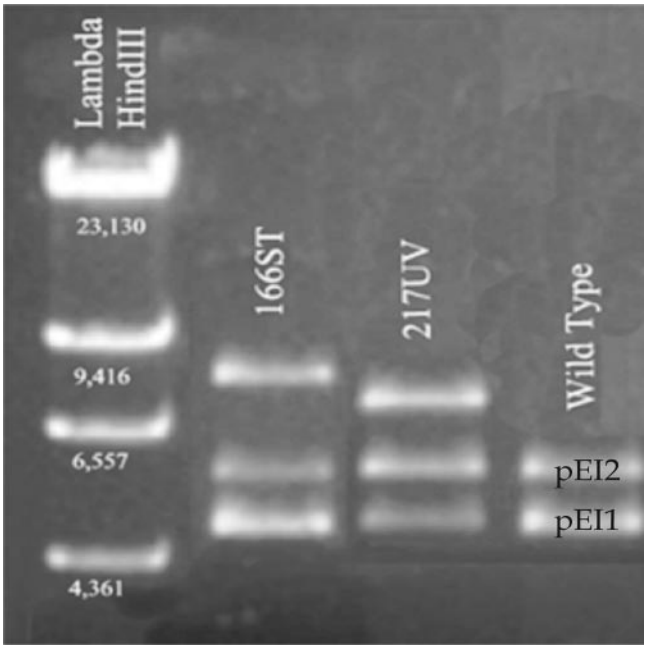

FIG. 2. Agarose gel showing plasmid preparations from wild-type *Edwardsiella ictaluri* and two mutant strains with transposon insertions in plasmids pEI1 and pEI2. Note the mutant pEI2 plasmid in 166ST carrying the 2,378-bp transposon insertion and the lower relative brightness of the wild-type pEI2 plasmid, suggesting an apparent reduction in copy number. Also note similar results for pEI1 in 217UV. The left lane is a size ladder with numbers of base pairs indicated.

YopM in *Yersinia*, Y4FR in *Rhizobium*, and ID431 and blr1676 in *Bradyrhizobium*.

The identification of an attenuated phenotype as a result of insertions in the plasmids by STM is somewhat surprising. Both pEI1 and pEI2 are multicopy plasmids (data not shown), and not all of the copies would be expected to carry the transposon if an insertion occurs in one of the plasmids. The examination of plasmid profiles for 166ST and 217UV confirms that both wild-type and mutant plasmids persist, although differences in band intensity suggest a reduction in copy number for the wild-type plasmid that corresponds to the plasmid in which the insertion occurred (Fig. 2). The intact genes and associated promoters in the remaining wild-type plasmids, however, would be expected to complement the activity lost in association with either mutation. The low CI values of both 166ST and 217UV, however, suggest that a reduction in the number of copies of the genes in question results in attenuation, possibly due to competitive binding by nonfunctional mutant proteins or to a need for a threshold number of protein molecules to mediate function.

Insertions in genes with no known genetic matches. The most intriguing mutants uncovered in this study are those that carry insertions in genes with no matches in the bacterial databases at NCBI (Table 3). Most of the mutants with insertions in genes with no matches have very low CI values, and several have very low G+C content, suggesting that they are located in *E. ictaluri* pathogenicity islands. Further work to identify the roles of these unknown proteins may lead to the identification of factors unique to the pathogenesis of *E. ictaluri*.

CI assays. The completion of the CI assays resulted in CI values ranging from 0.0 to 0.079 (Tables 2 and 3). Of the mutants with insertions in known virulence genes, three had CI values of 0.0, including one in a putative LPS biosynthesis gene, one immediately upstream of a putative TTSS chaperone and effector, and one in a TTSS apparatus protein. All of the mutants with insertions in genes encoding hypothetical proteins or proteins with no matches in the databases had CI values of less than 0.079, with six values of 0.0.

