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Fur-Regulated Iron Uptake System of *Edwardsiella ictaluri* and Its Influence on Pathogenesis and Immunogenicity in the Catfish Host

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The ability of bacterial pathogens to take up iron from the host during infection is necessary for their multiplication within the host. However, host high-affinity iron binding proteins limit levels of free iron in fluids and tissues. To overcome this deficiency of iron during infection, bacterial pathogens have developed iron uptake systems that are upregulated in the absence of iron, typically tightly controlled by the ferric uptake regulator (Fur) protein. The iron uptake system of *Edwardsiella ictaluri*, a host-restricted pathogen of channel catfish (*Ictalurus punctatus*) and the main pathogen of this fish in aquaculture, is unknown. Here we describe the *E. ictaluri* Fur protein, the iron uptake machinery controlled by Fur, and the effects of *fur* gene deletion on virulence and immunogenicity in the fish host. Analysis of the *E. ictaluri* Fur protein shows that it lacks the N-terminal region found in the majority of pathogen-encoded Fur proteins. However, it is fully functional in regulated genes encoding iron uptake proteins. *E. ictaluri* grown under iron-limited conditions upregulates an outer membrane protein (HemR) that shows heme-hemoglobin transport activity and is tightly regulated by Fur. *In vivo* studies showed that an *E. ictaluri* Δ*fur* mutant is attenuated and immune protective in zebrafish (*Danio rerio*) and catfish (*Ictalurus punctatus*), triggering systemic immunity. We conclude that an *E. ictaluri* Δ*fur* mutant could be an effective component of an immersion-oral vaccine for the catfish industry.

Vertebrates sequester iron from invading pathogens as a means of nutritional immunity, using high-affinity iron binding proteins to limit levels of free iron in biological fluids and tissues in order to deprive pathogens of this key nutritional component. Invading bacterial pathogens sense this iron depletion as a signal that they are within a host and induce the expression of genes that allow iron uptake in order to overcome the host defenses. To obtain this host sequestered iron, most pathogenic bacteria have developed iron uptake systems that usually are siderophore mediated or that directly take up iron from host proteins (50). Siderophore-mediated systems typically involve low-molecular-weight siderophores released by the bacteria that chelate iron and subsequently transfer it to iron-regulated outer membrane proteins (IROMPs) that function as receptors of the iron-siderophore complexes (39, 50). These siderophore mechanisms of iron acquisition have been linked to the virulence of different fish bacterial pathogens, such as *Vibrio anguillarum* (*Listonella anguillarum*) (38, 73), *Aeromonas salmonicida* (24, 27), *Photobacterium damselae* subsp. *piscicida* (13), *Edwardsiella tarda* (25), and *Tenacibaculum maritimum* (2). Direct iron uptake systems from host proteins rely on the interaction between specific microbial receptors and host transferrin or heme-containing compounds (31, 41) and often involve bacterial hemolytic or proteolytic activity (10). Usually, both siderophore-mediated and direct iron uptake systems are controlled by the ferric uptake regulator (Fur) protein (10).

Fur is a dimeric metal ion-dependent transcription regulator that controls the expression of genes involved in a diversity of cellular functions, including iron uptake. Fur monomers typically contain two structural domains, the N-terminal DNA binding domain and the C-terminal dimerization domain (26, 28, 29). When Fur monomers are bound to Fe^{2+}, they form a dimer that binds to promoter DNA regions (Fur boxes), repressing gene expression (29). During oral-gastric infection, it is thought that the small intestine conditions are anaerobic and therefore replete with free Fe^{2+}, leading to an active Fur protein that represses genes involved in iron uptake. The iron uptake system is induced upon invasion, when iron is presumably sequestered by host iron binding proteins (26, 28).

