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Attenuation, Persistence, and Vaccine Potential of an *Edwardsiella ictaluri purA* Mutant†

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In this study, an adenine-auxotrophic strain of *Edwardsiella ictaluri* was constructed and its virulence, tissue persistence, and vaccine efficacy were evaluated. A clone containing the *purA* gene was isolated from an *E. ictaluri* genomic library, sequenced, and shown to have an overall sequence identity of 79.3% at the nucleotide level and 85.7% at the amino acid level with the *Escherichia coli purA* gene. The cloned *E. ictaluri purA* gene was mutated by deleting a 598-bp segment of the gene and inserting the kanamycin resistance gene from Tn*903* into the gap. The Δ*purA*::Km^r gene was subcloned into the suicide plasmid pGP704, and the resulting plasmid was used to deliver the modified gene into a virulent strain of *E. ictaluri* by conjugation. Homologous recombination replaced the chromosomal *purA* gene with the mutated gene to create an adenine-auxotrophic strain (LSU-E2). Compared to wild-type *E. ictaluri*, LSU-E2 was highly attenuated by the injection, immersion, and oral routes of exposure. By the injection route, LSU-E2 had a 50% lethal dose (LD_{50}) that was greater than 5 $logs_{10}$ higher than the LD_{50} for wild-type *E. ictaluri*. In a tissue persistence study, LSU-E2 was able to invade channel catfish by the immersion route and persist in internal organs for at least 48 h. Channel catfish that were vaccinated with a single immersion dose of LSU-E2 had mortality significantly lower ($P < 0.01$) following a wild-type *E. ictaluri* challenge than that of nonvaccinated fish.

The largest aquaculture industry in the United States is the channel catfish industry, and the most serious disease affecting this industry is enteric septicemia of catfish (ESC) (32, 36). The causative agent, *Edwardsiella ictaluri*, was first described by Hawke in 1979 after it was isolated from catfish farms in Georgia and Alabama (15). It has since been reported in every state that produces channel catfish (61). A recent summary of catfish diagnostic cases for the years 1991 to 1995 in the southeastern United States indicates that 3,651 of 12,723 cases, or 28.7%, had *E. ictaluri* as the primary etiology (36).

E. ictaluri strains are serologically and biochemically homogeneous (6, 43, 44, 63). As a result, numerous researchers have evaluated killed bacterins for protection against ESC (29, 45–47, 50, 60, 62), with equivocal results. Many reported successes are difficult to evaluate due to a failure to control natural exposure to *E. ictaluri* in many of the studies, resulting in positive antibody titers in nonvaccinated fish. A protective response was suggested in field trials using a killed preparation, but Thune et al. (60) suggested that subclinical exposure of vaccinated fish to *E. ictaluri* during periods in which temperatures were not conducive to disease was important in establishing the response.

Vaccination of laboratory-reared, specific-pathogen-free (SPF) fish by immersion and oral methods induced an antibody response in vaccinated fish, but little or no protection from disease (59), indicating that cell-mediated immunity (CMI) is important in developing protective immunity to ESC. This hypothesis is supported by several studies using a variety of antigen preparations that were effective in stimulating antibodies but that failed to protect against disease unless very high titers were achieved (29, 46, 47, 59, 62). In addition, injection of fish with purified *E. ictaluri* lipopolysaccharide did not provide protective immunity against ESC unless Freund's complete adjuvant was used, despite the development of high antibody titers in all lipopolysaccharide-injected fish (50). These data, combined with the suggested intracellular nature of *E. ictaluri* (4, 37, 39), all indicate that stimulation of a strong CMI response is required for effective vaccination against ESC.

Injection of killed preparations in combination with adjuvants is one way to stimulate increased CMI, but because of the large numbers, small size, and low economic value of individual fish, this route of vaccination is not practical for use by commercial catfish producers. Live attenuated strains of facultative, intracellular bacterial pathogens that maintain the ability to invade host tissues by natural routes should also generate strong CMI. Because virulent strains of *E. ictaluri* are invasive in channel catfish via oral and immersion routes of exposure (4, 40, 53), a live attenuated strain of *E. ictaluri* that invades and temporarily persists in catfish tissues when delivered by either of these two routes should effectively stimulate a CMI response.

Certain auxotrophic phenotypes of bacterial pathogens are consistently attenuated because of the limited availability of a required nutrient(s) in vertebrate tissues, making them suitable for use as live vaccines (13, 56). The best known of these are the *aro* mutants, which are effective as live vaccines for several bacterial species (7, 20, 21, 30, 49, 55). A thymine-auxotrophic strain of *Shigella flexneri* is also effective as a live vaccine (1). Purine auxotrophs are consistently attenuated in mammalian hosts as well (3, 5, 8, 14, 19, 23, 28), especially when they have a specific requirement for adenine (34). In mammalian hosts, however, adenine-auxotrophic *Salmonella purA* mutants are less effective as vaccines than *aroA* mutants (42, 54), possibly because *purA* mutants are overly attenuated by the extremely low availability of adenine in mammalian

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TABLE 1. Bacterial strains and plasmids

Bacterial 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* phage lysogen	10
SM10 λ*pir*	Km^r *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir* phage lysogen	10
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	Louisiana State University Aquatic Animal Diagnostic Laboratory
LSU-E2	As for 93-146 except Δ*purA*::Tn*903* (Km^r)	This study
Plasmids		
pEIl1	Ap^r, pBluescript derivative with 1,104-bp *E. ictaluri purA* PCR product inserted in *Eco*RV site	This study
pEIl4	Km^r, pBK-CMV derivative with 5.6-kb segment of the *E. ictaluri* chromosome containing the *purA* gene inserted in *Bam*HI site	This study
pEIl5	Ap^r, pBluescript derivative with 3.5-kb *Not*I fragment containing the *purA* gene	This study
pNK2859	Ap^r Km^r, derivative of pBR322 with mini-Tn*10*Km (Tn*903* Km), *Ptac*^{P}	25
pEIl6	Ap^r Km^r, pEIl5 derivative with 598-bp *Nar*I deletion in the *purA* gene and 1.7-kb *Bam*HI segment of Tn*903* inserted in *Nar*I deletion site	This study
pGP704	Ap^r, pBR322 derivative with R6K *ori*, *mob* RP4, polylinker from M13 tg131	35
pEIl7	Ap^r, Km^r, pGP704 derivative with 4.6-kb *Not*I fragment containing modified *E. ictaluri purA* gene inserted in *Eco*RV site	This study

tissues (34). Although direct measurements of adenine availability in fish tissues are not available, it is known that certain aspects of purine metabolism in fish differ from those in mammals (17, 31, 38, 65). These metabolic differences may result in increased adenine availability in fish tissues compared to mammalian tissues, which could make adenine auxotrophs more effective as live attenuated bacterial vaccines in fish than they are in mammals. Consequently, this study was undertaken to produce an adenine-auxotrophic strain of *E. ictaluri* and evaluate its invasiveness, infectivity, and ability to protect channel catfish from ESC.

MATERIALS AND METHODS

Bacterial media and antibiotics. Bacterial strains, plasmids, and their sources are listed in Table 1. *Escherichia coli* was grown at 37°C with Luria-Bertani (LB) broth and agar plates (51). *E. ictaluri* was grown at 28°C with brain heart infusion (BHI) broth and agar plates or with Trypticase soy agar (TSA) II plates with 5% sheep blood (BA). λZap Express bacteriophage (Stratagene, La Jolla, Calif.) were grown in *E. coli* XL1-Blue MRF′ (Stratagene) with NZYM agar plates and NZYM top agarose (51). *E. ictaluri* defined minimal medium (EIMM) broth and agar plates (9) with and without supplemental adenine (25 μg/ml) were used for nutritional characterization of *E. ictaluri* strains. The API 20E system (bioMérieux Vitek, Hazelwood, Mo.) was used for species identification and biochemical characterization of *E. ictaluri* strains. Conjugates between *E. ictaluri* and *E. coli* were grown at 28°C on LB plates.