***E. ictaluri* TTSS.** The hybridization of an *esaU* probe to the *E. ictaluri* λ library resulted in the identification of a 4,662-bp *E. ictaluri* genomic fragment carrying six complete ORFs encoding proteins with homology to the inner membrane components of the needle complex of TTSS of a number of gram-negative bacteria. Using this sequence, subsequent Blastx analysis of the partial *E. ictaluri* genome identified a 41,205-bp fragment containing a 26,135-bp pathogenicity island encoding 33 genes of a TTSS (GenBank accession no. DQ233733). The *E. ictaluri* TTSS is highly similar to the TTSS described for *Edwardsiella tarda* (81), and both are similar to the SPI-2 class of TTSS carried by *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Chromobacterium violaceum* (8). Gene alignment and comparisons are presented in Fig. 3 and Table 4.

Characterization of the TTSS mutant 65ST. The transposon insertion in *esaU* follows the sequence encoding amino acid residue 61 of 351 total amino acids. *esaU* is oriented in the same transcriptional direction as *esaR*, *esaS*, *esaT*, and *slt*, and these genes may be in the same transcriptional unit, because no obvious transcriptional termination sequences are found between them. There is an apparent sigma-70 transcription initiation site upstream of *esaR*, and *esaR* appears to be translationally coupled to *esaS*, *esaT*, *esaU*, and *slt* by hairpins associated with their translation initiation regions. Sequence analysis indicates that the transposon insertion in 65ST is after bp 184 of *esaU*, resulting in a frame shift that generates a stop codon 9 bp downstream. Consequently, it is unlikely that the *slt* gene, which is translationally linked, is expressed in 65ST.

The pathogenicity of *E. ictaluri* is dependent on its ability to infect and multiply within host cells (11). The genetic similarity to the SPI-2 class of TTSS, which are involved in intracellular survival and replication in *Salmonella* (18, 38–40) and *E. tarda* (81), suggests a similar role for the *E. ictaluri* TTSS. Consequently, the *esaU* insertion mutant, 65ST, was evaluated for its ability to enter the host following immersion challenge and to enter and to survive and replicate in the CCO cell line and in HKDM. Before these studies were initiated, Southern blot analysis of 65ST genomic DNA confirmed that the attenuated phenotype was due to a single transposon insertion (data not shown). As indicated in Tables 5 and 6, the insertion in *esaU* resulted in markedly reduced replication but did not affect uptake by HKDM. The mutation in *E. ictaluri esaU* also did not affect entry into the nonphagocytic CCO cells (Table 5), but the mutant's replication was poor compared to that of the wild-type strain (Fig. 4; Table 6). Although the initial uptake into both HKDM and CCO cells was not significantly different between the TTSS mutant and the wild-type strain, uptake in the CCO cells was poor relative to that in the HKDM,