Channel catfish (*Ictalurus punctatus*) is the most important aquaculture species in the United States, accounting for more than 60% of all U.S. aquaculture production (20), and *Edwardsiella ictaluri* is one of the most important pathogens in this industry (61). The most highly upregulated group of catfish genes following *E. ictaluri* infection are the genes involved in iron homeostasis, including those for intelectin, haptoglobin, hemopexin (*Wap65*), ceruloplasmin, transferrin, and ferritin (34, 35, 45, 46, 63). This indicates that there is a "tug of war" for the iron between the catfish host and the bacterial pathogen *E. ictaluri*. While the iron acquisition system of *E. ictaluri* has not been previously characterized, we observed that *E. ictaluri* grown in the absence of iron upregulates synthesis of a specific outer membrane protein. This observation prompted us to investigate the iron uptake system of *E. ictaluri*.

In this study, we characterized the *E. ictaluri* Fur protein, the iron uptake system controlled by Fur, and the effects of the *fur* gene on virulence and immunogenicity in the fish host. We determined that fish isolates of *Edwardsiella* have a *fur* gene that is smaller than other *fur* family members, where its evolutionary pathway may have undergone genome degradation. We also established that *E. ictaluri* does not secrete detectable siderophores

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference(s)
Strains		
Escherichia coli		
χ6212	ϕ80d*lacZ*Δ*M15 deoR* Δ(*lacZYA-argF*)U169 *glnV44* λ^- *gyrA96 recA1 relA1 endA1* Δ*asdA4* Δ*zhf-2*::Tn*10 hsdR17* (r^- m^+); F^- Rec^- (UV^s) DAP^- Lac^- Nal^r Tet^s	12
χ7213	*thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1* RP4-2-Tc::Mu [λ*pir*] Δ*asdA4* Δ(*zhf-2*::Tn*10*); Km^r Tet^s Amp^s DAP^-	52
χ7232	*endA1 hsdR17* (r_K^- m_K+) *glnV44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)U169 λ*pir deoR* [ϕ*80dlac*Δ(*lacZ*)*M15*]; Nal^r UV^s Thi^- Lac^-	52
χ7122	O78	49
Edwardsiella ictaluri		
J100	Wild type; $pEI1^+$; $pEI2^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	47, 57
J135	J100 derivative; Δ*fur-35*; $pEI1^+$; $pEI2^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
J146	J100 derivative; Δ*hmuR36*; $pEI1^+$; $pEI2^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
J147	J135 derivative; Δ*fur-35* Δ*hmuR36*; $pEI1^+$; $pEI2^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
J135	J100 derivative; Δ*fur-35*; $pEI1^+$; $pEI2^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
Salmonella enterica serovar Typhimurium		
χ3761	UK-1	21, 36
χ11143	UK-1 Δ*fur-44*	This study
χ11143(pEZ116)	UK-1 Δ*fur-44*; pEZ116; Cm^r	This study
Plasmids		
pMEG-375	8,142 bp, Cm, Amp, *lacZ*, R6K *ori*, *mob*, *incP*, *sacR*, *sacB*	56
pR112	5173 bp, Cm, R6K *ori*, *oriT*, *oriV*, *sacR*, *sacB*	14
pACYC184	4,245 bp, Tet^r, Cm^r, p15A *ori*	7
pEZ151	4,065 bp, pSC101 *ori*, Gm^r GFP^+	55
pYA4807	Δ*fur-44*, Cm, pR112	This study
pEZ116	P_{fur}-*fur*, Cm^r, pACYC184	This study
pEZ123	Δ*fur-35*, pMEG-375	This study
pEZ155	Δ*hmuR36*, pMEG-375	This study
pEZ191	P_{fur}-*fur*, Gm^r, pEZ151	This study
pEZ192	P_{lac}-*hemR*, Gm^r, pEZ151	This study

but does contain a heme-hemoglobin uptake system regulated by Fur.