The F′ episome was maintained in *E. coli* XL1-Blue MRF′ with tetracycline selection at 12.5 μg/ml. Ampicillin at 200 μg/ml was used to maintain pBluescript (Stratagene), pGP704, and their derivatives. Kanamycin at 50 μg/ml was used to maintain plasmids derived from the pBK-CMV phagemid and plasmids carrying Tn*903*. Colistin at 10 μg/ml was used for counterselection against *E. coli* SM10 λ*pir* following conjugations. For blue-white screening of DNA cloned into pBluescript, *E. coli* XL1-Blue MRF′ was spread on LB plates with 100 μl of 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 μl of 2% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

DNA preparations and manipulations. *E. ictaluri* genomic DNA was prepared from 100-ml overnight cultures by a modification of the protocol of Ausubel et al. (2). Small- and large-scale preparations of plasmid DNA were made by alkaline lysis (51), and large-scale plasmid preparations were purified with Qiagen-tip 100 columns (Qiagen, Chatsworth, Calif.). Restriction endonucleases, DNA polymerase I Klenow fragment, calf intestinal alkaline phosphatase, and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's protocols. DNA fragments were purified from agarose gels with the Elu-Quick DNA Purification Kit (Schleicher & Schuell, Keene, N.H.).

Electroporation. Both *E. coli* and *E. ictaluri* were prepared for electroporation by the protocol of Ausubel et al. (2). Washed *E. coli* cells were transfected by electroporation in 0.2-cm cuvettes at 2.5 kV and 25 μF with the pulse controller set at 200 Ω, and washed *E. ictaluri* cells were electroporated by the same protocol at 1.75 kV. Bacteria were recovered for 1 h in BHI broth at 37°C (*E. coli*) or 28°C (*E. ictaluri*) before being spread on selective medium.

Amplification of a portion of the *E. ictaluri purA* gene. An 1,104-bp fragment of the *E. ictaluri purA* gene was amplified from genomic *E. ictaluri* DNA by PCR with primers derived from conserved regions of the *E. coli purA* gene based on alignments of published *purA* gene sequences (26, 33, 64). All PCRs were conducted on a Perkin-Elmer (Foster City, Calif.) DNA Thermal Cycler 480 with AmpliTaq DNA polymerase at pH 8.5 with a magnesium concentration of 1.5 mM and with 125 ng of template DNA per reaction mixture and at concentrations of 0.25 μM for each primer and 30 μM for each deoxynucleoside triphosphate. Cycle conditions were at 95°C for 30 s, 53°C for 45 s, and 72°C for 45 s for 35 cycles, with an initial denaturation step at 95°C for 2 min and a final extension step at 72°C for 10 min. To increase PCR product yield, a second PCR was carried out with the product from the first PCR as the template (0.5 μl of a 1/10 dilution) under the same conditions. Prior to ligation into pBluescript, the *purA* PCR product was purified with the Elu-Quick DNA Purification Kit to remove excess primers and deoxynucleoside triphosphates. Primers for both PCR and sequencing were synthesized by solid-phase cyanoethyl phosphoramidite chemistry on a Perkin-Elmer/Applied Biosystems (ABI) DNA Synthesizer Model 394.

DNA hybridization. Agarose gels were prepared for Southern hybridization by the protocol of Ausubel et al. (2), and DNA was transferred to Nytran Plus 0.45-μm-pore-size nylon membranes (Schleicher & Schuell) with the PosiBlot 30-30 pressure blotter and pressure control station (Stratagene, Inc.). The 1,104-bp *E. ictaluri purA* PCR fragment was labeled with the ECL direct nucleic acid labeling and detection system (Amersham Life Sciences, Arlington Heights, Ill.) and used as a probe for both plaque and Southern hybridizations. Prehybridization, hybridization, and stringency washes were all performed in tubes at 41°C according to the ECL protocol with a hybridization oven with an integral rotisserie device, followed by chemiluminescent detection.

Library screening. An *E. ictaluri* genomic library was constructed by cloning *E. ictaluri* genomic DNA that had been partially digested with *Sau*3AI into the *Bam*HI site of λZap Express (Stratagene, Inc.). *E. coli* XL1-Blue MRF′ was infected with the library according to the λZap Express protocol and spread on three plates containing approximately 11,000 PFU per plate. Plaques were transferred to Nytran Plus 0.45-μm-pore-size nylon membranes and screened with the labeled 1,104-bp probe. One positive plaque was purified, excised as a phagemid in *E. coli* XLOLR according to the Stratagene protocol, and confirmed to contain the *purA* gene in its insert by Southern hybridization.

DNA sequencing. The cloned 1,104-bp *purA* PCR fragment (carried on plasmid pEI11) was sequenced on both strands by the dideoxynucleotide chain termination method (52) with Taquence Version 2.0 (United States Biochemical, Cleveland, Ohio). The complete sequence of the remainder of the *purA* gene and flanking chromosomal sequence (carried on plasmid pEIl4) was determined with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit and an

ABI Prism 310 Genetic Analyzer according to the manufacturer's directions (Perkin-Elmer). The ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit was also used to sequence PCR products amplified from *E. ictaluri* LSU-E2 genomic DNA. Generated sequences were assembled, analyzed, and aligned with other sequences with the Wisconsin Sequence Analysis Package Version 8.0 (Genetics Computer Group, Madison, Wis.) and PC/Gene (IntelliGenetics, Mountain View, Calif.).

Bacterial conjugation. Conjugation was conducted between *E. coli* SM10 λ*pir* and *E. ictaluri* 93-146 by a modification of the protocol of de Lorenzo and Timmis (10) in which the two bacteria were mixed in 5 ml of 10 mM $MgSO_4$ in a 2:1 ratio (approximately 1.3×10^8 *E. ictaluri* cells and 6.5×10^7 *E. coli* cells), collected on a Gelman 0.45 μM Metricel filter, and incubated overnight at 28°C on LB agar plates. Bacteria were resuspended in 5 ml of 10 mM $MgSO_4$, and mutant *E. ictaluri* colonies were selected by spreading 100-μl aliquots of the suspension on LB-kanamycin-colistin plates. Two hundred fifty kanamycin-resistant colonies were screened for ampicillin sensitivity by patching colonies onto LB-ampicillin-kanamycin plates.

Phenotypic stability of LSU-E2 in nonselective media. The stability of the adenine-auxotrophic phenotype in *E. ictaluri* LSU-E2 was confirmed by passing the mutant through 30 overnight 5-ml BHI broth cultures. The final two 5-ml cultures were pelleted by centrifugation in a swinging bucket rotor at $4,750 \times g$ for 10 min, resuspended in 0.9 ml of sterile 0.9% saline, spread on EIMM plates, and checked for growth after 4 days. Totals of *E. ictaluri* cells screened were determined by dilution plate counts.

Genotypic confirmation of LSU-E2. The LSU-E2 Δ*purA*::Km^r construction was confirmed in a series of PCRs with genomic DNA from LSU-E2 and *E. ictaluri* 93-146 as templates. Plasmid pEII7 DNA was used as a positive control for the reactions. Primer pairs included 903Kan^+ and 903Kan^-, specific for the Tn*903* sequence; pBRAmp^+ and pBRAmp^-, specific for the ampicillin resistance gene sequence; PurAU1, specific for the *E. ictaluri purA* gene sequence, and 903Kan^+; and PurAM11, specific for the *E. ictaluri purA* gene sequence, and Tn903M2, specific for the Tn*903* sequence. The PurAU1/903Kan^+ PCR product and the PurAM11/Tn903M2 PCR product amplified from LSU-E2 DNA were both sequenced to confirm their identities.