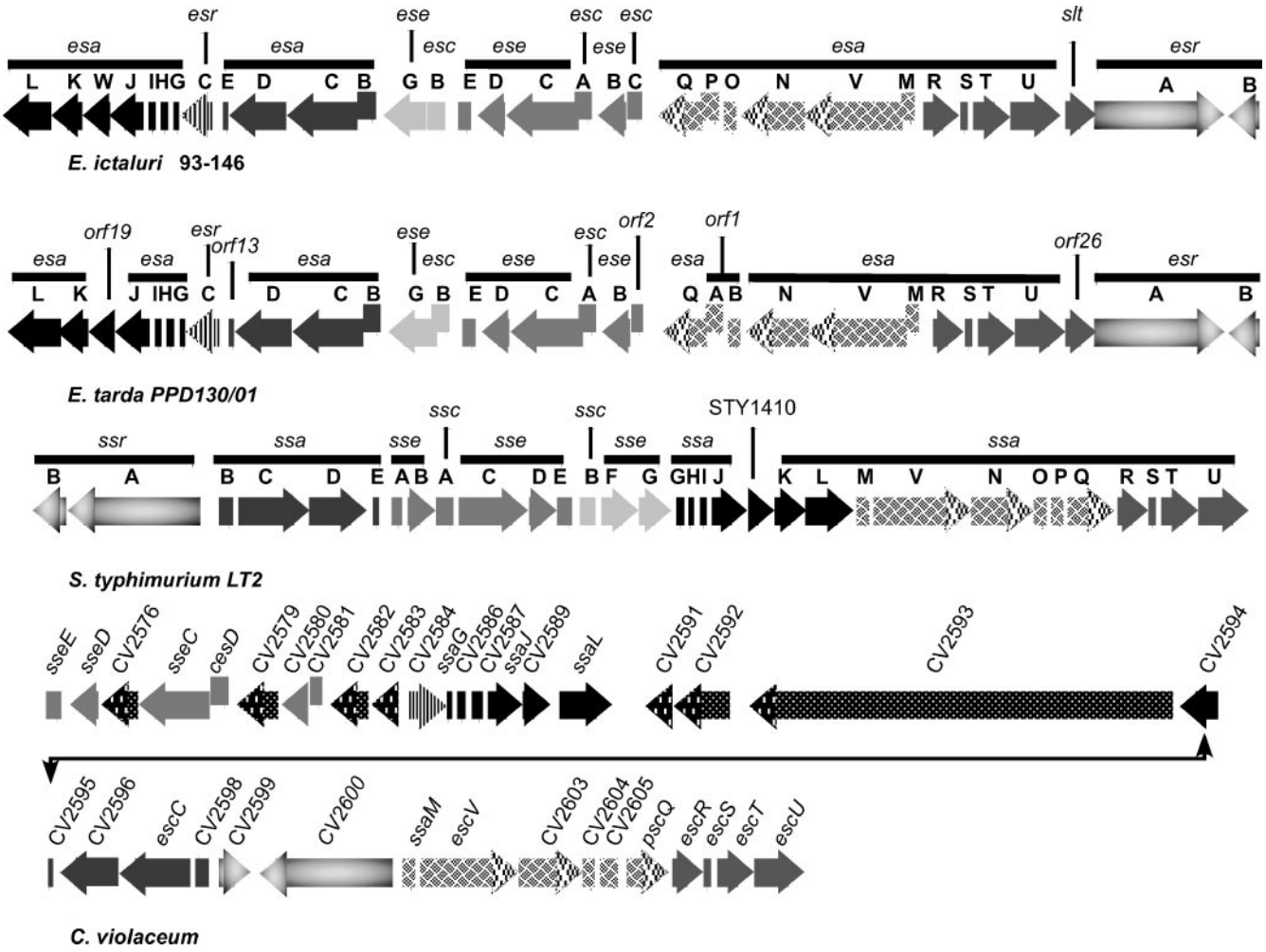

FIG. 3. Genetic organization of the type III secretion system genes of *E. ictaluri* compared to homologous regions of other TTSS. Arrows with the same pattern indicate homologous proteins. Note that *E. ictaluri* gene *escC* (88) was formerly known as *eseA*.

with less than 1% of the cells recovered after the gentamicin exposure compared to about 50% in the HKDM (Table 5).

As indicated in Fig. 5, there was no significant difference in vivo between the uptake or initial growth of the TTSS mutant in comparison to the uptake and initial growth of the wild-type *E. ictaluri* bacteria following immersion challenge. The tissue levels of the TTSS mutant strain, however, were significantly lower and declined for all times sampled after 24 h out to day 7, indicating slow clearance from the tissue, compared to increasing numbers of the wild type. Three fish challenged with the mutant had minimal bacteria remaining on day 8 (data not shown), but could not be compared to the fish challenged with the wild-type bacteria because five of the wild-type-challenged fish succumbed to the disease prior to sampling. No deaths occurred in fish challenged with the TTSS mutant. These results suggest that the *E. ictaluri* TTSS is not involved in the initial penetration of tissues from the environment but is required for survival and replication in the host. Fish mortality following challenge was 56.7% with the wild-type bacteria, with no deaths in fish challenged with 65ST.

DISCUSSION

The STM procedure provided a powerful tool for the identification of genes required for the survival of *E. ictaluri* in channel catfish. Every strain identified by STM is, by definition, attenuated in the catfish host. Transconjugants that can be transcomplemented by the production of a soluble virulence factor produced by another transconjugant in the pool or that carry the transposon in a gene required to produce disease but not required for survival and growth in the host will not be detected.