The Fur protein regulates not only iron uptake-related genes but also genes important to virulence. *Salmonella* Δ*fur* mutants are attenuated in mammals when administered orally (51) or intraperitoneally (16) but are not very immunogenic (11). We also evaluated the potential utilization of *E. ictaluri* Δ*fur* mutants as a live attenuated vaccine. *E. ictaluri* Δ*fur* mutants were attenuated in zebrafish and catfish hosts. When *E. ictaluri* Δ*fur* mutants were administered by immersion or orally, they conferred immune protection, triggering systemic and skin immune responses.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and reagents. The bacterial strains and plasmids used are listed in Table 1. Bacteriological media and components were from Difco (Franklin Lakes, NJ). Antibiotics and reagents were from Sigma (St. Louis, MO). LB broth (tryptone, 10 g; yeast extract 5 g; NaCl, 10 g; glucose, 1 g; double-distilled water [ddH_2O], 1 liter) (5), Bacto brain heart infusion (BHI) broth, chromoazurol S (CAS) broth (58), and Trypticase soy broth (TSB) were used routinely. When required, media were supplemented with 1.5% agar, 5% sucrose, colistin sulfate (Col) (12.5 μg/ml), ampicillin (Amp) (100 μg/ml), chloramphenicol (Cm) (25 μg/ml), kanamycin (Km) (50 μg/ml), $FeSO_4$ (150 μM) (Sigma), or 2,2′-dipyridyl (150 μM) (Sigma). Bacterial growth was monitored spectrophotometrically and/or by plating. Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England BioLabs. *Taq* DNA polymerase (New England BioLabs) was used in all PCR tests. Qiagen products (Hilden, Germany) were used to isolate plasmid DNA, gel purify fragments, or purify PCR products. T4 ligase, T4 DNA polymerase, and shrimp alkaline phosphatase (SAP) were from Promega.

Sequence analysis. A nucleotide Basic Local Alignment Search Tool (BLAST) search was performed based on the sequences of the putative *fur* and heme uptake genes present in the genome sequence of *E. ictaluri* 93-146 accessed from NCBI's Entrez Genome database (NC_012779). *fur* sequences were obtained from NCBI's Entrez Protein database for *Edwardsiella ictaluri* 93-146 (YP_002934295.1), *E. tarda* PPD 130/90 (AEO72442.1), *E. tarda* EIB202 (YP_003296656.1), *E. tarda* FL6-60 (ADM42454.1), *E. tarda* ATCC 23685 (ZP_06715756.1), *Escherichia coli* O157:H7 EDL933 (NP_286398.1), *Salmonella enterica* serovar Typhi Ty2 (NP_455254.1), *Yersinia pestis* KIM 10 (NP_668533.1), *Vibrio cholerae* (AAA27519.1), *A. salmonicida* A449 (YP_001143048.1), *Pseudomonas putida* (YP_001269900.1), and *Pseudomonas aeruginosa* (NP_253452.1).

Amino acid sequence alignments were performed using the CLC Free Workbench software tool (v. 6.1; CLC bio A/S, Aarhus, Denmark). Protein structure-based alignments were performed by using the web-based interface for ESPript v.2.2 located at http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi (18). The three-dimensional (3D) structures of the *E. ictaluri* Fur, HemP, HemR, HemT, HemU, HemV, and HemS proteins were predicted using position-specific iterative BLAST (PSI-BLAST) alignment and HHpred (62).

Construction of *E. ictaluri* mutants. The recombinant pEZ suicide vectors (Table 1) carrying the linked flanking regions to generate an in-frame deletion of the *fur* or *hmuR* gene were constructed as described previously (55–57). The defined deletion mutations encompass a deletion