SPF channel catfish. Egg masses were obtained from commercial channel catfish producers with no history of ESC outbreaks, disinfected with 100 ppm of free iodine, and hatched in closed recirculating systems in an SPF laboratory. Fish were maintained in the SPF laboratory through either the fingerling or the juvenile stage. Prior to experimental infection, SPF fish were restocked into 20-liter tanks in separate challenge laboratories and acclimated for 2 to 4 weeks. Flow rates in the challenge tanks were maintained at approximately 300 to 400 ml per min with dechlorinated municipal water, and temperatures were maintained at 25 ± 1°C.

Attenuation trial. Six hundred SPF channel catfish fingerlings, weighing approximately 5 g each, were stocked at a rate of 10 per tank, and three tanks were randomly assigned to each of 20 treatment groups. Treatment groups were experimentally infected with a specified dose of wild-type *E. ictaluri* or LSU-E2 *E. ictaluri* by one of three routes: immersion, injection, or oral administration. For the immersion challenge, the specified dose of either 93-146 wild-type or LSU-E2 bacterial culture was added directly to the tanks. For the wild type, two doses were used: a low dose of approximately 6.5×10^6 CFU/ml and a high dose of approximately 6.5×10^7 CFU/ml. Two doses were also used for LSU-E2: approximately 1.6×10^6 CFU/ml and 1.6×10^7 CFU/ml. Negative control treatment tanks were exposed to 200 ml of BHI broth. For the injection, challenge fish were anesthetized with 100 mg of tricaine methanesulfonate (MS-222) per liter and given intraperitoneal injections of 0.1 ml of the specified dose of 93-146 wild type or LSU-E2. Five treatment groups were injected with wild-type *E. ictaluri* at doses corresponding to approximately 1.3×10^2 to 1.3×10^6 CFU/fish, and four treatment groups were injected with LSU-E2 at doses corresponding to 1.6×10^5 to 1.6×10^8 CFU/fish. Negative control fish were each injected with 0.1 ml of BHI broth. Orally challenged fish were also anesthetized with MS-222, and a straight 1.5-in 22-gauge oral feeding tube was passed into the stomach of each fish to deliver 0.1 ml of the specified dose of 93-146 wild type or LSU-E2. Two treatment groups were infused with wild-type *E. ictaluri* at doses corresponding to approximately 1.3×10^8 and 1.3×10^9 CFU/fish, and two treatment groups were infused with LSU-E2 at doses corresponding to approximately 1.6×10^7 and 1.6×10^8 CFU/fish. Negative control fish were each infused with 0.1 ml of BHI broth.

Mortality was recorded for each 24-h period after experimental infection until three consecutive days passed without mortality. A sample of trunk kidney from each dead fish was streaked on BA to confirm *E. ictaluri* as the cause of death. Samples from fish exposed to wild-type *E. ictaluri* were cultured on BA, and samples from fish exposed to LSU-E2 *E. ictaluri* were cultured on both BHI plates and BA. For the injection challenge, a 50% lethal dose (LD_{50}) was calculated for wild-type and mutant *E. ictaluri* by the method of Reed and Muench (48).

Tissue distribution and persistence. Sixty SPF channel catfish fingerlings were stocked at a rate of 10 per tank and randomly divided into two treatment groups with three tanks per treatment. One of the treatment groups was experimentally infected with wild-type *E. ictaluri*, and the other was experimentally infected with LSU-E2. Both experimental infections were by immersion exposure. Wild-type *E. ictaluri* or LSU-E2 bacterial culture was added directly to the flowthrough tanks at doses corresponding to approximately 3.5×10^7 CFU/ml for wild type and 6.7×10^6 CFU/ml for LSU-E2. Water flow was stopped for 15 min following initial exposure and then resumed.

At 2, 6, 12, 24, 48, and 72 h postexposure, one fish was removed from each tank and euthanized by transfer to water containing 1 g of MS-222 per liter. In the wild-type treatment, the study was extended and fish were also collected at 96 and 120 h, although only two fish were sampled at 120 h. One fish was collected from each tank prior to experimental infections for a 0-h sample. By aseptic technique, samples were taken of liver, spleen, head kidney, and trunk kidney from each fish, suspended in 0.5 ml of sterile 0.9% saline solution, weighed, and pulverized. The resulting suspension was serially diluted in 0.9% saline solution in triplicate with 96-well plates, and 20-μl aliquots were dropped onto BHI plates for quantification. Colonies were counted after incubation for 48 h. *E. ictaluri* and other bacterial species were identified with the API 20E system.

The averages of the 3 $\log_{10}$-transformed bacterial concentrations for each tissue at each time were compared with SAS Version 6 (SAS Institute, Cary, N.C.) analysis of variance in the general linear models procedure. Tukey's studentized range test (per SAS program manual) was used for main effects, and the least-squares means Bonferroni adjusted *t* test was used for interaction effects.

Vaccine trial. One hundred eighty juvenile SPF channel catfish were stocked at a rate of 15 per tank and randomly divided into two treatment groups with six tanks per treatment. One treatment group was vaccinated with LSU-E2 *E. ictaluri* by immersion, and the other group was not vaccinated. Two hundred milliliters of an LSU-E2 overnight culture was added directly to the tanks designated for vaccinated fish, and water flow was stopped for 15 min following initial exposure. Bacterial concentration in the water was approximately 3.65×10^7 CFU/ml.

On day 27 postvaccination, both vaccinated and nonvaccinated treatment tank fish were experimentally infected with wild-type *E. ictaluri* by immersion exposure. *E. ictaluri* bacterial culture was added directly to the flowthrough tanks for a final bacterial concentration of approximately 5.3×10^7 CFU/liter in the water. Water flow was stopped for 15 min following initial exposure and then resumed. Mortality was recorded for each 24-h period after experimental infection until day 26 postexposure. Bacterial cultures were taken from the trunk kidney of each dead fish and cultured on BA to confirm *E. ictaluri* as the cause of death.

Cumulative daily percent mortality data for each tank was subjected to arcsine transformation, and each day's mean transformed percent mortality for vaccinated tanks was compared with each day's mean for nonvaccinated tanks by repeated-measures analysis with SAS Version 6 analysis of variance in a split-plot arrangement of treatments with treatment and tank on the main plot and time on the subplot.

Nucleotide sequence. The nucleotide sequence for the 1,544-bp *purA* operon containing the 1,299-bp *E. ictaluri purA* gene will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession no. AF026490.

RESULTS

PCR amplification and gene cloning. Primers based on conserved regions of the *E. coli purA* gene successfully amplified an 1,104-bp fragment of the *E. ictaluri purA* gene, which was cloned into pBluescript and sequenced to confirmed that a portion of the *E. ictaluri purA* gene had been amplified. The *E. ictaluri purA* PCR product had 79.1% identity with the *E. coli purA* gene. Screening of the *E. ictaluri* genomic library with the labeled PCR product yielded plasmid pEII4. Restriction analysis and Southern hybridization with the labeled *E. ictaluri purA* PCR fragment as a probe confirmed that pEII4 carried the *E. ictaluri purA* gene in its insert.

Sequencing results. Table 2 shows a comparison of the *E. ictaluri purA* gene sequence and its deduced amino acid sequence with known bacterial *purA* gene sequences. The *E. ictaluri purA* gene has high GC content (59.1%), which reflects the overall high GC content in the *E. ictaluri* chromosome (53 mol%) (16). The lengths of the *E. ictaluri*, *E. coli*, and *Haemophilus influenzae purA* genes are all identical at 1,299 bp, and most of the other genes are within 9 bp of this length.

The highest identity at the nucleotide and amino acid levels is with *E. coli*, at 79.3 and 85.7%, respectively. Amino acid similarity with *E. coli* is also very high, at 91.9%. *Vibrio parahaemolyticus* and *H. influenzae* also had high identity with *E. ictaluri* at the nucleotide and amino acid levels, with amino acid similarity comparable to that of *E. coli*. Identity is not as high with the other gram-negative bacteria or with *Bacillus subtilis* and *Spiroplasma citri*, but all had amino acid similarity with *E. ictaluri* that was >60%.