Miscellaneous chromosomal insertions. The *miaA* gene has been implicated in the regulation of the virulence gene *virF* in *Shigella flexneri* and of the *vir* regulon in *Agrobacterium tumefaciens* (24, 33). In both of these organisms, the expression of a variety of virulence-related genes is downregulated from 3- to 10-fold in *miaA* mutants, depending on the gene in question. Mutations in *miaA* in *A. tumefaciens*, however, had minimal effects on virulence in a potato challenge model and had no effects in tobacco, kalanchoe, or tomato (33). The relatively low level of attenuation in 69ST, with a CI of 0.014, is consis-

TABLE 4. Comparison of the *E. ictaluri* TTSS to the *E. tarda* and *Salmonella* pathogenicity island 2-encoded TTSS and putative functions

E. ictaluri gene[a]	Total no. of amino acids of product	% Identity/similarity at the amino acid level to gene product of:		Putative TTSS function
		E. tarda	*S. enterica* serovar Typhimurium	
esaL	341	88/92	28/47	Regulation of translocon expression
esaK	217	80/88	22/56	Apparatus protein with glutamine-rich region
esaW (*orf19*)	194	90/94	30/55	Apparatus protein with leucine-rich region
esaJ	243	88/94	41/59	Outer membrane protein of the needle complex
esaI	83	78/90	43/64	Apparatus protein
esaH	88	90/94	30/57	Apparatus protein
esaG	73	90/98	46/69	Apparatus protein
esrC	230	96/97		AraC family regulatory protein
esaE (*orf13*)	74	88/94	25/45	Hypothetical protein
esaD	399	82/87	24/42	Periplasmic channel
esaC	494	93/95	46/67	Oligomeric outer membrane secretin
esaB	159	89/91	27/77	Outer membrane component
eseG[b]	301	70/79	26/44	Effector protein
escB	163	88/92	30/46	*sseF* and *sseG* chaperone
eseE	127	92/96	25/49	Chaperone
eseD	193	78/89	27/54	Translocon protein
eseC	508	78/88	27/48	Translocon protein
escA	155	92/96	30/53	Chaperone
eseB	198	77/87	33/52	Translocon filament protein
escC[c] (*orf2*)	140	80/86	25/58	Unknown
esaQ	304	86/90	39/57	Apparatus protein that regulates translocation
esaP (*orf1A*)	159	91/95	17/49	Apparatus protein, integral membrane protein
esaO (*orf1B*)	122	80/83	24/64	Apparatus protein containing leucine zipper
esaN	438	90/94	55/68	Apparatus protein, ATP synthase
esaV	685	93/96	52/69	Apparatus membrane protein
esaM	126	90/93	29/47	Apparatus protein, secretion regulator
esaR	250	96/99	58/76	Integral apparatus membrane protein
esaS	89	96/97	52/71	Integral apparatus membrane protein
esaT	260	93/96	43/64	Integral apparatus membrane protein
esaU	352	89/94	43/60	Integral apparatus membrane protein
slt (*orf26*)	220	81/85	30/65	Soluble lytic murein transglycosylase
esrA	937	90/93	32/48	Regulatory sensor kinase
esrB	214	96/98	48/66	Response regulator

[a] Where named differently, comparable *E. tarda* genes are in parentheses. L-ALIGN and protein structure analysis of the *E. ictaluri* homologues of *E. tarda orf2*, *orf1A*, *orf1B*, *orf13*, *orf19*, and *orf26* demonstrated significant similarity to the *S. enterica* serovar Typhimurium homologues, and the *E. ictaluri* homologues are named accordingly.

[b] Based on analysis of sixfold coverage of the *E. ictaluri* genome in this region, *E. ictaluri* encodes an *eseG* gene that has a truncated amino terminus due to the loss of an A at position 11382 of the DQ233733 sequence that introduces a stop codon. The *E. ictaluri ureG* gene starts at bp 120 of the *E. tarda ureG* gene.

[c] Formerly *eseA* (see reference 88).

tent with these results but does not rule out an effect on the expression of an unknown virulence gene.