TABLE 2 Primers used in this study

Primer	Sequence[a]
Δ*fur-35* (SphI) F1	5′-ACAT**GCATGC**TGGGTTAATGTCTGCCGCC-3′
Δ*fur-35* (XhoI) R1	5′-CCG**CTCGAG** ATCGATTAGCTTCTTGTACA-3′
Δ*fur-35* (XhoI) F2	5′-CCG**CTCGAG**TGAGTGACCTGGCGCCCACA-3′
Δ*fur-35* (XbaI) R2	5′-TCG**TCTAGA** TAAATACGGTCTATGTCATA-3′
Δ*hmuR* (SphI) F1	5′-ACAT**GCATGC** AAACTGCTTGCCGTCATCGG-3′
Δ*hmuR* (XhoI) R1	5′-CCG**CTCGAG** GGCTCAACACTCCAAATGTA-3′
Δ*hmuR* (XhoI) F2	5′-CCG**CTCGAG** CGCGATTTTCACCCCGGGGC-3′
Δ*hmuR* (XbaI) R2	5′-TCG**TCTAGA** AAAGGCGGCGTGCCAGTGCT-3′
Δ*fur-44* (BglII) F1	5′-GGA**AGATCTT**GTGTAAATCTTTCGAAGAGCC AACCG-3′
Δ*fur-44* (XmaI) R2	5′-TCC**CCCCGGG**TATACCCAGTATGGAGGCGGT ACTGG-3′
hmuR-F	5′-GACATCAACGCGGACAAATGGTCA-3′
hmuR-R	5′-GTTGGGACGCCAGTAGTTGACAAA-3′
mntH-F	5′-TATCTGCATTCGTCGCTGACCCAA-3′
mntH-R	5′-TGATCCCACTGTGCCCGTTAAAGT-3′
hemF-F	5′-ACCGCCTCATTGTTCAACTGCGTA-3′
hemF-R	5′-ATCAAAGGTCTGGATGCCGATGGA-3′
mgtB-F	5′-AGTCGACGAGCTTCCGTTTGACTT-3′
mgtB-R	5′-CGTGATCTTCAATGTGGCTGGCAA-3′
fadR-F	5′-AGCTGATTGGCGTTACCCGAACTA-3′
fadR-R	5′-AATATTCAGCCCGGAGGTTTCCCA-3′
gmpA-F	5′-ATCGCCTACACCTCCGTGCTTAAA-3′
gmpA-R	5′-ATAGCTTGACCTGTTCATCGCCGT-3′
rrn (16S)-F	5′-TCGACATCGTTTACAGCGTGGACT-3′
rrn (16S)-R	5′-TGCATCCAAGACTGGCAAGCTAGA-3′

[a] Boldface indicates restriction enzyme sites.

including the ATG start codon but not including the TAG stop codon. The primers used to construct the suicide vectors are listed in Table 2. Primers 1 and 2 were designed to amplify the upstream gene-flanking regions. The downstream gene-flanking regions were amplified by primers 3 and 4. The flanking regions were ligated and cloned into pMEG-375 digested with SphI and XbaI. To construct *E. ictaluri* mutants, the suicide plasmid was conjugationally transferred from *Escherichia coli* χ7213 (52) to *E. ictaluri* strains. Strains containing single-crossover plasmid insertions were isolated on BHI agar plates containing Col and Amp. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity counterselection system (14) adapted to *E. ictaluri* (55, 57). The colonies were selected for Amp^s and Col^r and screening by PCR using primers 1 and 4. Biochemical profiles of *E. ictaluri* strains were determined using the API 20E system (bioMérieux, Marcy L'Etoile, France).

Construction of *Salmonella* Typhimurium Δ*fur-44*. The *fur* gene of χ3761 *Salmonella enterica* serovar Typhimurium UK-1 (Table 1) was deleted in frame mediated the previous suicide vector methodology (55, 57). The primers used to construct the suicide vectors are listed in Table 2. The flanking regions were ligated and cloned into pRE112 (14) digested with BglII and XmaI. The *S.* Typhimurium Δ*fur-44* mutation consisted of a deletion of 706 bp, including 453 bp of the *fur* gene and 253 bp of the *fur* promoter region, with the Crp binding and the OxyR binding sites.