TABLE 2. Comparison of the *E. ictaluri purA* gene with the *purA* genes from other bacterial species

Bacterial sp.	Gene length (bp)	GC content (%)	Nucleic acid identity[a]	Amino acid identity[a]	Amino acid similarity[a]	Source or reference
Edwardsiella ictaluri	1,299	59.1				This study
Escherichia coli	1,299	53.8	79.3	85.7	91.9	64
Vibrio parahaemolyticus	1,317	46.0	69.4	75.5	88.2	33a
Haemophilus influenzae	1,299	41.3	65.5	73.7	88.0	57a
Thiobacillus ferrooxidans	1,290	59.5	58.9	59.1	76.3	26
Brucella abortus	1,206	60.6	57.9	50.9	68.3	GenBank[b]
Bacillus subtilis	1,293	47.1	52.6	44.9	64.7	33
Spiroplasma citri	1,308	30.9	45.2	39.0	61.0	GenBank[c]

[a] Percent identities and similarities based on comparison to the *E. ictaluri purA* gene.
[b] GenBank accession no. L43054.
[c] GenBank accession no. L22971.

The *E. coli purA* operon has been characterized elsewhere (64), and the *E. ictaluri purA* operon appears to have similar features (Fig. 1). As in *E. coli*, the *E. ictaluri purA* gene appears to be in a monocistronic operon. The promoter sequence for initiation of transcription for the *E. coli purA* gene was identified as CTGTAA-(17 bp)-TAGAAT-(7 or 8 bp)-RNA start. The −10 and −35 regions upstream of the *E. ictaluri purA* gene are very similar to those of *E. coli*, with CTGAAA-(17 bp)-TAGAAT located in the same position relative to the start codon. Both align well with the consensus promoter sequence for transcription in *E. coli*, which is TTGACA-(17 bp)-TATAAT-(5 to 9 bp)-RNA start (22). In addition, a 6-bp sequence (GGTGAA) that is similar to the *E. coli purA* Shine-Dalgarno sequence (GGTGAT) is present 8 bp upstream of the start codon in *E. ictaluri*. *E. coli* has two regions downstream of its stop codon that have the potential to form hairpin loops, and the second of these has the features of a ρ-independent terminator. *E. ictaluri* has a 13-bp-long inverted repeat beginning 17 bp downstream of the stop codon that also has features that suggest that it is capable of ρ-independent termination, including a region of GC-rich split dyad symmetry followed by a string of T residues. The presence of this terminator is a good indication that the *purA* mRNA transcript contains no downstream genes.

Initiation of the *purA* transcript in *E. coli* is under the control of the PurR repressor protein and two 16-bp palindromic operator sequences, one of which is located at the 5′ end of the mRNA transcript and the other of which is located 90 bp upstream of the mRNA transcript (Fig. 1) (66). *E. ictaluri* does not have a similar sequence at either of these locations, but it does have a 16-bp sequence located approximately 50 bp upstream of the start of the *purA* mRNA transcript that aligns well with the 16-bp consensus *pur* operator sequence in *E. coli* (Fig. 1).

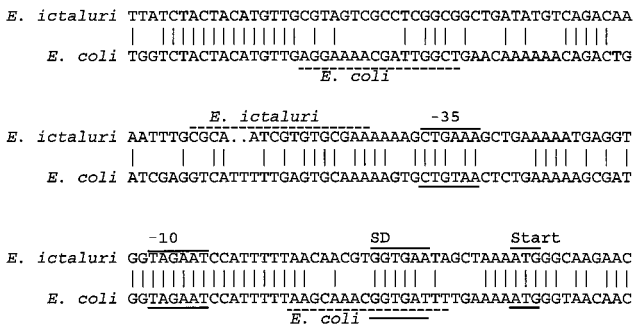

FIG. 1. Alignment of the *E. ictaluri purA* and *E. coli purA* promoter regions (64). Shine-Dalgarno sequences, −10 and −35 regions, and start codons are indicated for both *E. ictaluri* and *E. coli* by solid lines. Sixteen-base-pair palindromic *purA* operator sequences are indicated by dashed lines (66).

Construction of pEII7. The strategy used to construct plasmid pEII7 is shown in Fig. 2. First, a 3.5-kb *Not*I fragment of the pEII4 insert was subcloned into pBluescript to facilitate mutagenesis of the *E. ictaluri purA* gene, and the resulting 6.5-kb plasmid was designated pEII5. Next, the *E. ictaluri purA* gene in pEII5 was interrupted by deletion of a 598-bp *Nar*I fragment and insertion of the kanamycin resistance gene from Tn*903*, obtained as a 1.7-kb *Bam*HI fragment from plasmid pNK2859 (25). The resulting 7.6-kb plasmid was designated pEII6. Finally, the 4.6-kb *Not*I pEII6 insert was blunt end

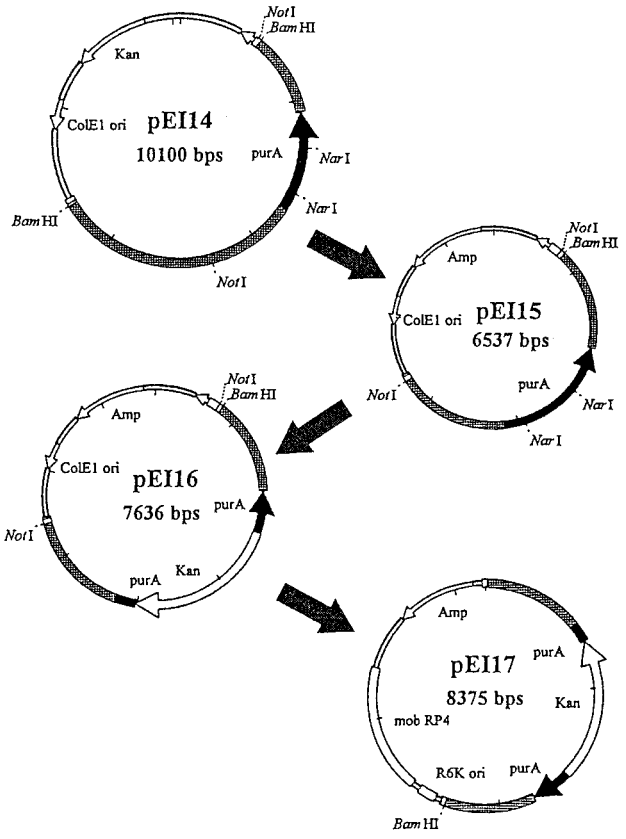

FIG. 2. Plasmids involved in the construction of pEII7, the plasmid used to transfer a mutated copy of the *purA* gene into wild-type *E. ictaluri* by conjugation.

ligated into the *Eco*RV site of the suicide plasmid pGP704 (35), and the resulting 8.5-kb plasmid carrying the Δ*purA*::Km^r construct and over 2,200 bp of flanking *E. ictaluri* chromosomal sequence was designated pEIl7.

Construction and characterization of a *purA* mutant strain of *E. ictaluri*. Transfer of pEIl7 into *E. ictaluri* by conjugation with *E. coli* SM10 λ*pir* as the donor strain was successful. In a single conjugation, 1,865 kanamycin-resistant *E. ictaluri* colonies were isolated from approximately 9.8×10^6 *E. ictaluri* CFU spread on selective media. Of 250 kanamycin-resistant colonies screened, four ampicillin-sensitive colonies were identified, indicating the occurrence of a double-crossover recombination event and incorporation of the deletion-insertion mutation in the chromosome with concurrent loss of the suicide plasmid. All four mutants failed to grow in EIMM alone but grew in EIMM supplemented with 25 μg of adenine per ml. Biochemically, the mutants maintained the wild-type phenotype, as determined with API 20E strips and sensitivity to antibiotics other than kanamycin, and showed no evidence of suicide plasmid persistence in plasmid preparations. Other than adenine auxotrophy, the only apparent change was a substantial reduction in colony size on BA, presumably due to the low availability of adenine in mammalian blood, which allows the mutants to be differentiated from wild-type strains. One of the mutants, designated LSU-E2, was selected for further characterization. Following 30 consecutive passages of LSU-E2 in BHI, no revertants to the prototrophic phenotype were detected after 7.5×10^{10} CFU from the final pass was spread on EIMM, indicating the stability of the mutant phenotype.