Although the CI for 197GH is only 0.16, indicating a negligible effect on virulence, the 197GH insertion is only 51 bp from the stop codon of *adiA*, which could result in the production of a truncated, partially functional enzyme. The insertion in 234AB, however, is in the amino terminus, which would result in the production of a severely truncated product that would be unlikely to have enzymatic activity. The lower CI for 234AB, at 0.00013, indicates that the mutation in 234AB has a significant attenuating effect and suggests that AdiA plays a role in *E. ictaluri* pathogenesis. The metabolism of arginine in eukaryotic cells is a complex process involving inducible nitric oxide synthase, arginases, and AdiA (9). Significant interregulation of these pathways is involved in the control of a range of metabolic functions, including inducible nitric oxide synthase activity and programmed cell death, which are also important in relation to intracellular bacterial infections (42). Although bacterial arginase activity is known to be involved in the inhibition of host cell nitric oxide production by *Helicobacter pylori* (32), there are no reports connecting bacterial AdiA and bacterial virulence, except for several reports that evaluate the role of AdiA in environmental acid resistance (16, 52, 53). There is a report, however, that elevated dietary arginine significantly reduces catfish mortality following an *E. ictaluri* im-

TABLE 5. Percentage of bacteria initially added to wells that were recovered from CCO cells or HKDM

Strain	MOI (CFU/cell)	% CFU recovered from[a]:	
		CCO cells	HKDM
Wild type	1:10	0.37 ± 0.12	56.0 ± 15.95
	1:1	0.41 ± 0.15	ND
65ST	1:10	0.42 ± 0.05	37.3 ± 5.49
	1:1	0.55 ± 0.03	ND

[a] Percentage of bacteria recovered following a 30-min exposure to either wild-type *E. ictaluri* or the EsaU mutant 65ST and a 60-min exposure to a killing dose of gentamicin. Values are the means ± standard errors of the results of three experiments. There were no significant differences between the results for the mutant and wild-type strains. ND, not determined.

TABLE 6. Increase in numbers of CFU over time for wild-type *Edwardsiella ictaluri* and the EsaU mutant, 65ST, following infection of HKDM and CCO cells[a]

Cell type and time (h) postinfection	No. of CFU of wild type/well	Fold increase from time zero[b]	No. of CFU of 65ST/well	Fold increase from time zero[b]
HKDM				
0	1,483 ± 33.3		616 ± 83.3	
5	6,500 ± 2,179.4	4.3	517 ± 120.2**	0.85
10	53,333 ± 23,154.1	35.3	817 ± 183.3***	1.3
CCO cells				
0	413 ± 48.1		477 ± 8.8	
4	1,793 ± 218.3	4.3	283 ± 18.6***	0.59
8	5,233 ± 611.9	12.7	226 ± 21.9***	0.47

[a] CCO and HKCM cells were infected in 24-well plates at MOIs of 1 CFU:10 HKDM cells and 1 CFU:1 CCO cell. CFU data are the means ± standard errors of the results for triplicate wells and are representative of one of three experiments. A significant difference for mutant compared to wild type following a log transformation of the CFU data are indicated by asterisks. **, $P < 0.01$; ***, $P = 0.001$.

[b] Time zero is at 90 min postinfection (30 min of exposure and 60 min of gentamicin killing).

mersion challenge. The dietary arginine levels for the maximum growth of catfish were determined to be 0.8 to 0.9% of a 24%-protein diet (12). When evaluated relative to exposure to *E. ictaluri*, however, providing arginine at 0.5, 1, 2, and 4% of the diet resulted in cumulative percent mortalities of 16, 15, 7, and 7%, respectively, after 28 days (12). The fact that doubling dietary arginine increased the resistance of catfish to *E. ictaluri* infection further indicates that arginine metabolism, possibly involving AdiA, is important in *E. ictaluri* pathogenesis. Booth (10) reported that *E. ictaluri* produces an acid-inducible urease enzyme that is required for replication in catfish macrophages and speculated that AdiA could be involved in the de novo synthesis of urea, which could be metabolized to ammonia by the urease enzyme, resulting in an increase in environmental pH.