Complementation of the *fur* and *hemR* genes. The *fur* gene of *E. ictaluri*, with its own promoter, was cloned into the pAYCY184 vector (7) at the XbaI and HindIII restriction sites and into pEZ151 at the AdhI restriction site. The primers used to amplify *fur* were 5′-TCGTCTAGATGTCTGCCGCCTGCCGGCGC-3′ (upstream) and 5′-CCCAAGCTTTCAGGCCTTTTCATCGTGCA-3′ (downstream). XbaI and HindIII sites, respectively, were included in these primers (underlined). The resulting plasmid, pEZ136, was used to complement *Salmonella* Δ*fur-44* mutant strains, and pEZ191 was used to complement *E. ictaluri*. The primers used to amplify *hemR* were 5′-TCGTCTAGAATTTACACTTTATGCTTCCGGCTCGTATGTTATGCCTACGCTTTTCTCCCCTTCCCTACGC-3′ (upstream) and 5′-CCCAAGCTTCCGCTCGAGCTACCACTGGTAGCTGAGCAGCAGACGGGC-3′ (downstream). XbaI and HindIII sites, respectively, were included in these primers (underlined). The lactose promoter (P_{lac}) was used to express the *hemR* gene. The resulting plasmid, pEZ192, was used to complement *E. ictaluri*.

SDS-PAGE and Western blotting. To evaluate the synthesis of Fur, the strains were grown in 3 ml of BHI broth or LB broth at 28°C with aeration (180 rpm). The samples were collected when the culture reached an absorbance of 0.85 (optical density at 600 nm [OD_{600}] of 1.0 ~ 1.0 × 10^8 CFU/ml). One milliliter of culture was collected and prepared for Western blot analysis (54). The total proteins were normalized using a NanoDrop spectrophotometer (ND-1000) at 25 μg/μl, separated by 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (54). A fat-free milk powder solution (5%, wt/vol) in phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20 (PBS-T) was used for blocking. The membrane was incubated individually with a primary rabbit polyclonal anti-GroEL antibody (Sigma) (1:10,000) or rabbit polyclonal anti-Fur antibody (1:10,000) (60) for 1 h at room temperature, washed three times with PBS-T, and then incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma). Color was developed with a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate mixture (NBT-BCIP) (Amaresco), chromogenic substrates for alkaline phosphatase.

OMP isolation. Sarkosyl-insoluble outer membrane proteins (OMPs) were obtained as previously described (55). OMPs were isolated from *E. ictaluri* grown under iron-replete conditions (BHI broth and BHI broth supplemented with 150 μm $FeSO_4$). Iron-regulated outer membrane proteins (IROMPs) were isolated from *E. ictaluri* grown in BHI broth supplemented with 2′2′-dipyridyl (150 μM) (iron-depleted conditions). The outer membrane proteins were normalized to 25 μg/μl by using the NanoDrop spectrophotometer (ND-1000) and separated by 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Coomassie blue staining was performed to visualize proteins.

Protein identification. The single protein band of <72 kDa synthesized in the absence of iron or in *E. ictaluri* Δ*fur* mutants was excised from the SDS-polyacrylamide gel for peptide sequencing analysis (ProtTech Inc., Norristown, PA) by using NanoLC-MS/MS peptide sequencing technology. The protein gel band was destained, cleaned, and digested in-gel with sequencing-grade modified trypsin (Promega, Madison, WI). The resulting peptide mixture was analyzed with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system, in which a high-performance liquid chromatograph (HPLC) with a 75-μm-inner-diameter reverse-phase C_{18} column was coupled to an ion trap mass spectrometer (Thermo, Palo Alto, CA). The mass spectrometric data were utilized to search the nonredundant protein database at the National Center for Biotechnology Information (NCBI).