The genotype of LSU-E2 was confirmed by PCR (Fig. 3). Amplification of a portion of the Tn*903* kanamycin resistance gene with primers 903Kan$^+$ and 903Kan$^-$ yielded the predicted 624-bp band from mutant chromosomal DNA, indicating that the Tn*903* gene had been incorporated into the LSU-E2 chromosome. Only nonspecific bands were obtained from wild-type *E. ictaluri*. Amplification with primers pBRAmp$^+$ and pBRAmp$^-$ yielded only nonspecific bands from both LSU-E2 and wild-type *E. ictaluri*, indicating that the ampicillin resistance gene from pEIl7 had not been incorporated into the chromosome. Plasmid pEIl7 DNA that was used as a positive control for this reaction yielded the predicted 709-bp band. Primers PurAU1 and 903Kan$^+$ amplified the 3′ end of the Tn*903* insert and flanking *purA* gene sequence, resulting in an 1,191-bp fragment from LSU-E2 DNA and no amplification for wild-type DNA. Primers PurAM11 and Tn903M2 yielded a 587-bp fragment from LSU-E2 DNA containing the 5′ end of the Tn*903* insert and flanking *purA* gene sequence, and results were again negative for wild-type DNA. Sequencing of the 1,191-bp PurAU1/903Kan$^+$ fragment and the 587-bp PurAM11/Tn903M2 fragment demonstrated *purA* gene sequence up to the Tn*903* ligation site, after which the Tn*903* gene sequence began for both fragments.

Attenuation trial. Except as noted below, dead fish in the attenuation trial had external and internal lesions that were consistent with ESC, and *E. ictaluri* was isolated from all suspected ESC-killed fish. All of the isolates from fish exposed to LSU-E2 were positive for *E. ictaluri* on BHI plates, and no revertants to wild-type phenotype were detected on BA.

Final percent mortalities in the attenuation trial indicate that LSU-E2 is highly attenuated compared to the wild type by all three routes of exposure (Table 3). LSU-E2 had a calculated LD_{50} of 5.1×10^7 CFU per fish for the injection route of exposure, with mortality occurring only at the two highest doses. An LD_{50} could not be calculated for the wild-type injection exposure because the lowest dose, of 130 CFU/fish,

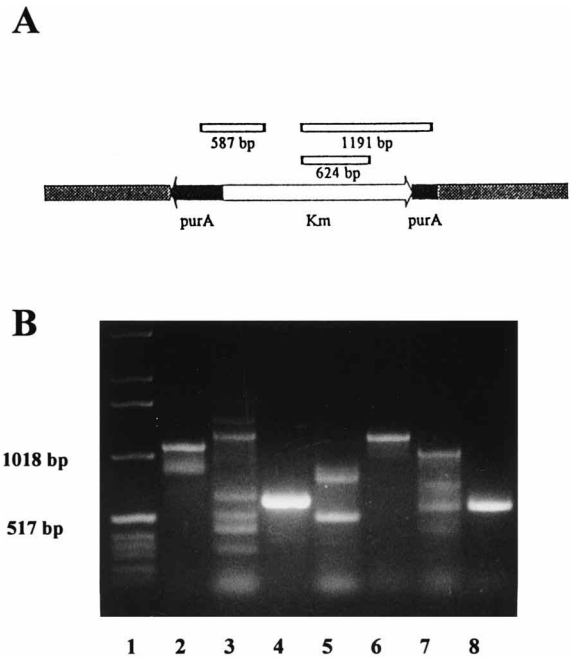

FIG. 3. Genotypic confirmation of the mutant LSU-E2 Δ*purA*::Km^r construct by PCR. (A) Graphic representation of the PCR amplifications used; the open bars above the LSU-E2 *purA*::Km^r construct represent the locations of the PCR products shown in panel B. (B) Agarose gel showing the PCR products. Lane 1 is a 1-kb ladder (Life Technologies, Gaithersburg, Md.). Lane 2 is the 1,104-bp PCR fragment amplified from wild-type *E. ictaluri* genomic DNA with primers inside the *purA* gene. Lane 3 is the results of PCR amplification from wild-type *E. ictaluri* genomic DNA with primers specific for the Tn*903* kanamycin resistance gene. Only nonspecific bands are present. Lane 4 is the 624-bp PCR fragment amplified from mutant LSU-E2 genomic DNA with the same two primers specific for Tn*903* used in lane 3. Lanes 5 and 7 are the results of PCR amplifications from wild-type genomic DNA with one primer specific for the *purA* gene plus strand and one primer specific for Tn*903*. Only nonspecific bands are present in both. Lane 6 is the 1,191-bp PCR fragment amplified from mutant LSU-E2 genomic DNA with the same primers used in lane 5. Lane 8 is the 587-bp PCR fragment amplified from mutant LSU-E2 genomic DNA with the same primers used in lane 7.

resulted in over 50% mortality. It can be concluded, however, that the LD_{50} for the wild type is <130 CFU/fish, which is more than 5 $\log_{10}$ lower than the LD_{50} for LSU-E2. The 1.3-×-10^5- and 1.3-×-10^6-CFU/fish doses of injected wild type caused 96.7 and 100% mortality, respectively, compared to 0% mortality at the equivalent doses for LSU-E2. No mortality was seen even at the high doses for LSU-E2 in immersion and oral exposures (Table 3). In contrast, 54.8% of fish exposed to wild-type *E. ictaluri* died with the highest oral dose and 63.3% died in the high-dose immersion exposure.

The kinetics of the injection exposure mortalities for wild-type and LSU-E2 *E. ictaluri* are shown in Fig. 4. In the high-dose LSU-E2 injection exposure (1.6×10^8 CFU), all of the deaths except for one occurred in the first 2 days following exposure. At the next dose of LSU-E2 (1.6×10^7 CFU), half of the deaths occurred in the first 2 days, and the other deaths occurred from day 4 to day 17 postexposure. Five of the seven deaths that occurred from days 4 to 17, however, had severe external lesions characteristic of *Cytophaga columnaris* infection. In contrast, deaths in the wild-type exposures occurred after a distinctive lag period that was more typical of a natural *E. ictaluri* infection. The length of the lag effect was dose dependent, as illustrated in Fig. 4. At the highest dose, deaths began very rapidly, similar to the high-dose challenge with

TABLE 3. Results of experimental infection of channel catfish fingerlings with wild-type *E. ictaluri* and LSU-E2 by three different routes of exposure

Treatment	Dose (CFU[a])	% Mortality
Immersion		
Wild type	6.5×10^6	16.7
Wild type	6.5×10^7	63.3
LSU-E2	1.6×10^6	0.0
LSU-E2	1.6×10^7	0.0
Control		0.0
Oral		
Wild type	1.3×10^8	20.7
Wild type	1.3×10^9	54.8
LSU-E2	1.6×10^7	0.0
LSU-E2	1.6×10^8	0.0
Control		0.0
Injection		
Wild type	1.3×10^2	55.6
Wild type	1.3×10^3	56.7
Wild type	1.3×10^4	72.4
Wild type	1.3×10^5	96.7
Wild type	1.3×10^6	100.0
LSU-E2	1.6×10^5	0.0
LSU-E2	1.6×10^6	0.0
LSU-E2	1.6×10^7	46.7
LSU-E2	1.6×10^8	53.3
Control		0.0

[a] For immersion route, doses are expressed as CFU per milliliter.