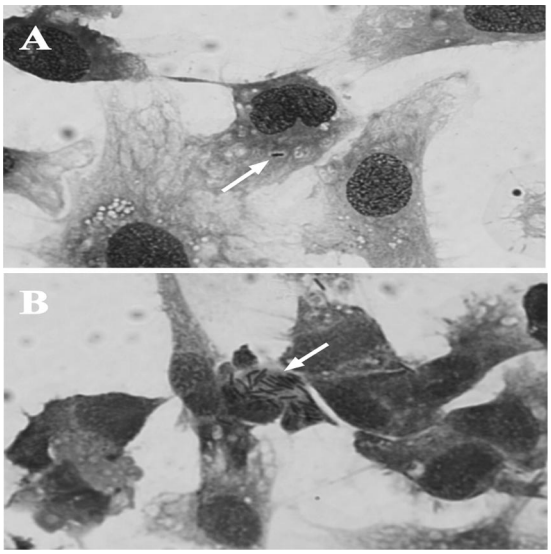

FIG. 4. CCO cells infected with *E. ictaluri* at 8 h postinfection in a gentamicin survival assay. (A) Cells infected with an *E. ictaluri esaU* mutant, 65ST, showing typical single bacterium per cell. (B) Cells infected with wild-type *E. ictaluri* strain 93-146, showing typical large number of bacterial cells per infected CCO cell. Both the mutant and the wild type typically had 1 to 2 bacterial cells per CCO cell immediately following infection.

Insertions in hypothetical genes in other pathogens. A role for MCP in *Vibrio cholerae* and *Vibrio vulnificus* virulence is suspected because MCP expression was induced in vivo following the infection of mice with *V. cholerae* (14) and in humans with *V. vulnificus* (45). In *Vibrio anguillarum*, the chemotactic gene *cheR* was important to virulence in fish following immersion challenge, but not following intraperitoneal injection (66). *Vibrio cholerae* encodes TcpI, a 620-amino-acid protein with similarity to the highly conserved regulatory domain of MCPs. TcpI is thought to regulate the major pilin subunit of the toxin-coregulated pilus while at the same time reducing chemotactic motility, possibly to facilitate *V. cholerae* microcolony formation (35). Motility and pilus formation are also coregulated by an MCP in *Pseudomonas aeruginosa* (35). All of these studies indicate an important role for MCP in the initiation of infection, and the high degree of attenuation of 168AB in catfish may indicate a similar role for MCP in the initiation of an *E. ictaluri* infection in catfish, especially when exposure to the host is via a waterborne, immersion route.

The presence of pentapeptide repeats in 72NO places the putative protein in a family of proteins that have predicted β-helical structures. The pentapeptide-repeat family of proteins are both cytoplasm and membrane bound. They are most

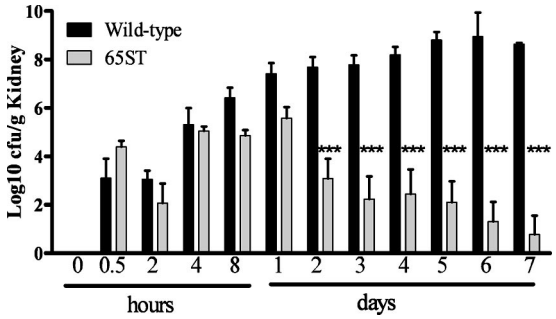

FIG. 5. Comparison of the early invasion and persistence of wild-type *Edwardsiella ictaluri* and an *esaU* mutant, 65ST, in channel catfish. Head kidney samples were taken from each of five fish at each time point, and the data represent the means of the results, with bars representing the standard errors of the means. ***, significant difference from the corresponding wild type ($P > 0.001$).

common in cyanobacteria but have also been identified in *Bacillus subtilis*, *E. coli*, and *Erwinia stewartii*, as well as plant proteins (43). The function of the repeats is uncertain, as is the molecular activity of the proteins, but the predicted structure suggests a targeting or structural function (7). Analysis in the PSORT II server indicates a predicted cytoplasmic or nuclear location for the *E. ictaluri* protein. In any case, the extremely low CI of 0.00000062 suggests an important role in *E. ictaluri* virulence.