LSU-E2, but at the lower doses, almost all of the deaths occurred on day 6 or later.

Tissue distribution and persistence. All fish tested before the experimental infections were negative for isolation of *E. ictaluri* from any tissues. Adenine-auxotrophic *E. ictaluri* LSU-E2 was isolated from at least one internal organ in all of the immersion-exposed channel catfish samples from 2 to 48 h postexposure, indicating that its invasive capabilities are intact (Table 4). However, the infection was limited and no viable LSU-E2 cells were detected in any organs at 72 h. At every sampling time, bacterial concentrations in the tissues were significantly higher for wild-type *E. ictaluri* than for LSU-E2 (Fig. 5).

Head and trunk kidneys had the highest numbers of bacteria per gram of tissue, with about 10^4 CFU per g of tissue at 2 h postexposure (Fig. 5). Liver and spleen contained about 3 $\log_{10}$ fewer cells at 2 h. Numbers in the spleen continued to increase up to 24 h postinfection, while numbers in the liver remained low. Head and trunk kidney were also most likely to carry LSU-E2, with only one fish negative for isolation at 48 h (Table 4). In contrast, spleen was culture negative for at least one fish at 2, 6, and 48 h, while liver was culture negative for at least one fish at all times. In fact, the peak in average numbers of bacteria per gram of spleen at 24 h postexposure (Fig. 5) appears to result more from an increase in the number of fish culturing positive from the spleen rather than an increase in bacterial numbers in individual fish.

Similar to the attenuation trial, secondary bacterial infections were detected in some fish exposed to LSU-E2, except that they were detected on days 2 and 3 postexposure. Although no pathology or clinical signs were grossly apparent, three isolates of *Pseudomonas* sp. and one of *Aeromonas hydrophila* were identified.

In the wild-type exposure, there was approximately 10^4 CFU/g of tissue by 2 h postexposure, and by 6 h postexposure, numbers had increased to approximately 10^6 CFU/g (Fig. 6). Numbers increased to approximately 10^7 per g from 3 to 5 days postexposure, and all fish cultured positive for *E. ictaluri* at all sampling times (Table 4). Bacterial counts in the liver did not rise as quickly and were significantly lower than those in the other tissues, similar to the results for LSU-E2.

Vaccine trial. Feeding activity remained normal following immersion vaccination of channel catfish with LSU-E2 *E. ictaluri*, and there were no deaths. All fish that died following subsequent immersion challenge with wild-type *E. ictaluri* cultured positive for *E. ictaluri* from the trunk kidney. Results of the vaccine trial are presented in Table 5, and graphs of the mortalities for both vaccinated and nonvaccinated fish are shown in Fig. 6. Tanks containing nonvaccinated fish had a final average mortality of 33.3%, which is significantly higher

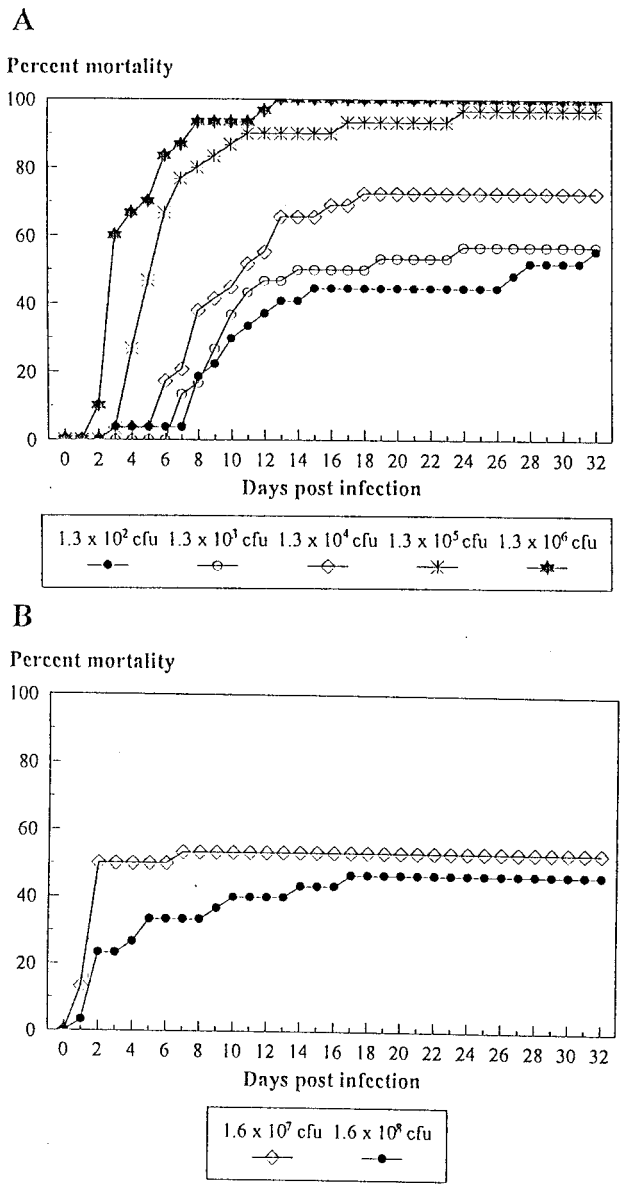

FIG. 4. Percent mortalities that resulted from intraperitoneal injection of wild-type *E. ictaluri* (A) and LSU-E2 (B) in channel catfish fingerlings at various doses over time. Doses represent the number of bacteria (CFU) injected per fish.

TABLE 4. Number of fish culturing positive from various tissues at different sampling times after immersion exposure to wild-type and LSU-E2 *E. ictaluri*[a]

Tissue	Wild type								LSU-E2						
	0 h	2 h	6 h	12 h	24 h	2 days	3 days	4 days	0 h	2 h	6 h	12 h	24 h	2 days	3 days
Spleen	0	3	3	3	3	3	3	3	0	1	2	3	3	2	0
Liver	0	3	3	3	3	1	3	3	0	1	2	2	1	2	0
Head kidney	0	3	3	3	3	3	3	3	0	3	3	3	3	2	0
Trunk kidney	0	3	3	3	3	3	3	3	0	3	3	3	3	2	0
Fish[b]	0	3	3	3	3	3	3	3	0	3	3	3	3	3	0

[a] Out of three fish per sampling time.
[b] Number of fish out of three that cultured positive from one or more tissues.

($P < 0.01$) than the average mortality of 11.1% in the tanks containing vaccinated fish. In fact, the average percent mortality in the tanks containing nonvaccinated fish was significantly higher ($P < 0.01$) than the average for the tanks containing vaccinated fish on each day from day 7 postexposure to the end of the study. The relative percent survival was 66.3 {relative percent survival = $100 \times [1 - (\%$ mortality vaccinated/% mortality controls)]}.

The first deaths occurred in both nonvaccinated and vaccinated fish on day 6 post-immersion exposure to wild-type *E. ictaluri* and steadily increased in nonvaccinated fish through day 19 postexposure (Fig. 6). In vaccinated fish, however, additional deaths were delayed until after day 10 postexposure, and almost all vaccinated fish deaths occurred between days 10 and 18 postexposure. As a result, the mean time of death for vaccinated fish was 13.9 days, compared to only 11.5 days for nonvaccinated fish.