Insertions in known virulence-related genes in other pathogens. Interestingly, two of the mutants found in this study had insertions in genes encoding a urease complex, even though *E. ictaluri* is reported as urease negative in standard biochemical testing (85). The urease enzyme is a multisubunit molecule that catalyzes the breakdown of urea to form carbonate and ammonia, resulting in a net increase in pH. Urease is known to play a significant role in the virulence of several pathogens, including *H. pylori*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *E. coli* O157:H7 (13, 30, 56, 62). Sequence analysis, however, links the nine genes of the *E. ictaluri* urease enzyme complex most closely to the complexes of *Y. enterocolitica*, *M. morganii*, and *Lactococcus fermentum*, with the *ureABCEFGD* gene sequence maintained and 67.1 to 91.2% amino acid identity to the *Y. enterocolitica* urease proteins. These three ureases are distinct among bacterial ureases because they are acid activated, with a pH optimum of around 3 to 4, and they encode a conserved acid activation motif in the catalytic site of the urease α subunit (UreC) (80, 87). This acid activation motif is conserved in *E. ictaluri*, and a requirement for acidic conditions may explain the fact that *E. ictaluri* is urease negative in standard biochemical tests (85), which are done at neutral pH. For a facultative intracellular pathogen capable of surviving and replicating in catfish HKDM (11), the ability to increase the environmental pH could be important in passage through the gastrointestinal tract or in survival in the acid phagosomes of macrophages.

The strong similarity in structure and amino acid sequence of Orf1 of pEI1 and the STEs suggests a similarity in function. In general, LRR proteins are structural in nature and are involved in diverse protein-protein interactions, including signal transduction, cell adhesion, cell development, DNA repair, and RNA processing (46, 47). Of the three STEs with the greatest similarity to pEI1 Orf1, the most studied are SspH1 and SspH2, both of which are involved in *S. enterica* serovar Typhimurium virulence in cattle (59). In calves infected with Δ*sspH1* or Δ*sspH2* mutants, severe diarrhea and death occurred 1 to 4 days postinfection, similar to those infected with the wild type. An Δ*sspH1*-Δ*sspH2* double mutant, however, did not cause diarrhea or death (59). Although Blastn analysis of the *E. ictaluri sspH1-sspH2* gene homologue and the *E. ictaluri* genome database indicates the presence of at least three additional effectors with SspH1/2 homology that vary in the number of LRR and vary in amino acid homology with SspH1/2 from 51 to 71%, the high level of attenuation associated with the single mutation in *orf1* of pEI1 (CI = 0.0000062) indicates that the level of attenuation is similar to that of the *S. enterica* serovar Typhimurium double mutants in calves. This suggests either a more integral role in pathogenesis for *orf1* in *E. ictaluri* than for either *sspH1* or *sspH2* in *Salmonella* or that the TTSS effectors of *E. ictaluri* are simpler and less redundant than those of *Salmonella*.

Further sequence analysis identified a gene immediately downstream from the *spa15* homologue encoded by *orf1* of pEI2 that encodes a protein with high homology to OspB. OspB is a secreted effector of *Shigella* with unknown function (55, 71), although a role in invasion, intracellular replication, induction of macrophage apoptosis, or decreased intracellular ATP could not be demonstrated (71). The operon structure of *orf1* and the OspB homologue is typical of chaperone/effector gene arrangements, suggesting that *orf1* is a chaperone for the OspB homologue.

The *E. ictaluri* TTSS. Both *Edwardsiella* TTSS differ from the other SPI-2 class of TTSS in that they encode an AraC-type regulator, EsrC. In *E. tarda*, the expression of EsrC is under the control of the EsrA/EsrB two-component regulators, both of which then act in concert to regulate the expression of different components of the TTSS (89). Both systems also encode a soluble lytic murein transglycosylase (*slt*) that may be cotranscribed and translationally coupled to the *esaR-U* inner membrane components of the TTSS apparatus and is presumably involved in local rearrangement of the peptidoglycan to allow needle complex formation in the periplasmic space (48, 69). Although peptidoglycan modulation is important in the formation of the *Salmonella* SPI-1 needle complex (48, 69), the *slt* genes of *S. enterica* serovar Typhimurium and *E. coli* are located at centisomes 99.621 and 99.802, substantially removed from the SPI-1-type TTSS at centisomes 61.892 to 62.6759 for *Salmonella* and 84.26 to 84.767 for *E. coli*.