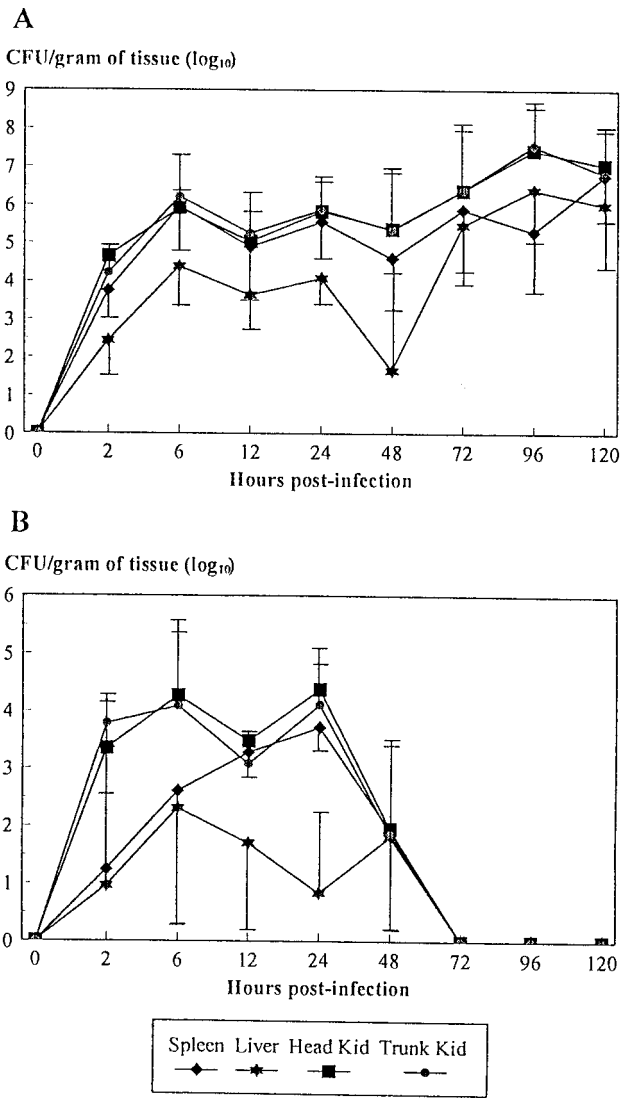

FIG. 5. Concentrations of wild-type *E. ictaluri* (A) and LSU-E2 (B) in different internal organs of channel catfish at various sampling times following immersion exposures. Numbers shown represent the averages of the $\log_{10}$ transformations of the number of bacteria per gram of tissue for the three tanks in each treatment. Kid, kidney.

DISCUSSION

The primary objective of this study was to develop a defined, rationally attenuated mutant strain of *E. ictaluri* that would remain invasive following immersion exposure for evaluation as a live vaccine. As the first step in this process, the *E. ictaluri purA* gene was successfully cloned and sequenced, resulting in the first published report of gene structure and sequence data for this important pathogen. Sequence results revealed that the *E. ictaluri purA* gene has greater sequence identity with the *E. coli purA* gene than with other known bacterial *purA* gene

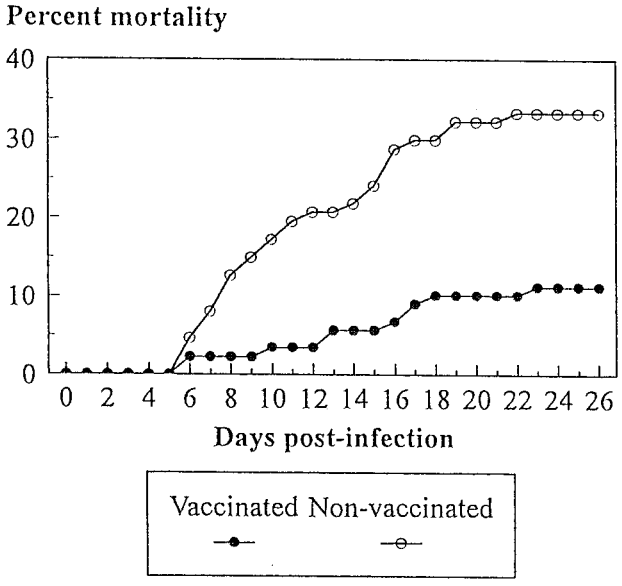

FIG. 6. Percent mortalities over time resulting from experimental *E. ictaluri* infection by immersion exposure for nonvaccinated channel catfish and for channel catfish that were vaccinated with LSU-E2 by immersion.

TABLE 5. Results from a single-dose LSU-E2 immersion vaccine trial with wild-type *E. ictaluri* immersion challenge

Fish	Tank	Mortality (no.)	Total no. of fish	% Mortality	Mean	SD
Nonvaccinated	1	6	15	40.0		
	2	7	15	46.7		
	3	8	14	57.1		
	4	3	15	20.0		
	5	3	14	21.4		
	6	2	14	14.3	33.3	17.2
Vaccinated	1	1	15	6.7		
	2	1	15	6.7		
	3	0	14	0.0		
	4	4	15	26.7		
	5	3	15	20.0		
	6	1	15	6.7	11.1	10.0

sequences (79.3%), which is expected because *E.* and *E. ictaluri* are both members of the family *Enterobacteriaceae*, and *E. coli* has greater overall identity with the *E. ictaluri* chromosome than most species within this family (relative binding ratio of 31% at 60°C) (16). The degree of identity between *E. coli* and *E. ictaluri* in the *purA* gene sequence is an indication that other genes critical to survival are conserved between the two species.

In *E. coli*, *B. subtilis*, and *Thiobacillus ferrooxidans*, the *purA* gene is under the control of its own promoter and is the only gene on its mRNA transcript (26, 33, 64). The *purA* gene is located at approximately min 95 on the *E. coli* chromosome, and it is unlinked to the *purB* gene at min 25 and the other *pur* genes involved in the synthesis of IMP from PRPP (5-phospho-α-D-ribosyl 1-pyrophate) (64). In fact, except for the *purHD*, *purMN*, *purEK*, and *guaBA* operons, the purine biosynthetic genes are all in monocistronic operons and dispersed on the *E. coli* chromosome (66). The *purA* gene in *E. ictaluri* also appears to be in a monocistronic operon and has features in common with the *E. coli purA* gene. The Shine-Dalgarno sequence and −10 and −35 regions are highly conserved between the species, and both have transcription terminators that appear to be ρ independent. In addition, the presence of a 16-bp sequence upstream of the *E. ictaluri purA* gene that is very similar to the 16-bp operator sequences upstream of the *E. coli purA* genes suggests that *E. ictaluri* uses a similar regulatory mechanism to control *purA* expression.

The mating and transfer of pEIl7 from SM10 λ*pir E. coli* into *E. ictaluri* are the first report of a genetic transfer system for *E. ictaluri*. The number of mutants obtained by transfer of pEIl7 into *E. ictaluri* (approximately 1 in >5,000 CFU screened) indicates the presence of an efficient system for homologous recombination in *E. ictaluri*. This conjugation system has also proven useful for the generation of random transposon mutants in our laboratory with the pGP704-based pLOF vectors (18).

The results of the experiments conducted to construct and characterize *E. ictaluri* mutant strain LSU-E2 demonstrated that the mutant meets several criteria important for a live attenuated vaccine against ESC. First, LSU-E2 is very stable and will not revert to the wild-type phenotype. Second, because precise genetic methods were used to construct LSU-E2, only the *purA* gene is mutated and genes that control pathogenesis are intact. Consequently, LSU-E2 maintains the ability to invade and infect fish following immersion exposure. In addition, all of the surface antigens of the virulent parent strain should be maintained in LSU-E2 for presentation to the host immune system. Finally, the genetic defect causing the adenine-auxotrophic phenotype in LSU-E2 is well characterized, and isolates from fish can be easily distinguished from wild-type *E. ictaluri* by growth in EIMM, by colony size on BA, or by PCR.

An effective live attenuated vaccine must also be safe. In the attenuation trial, it was shown that LSU-E2 has an LD_{50} that is greater than 5 $\log_{10}$ higher than the LD_{50} for wild-type *E. ictaluri* for the injection route of exposure. This difference is large enough to allow LSU-E2 to be used as a live vaccine, but it is less than the difference in LD_{50} seen between adenine-auxotrophic *Salmonella typhimurium* and wild-type *S. typhimurium* in mice. The LD_{50} for wild-type *S. typhimurium* ranged from 14 to <10 CFU by injection, while the LD_{50} for adenine-auxotrophic strains ranged from 10^7 to $10^{8.6}$ CFU (12, 42). Attenuation of adenine auxotrophs in mammals is apparently due to limited availability of adenine and adenosine in host tissues (34). Although direct measurements of adenine availability in fish tissues are not available, fish differ from mammals in certain aspects of purine metabolism, including the use of adenine in uricolysis (17) and in the purine nucleotide cycle (31), which produces ammonia for nitrogen excretion (38, 65). These metabolic differences may result in increased adenine availability in fish tissues compared to mammalian tissues and account for the lower degree of attenuation for the *purA* auxotroph in catfish. The results of this study indicate that the availability of adenine and adenosine in fish is limited, although possibly to a lesser extent than in mammals.

Mortality in the LSU-E2 injection treatments occurred only at the two highest doses. At the highest injection dose of 1.6×10^8 CFU/fish, all of the deaths except for one occurred in the first 2 days postexposure. Injection of LSU-E2 at 1.6×10^8 CFU per 5-g fish would result in a minimum of 3.2×10^7 CFU/g of tissue. This concentration is comparable to the concentrations of wild-type *E. ictaluri* seen in the internal organs on the final day of the tissue distribution study reported here (1×10^6 to 3.5×10^7 CFU/g of tissue), when the fish were entering the final stage of infection. Similar results were reported for a purine-auxotrophic strain of *Pseudomonas pseudomallei* that caused deaths in mice only within 2 days of intraperitoneal injection, presumably as a result of the toxicity of the bacterial suspensions (28). At the next highest injected dose for LSU-E2 (approximately 1.6×10^7 CFU), half of the deaths occurred in the first 2 days and half occurred between days 4 and 17 postexposure. Most of the later dead fish had severe external lesions characteristic of infection with *C. columnaris*, an opportunistic pathogen often associated with stress in channel catfish (58). It appears that this dose of LSU-E2 was stressful enough to predispose them to secondary infection with *C. columnaris*. No morbidity, mortality, or external lesions were apparent at the two lower doses. This study also demonstrated that LSU-E2 is attenuated compared to wild-type *E. ictaluri* by immersion and oral routes of exposure, because no morbidity or mortality occurred in fish exposed to LSU-E2 by either route. Based on the numbers of LSU-E2 cells present in the tissues at 12 h, it is unlikely that LSU-E2 is able to achieve numbers of bacteria in the tissues by the oral and immersion routes that are equivalent to the numbers achieved in the two high-dose injection exposures.

Only the immersion route of exposure was tested in the tissue distribution and persistence study because of its importance as a potential route of vaccination for commercial producers. This study demonstrated that the adenine-auxotrophic strain LSU-E2 has retained the ability to penetrate the host and colonize internal organs, indicating that attenuation of this strain in the immersion route of exposure is not due to its

inability to invade the host. LSU-E2 was isolated from at least one internal organ in all of the fish sampled from 2 to 48 h postexposure (Table 4).

Although wild-type *E. ictaluri* achieved higher tissue concentrations than LSU-E2 in the internal organs following immersion exposure, the relative tissue distributions for wild type and LSU-E2 were similar. Head and trunk kidneys were the most consistently colonized and had the highest tissue concentrations for both (Fig. 5). Wild-type concentrations were approximately 2 $\log_{10}$ higher than LSU-E2 in these organs until 24 h postinfection, when wild-type numbers began to increase, while mutant numbers began to decrease. The spleen had the next highest concentrations of wild type and LSU-E2, followed by the liver.

Baldwin and Newton (4) also saw higher concentrations of *E. ictaluri* in the trunk kidney than the liver following experimental infection of channel catfish fingerlings by oral exposure. By 96 h postinfection, bacterial concentrations in the liver and trunk kidney were similar in both studies (approximately 10^6 CFU/g of tissue in the liver and 10^7 CFU/g of tissue in the trunk kidney). By contrast, Nusbaum and Morrison (41) reported that counts of radiolabeled *E. ictaluri* cells were positive earlier, persisted longer, and were higher in the liver than in the trunk kidney following immersion exposure. The different tissue distribution reported by Nusbaum and Morrison may have resulted from an altered disease pathogenesis due to the low temperature at which the experiment was conducted (20°C), or it may reflect the detection of nonviable *E. ictaluri* antigens accumulated in the liver.

The *purA* mutant strain of *E. ictaluri* reported in this study was able to invade channel catfish by immersion exposure much more efficiently than a *Salmonella dublin purA* mutant was able to invade mice by oral exposure (54). Only one mouse cultured positive on day 1 postexposure, based on examination of the internal organs of mice exposed to the *S. dublin* mutant, and none were positive after that. However, *S. typhimurium purA* mutant strains injected intravenously in mice persisted at a level of about 10^4 CFU per organ in the liver and spleen until day 21 postexposure, after which they gradually cleared (42). Liver and spleen cultures were still positive for the mutant at 49 days postexposure. One possible explanation for the relatively brief persistence of the *E. ictaluri purA* mutant in channel catfish compared to the *S. typhimurium purA* mutant in mice is that different routes of exposure were used in the two studies. However, LSU-E2 concentrations in the head and trunk kidneys at 24 h following immersion exposure (approximately 10^4 CFU/g of tissue) were equivalent to concentrations of the *S. typhimurium purA* mutants in the liver and spleen at 24 h following injection exposure, indicating that the lack of LSU-E2 persistence is not due to a failure to achieve sufficient tissue concentrations. A more likely possibility is that the *E. ictaluri purA* mutant is more easily killed by macrophages than is the wild type, similar to guanine auxotrophs of *Yersinia pestis* and purine auxotrophs of *S. typhimurium* (11, 57), and is consequently cleared from the tissues quickly. Several investigators have suggested that *E. ictaluri* is capable of intracellular survival and replication in catfish macrophages (4, 37, 39), while others have reported an apparent inability to invade nonphagocytic cells (4, 24). *S. typhimurium*, on the other hand, is capable of penetration, survival, and replication inside diverse eukaryotic cell types, including epithelial and phagocytic cells (27). The extended persistence of the *S. typhimurium purA* mutant compared to the *E. ictaluri purA* mutant may be due to its ability to survive in cell types other than macrophages.

The results of the vaccine trial indicate that immersion vaccination of *E. ictaluri*-free channel catfish with a *purA* mutant strain of *E. ictaluri* provides significant protection against experimental infection with wild-type *E. ictaluri*. This is in contrast to results reported with other species. An *S. typhimurium purA* mutant was protective in mice following intraperitoneal injection of 2.5×10^5 or greater CFU but not at a dose of 2.5×10^3 CFU (34). In a subsequent study, an *S. typhimurium purA* mutant provided only minimal protection in mice following intravenous injection and failed to protect following oral vaccination (34). It was not determined, however, whether the failure to protect was due to a failure to invade or to a failure to induce protective immunity. Sigwart et al. (54), however, indicated that only one of three mice was culture positive for a *purA* mutant of *S. dublin* at day 1 postexposure and that all mice were culture negative on subsequent sampling days. They further reported that the *purA* mutant induced significantly less serum and mucosal antibody compared to an *aroA* mutant. Our study indicates that the *E. ictaluri purA* mutant retains its ability to invade from the water, persists in the host for at least 48 h, and provides significant protection of channel catfish from ESC by immersion vaccination under controlled laboratory conditions with *E. ictaluri*-free channel catfish. Because immersion vaccination is a practical method of vaccinating channel catfish fry and fingerlings, our adenine-auxotrophic LSU-E2 has potential as a live vaccine for use by commercial catfish producers.

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