The comparison of nucleotide content relates the *E. ictaluri* TTSS more closely to *C. violaceum*, with 60.4 and 56.4% G+C content, respectively, than to the *S. enterica* serovar Typhimurium and *E. coli* systems, with 46.6 and 40.4% G+C content, respectively. This high G+C content is unusual for TTSS clusters of animal pathogens, which are generally lower in G+C content than the surrounding genome at 40 to 48% (4). At 60.4% G+C content, the *E. ictaluri* TTSS G+C content is higher than that of the surrounding genome (4, 37) and is more similar to the TTSS G+C content of plant pathogens (4), despite its obvious similarity to the SPI-2 class of TTSS. The *E. tarda* TTSS has an even higher percent G+C content, at 63.8%. The *Edwardsiella* TTSS genes should provide interesting new information in the evolutionary analysis of TTSS.

Further analysis indicated that *E. ictaluri* also carries sequences similar to *orf29* and *orf30* of *E. tarda* (89). Because *E. tarda orf29* and *orf30* are only 591 bp upstream from *esrB* and are reportedly under the regulatory control of both EsrA-EsrB and EsrC, Zheng et al. (89) considered them part of the TTSS island of *E. tarda*. In addition, based on the presence of a predicted coiled-coil secondary structure that is common in TTSS effectors (21), Zheng et al. (89) suggested that Orf29 and Orf30 are *E. tarda* effectors. In comparison, *orf29* and *orf30* of the *E. ictaluri* TTSS are located 1,667 bp upstream from the start of *esrB*, and several ORFs whose products have similarity to a variety of transposases of gram-negative transposons are encoded in the intervening sequence. In addition, based on sixfold coverage of this region in the *E. ictaluri* genome project, the region carrying *orf29* and *orf30* appears to encode a single protein, Orf29/30. Putative transcription and translation initiation regions are found upstream from the start of *orf29-30*,

but a putative translation initiation region was not apparent immediately upstream of the putative *orf30* start codon of *E. ictaluri*. Although Orf29/30 of *E. ictaluri* has extensive coiled domains, a role as an effector remains to be determined. Additional work is required to evaluate the relationship of Orf29/30 to the *E. ictaluri* TTSS and its role in pathogenesis.

The severe attenuation of the *E. ictaluri* TTSS mutants is consistent with data for *S. enterica* serovar Typhimurium showing that SPI-2-encoded TTSS mutants have at least a fivefold increase in 50% lethal dose values in mice (72). The ability of the *E. ictaluri* TTSS mutants to cross the epithelial barrier is also consistent with *S. enterica* serovar Typhimurium SPI-2 TTSS mutants, which are equally attenuated by either oral or intraperitoneal exposure (72), indicating that SPI-2 is required for events in the infectious process that occur after penetration. Mutations in the TTSS of *E. tarda* resulted in a 1-log increase in the 50% lethal dose value, but neither efficiency of entry nor persistence was evaluated (81).

In conclusion, the identification of 50 mutants that are important for the survival of *E. ictaluri* in channel catfish offers significant insight into the pathogenesis of this pathogen, although a number of them are in housekeeping genes that have predictable growth- or replication-related phenotypes. The identification of effectors with close homology to the SspH1 and SspH2 effectors of the *Salmonella* SPI-2-encoded TTSS on plasmid pEI1 and in the chromosome implies a role in intracellular replication. Characterization of the TTSS *esaU* apparatus mutant demonstrates the importance of the secretion system in the pathogenesis of *E. ictaluri* in catfish and in intracellular survival. The variation in effectors compared to those of the *Salmonella* SPI-2 TTSS, in combination with the identification of a TTSS effector and its putative chaperone on plasmid pEI2 that have homology to an uncharacterized effector from *Shigella*, suggests that the *E. ictaluri* TTSS operates with some variation on the SPI-2 TTSS model.

ACKNOWLEDGMENT

This research was supported by the National Research Initiative of the United States Department of Agriculture Cooperative State Research, Education, and Extension Service with grant number 2002-35204-12605 to Ronald L. Thune.

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