Detection of secreted siderophores. Production of compounds with siderophore activity was tested by the chemical assays described by Schwyn and Neilands (58) in solid and liquid media. The assays were performed by spotting 10 μl of each bacterial culture grown under iron-limiting conditions onto a modified chromoazurol S (CAS) (Sigma) agar. The siderophore levels produced by the strains on plates were visualized by a yellow-orange halo around the bacterial colony after 18 to 72 h of incubation. For siderophore detection in liquid, supernatants from bacterial cultures grown in BHI broth or LB broth supplemented with 2′2′-dipyridyl (150 μM) were mixed with CAS solution, and the absorbance of the mixture was measured at 630 nm.

Iron-free siderophores were obtained by the following method. Bacterial cultures grown in 5 ml of LB broth supplemented with 2′2′-dipyridyl (150 μM) or $FeSO_4$ (150 μM) for 18 to 24 h at 28°C (OD_{600} of 1.0) were harvested by centrifugation at 5,000 × *g* for 10 min. The supernatants were filtered through a 0.2-μm-pore-size membrane filter to completely remove the cells, acidified with 25 μl of 10 N HCl, and extracted twice with a total of 4 ml of ethyl acetate for catechols and benzyl alcohol for hydroxamates (44). The aqueous phase was dried with gaseous N_2 and resuspended in 40 μl of methanol, and 10 μl was spotted onto 250-μm-layer (20 by 20 cm) flexible PE SIL G/UV254 plates (Whatman). The

plates were developed with benzene-glacial acetic acid-water (125:72:3, vol/vol/vol) in a closed chamber. Plates were then removed from the chamber, allowed to dry, and then immersed briefly in 0.1% $FeCl_3$ to visualize Fe binding compounds. Avian-pathogenic *Escherichia coli* χ7122, *S.* Typhimurium UK-1 χ3761 (36, 52), and *S.* Typhimurium χ11143 Δ*fur-44* were used as controls (Table 1).

Utilization of heme and hemoglobin by *E. ictaluri*. Sterile filter paper disks were placed onto a BHI agar plate supplemented with 2′2′-dipyridyl (150 μM) and swab inoculated with an *E. ictaluri* strain. The disks were inoculated with 5 μl of water (control), heme (10 μM; Sigma), and hemoglobin (~9.3 μM; Sigma). The plates were incubated for 48 h at 28°C. Growth around the paper disk indicated positive utilization of the iron source.

Semiquantitative RT-PCR. Expression of putative Fur-regulated genes was evaluated by reverse transcription-PCR (RT-PCR). Total RNA extraction was performed with an RNeasy QIAgene kit from wild-type *E. ictaluri* grown in the presence and absence of iron and from *E. ictaluri* J135 Δ*fur-35* grown in BHI. The cells were grown until late exponential phase (OD_{600} of 0.85; ~1×10^8 CFU/ml). cDNA synthesis was performed with the SuperScript III first-strand synthesis system (Invitrogen) using random hexamer primers. Semiquantitative PCR was performed using the specific primers listed in Table 2. The number of PCR cycles was normalized to 27. The 16S rRNA (*rrn*) gene was used as an expression control, and *fadR* was used as a single-gene expression control.

Bacterial inoculate preparation. Bacterial strains were grown overnight in standing cultures that were diluted 1:20 in prewarmed BHI broth with mild aeration (180 rpm) at 28°C to an OD_{600} of 0.85 (~10^8 CFU/ml). Bacteria were sedimented for 10 min by centrifugation (5,865 × *g*) at room temperature and resuspended in sterile phosphate-buffered saline containing 0.01% gelatin (BSG) (12) to densities appropriate for the inoculation.

Infection and immunization of zebrafish (*D. rerio*). The zebrafish challenges were performed by the methodology described earlier (47), with modifications (55, 57). The water temperature was 26 ± 1°C, and the fish were acclimated for 2 weeks prior to the experiment. Groups of 12 adult zebrafish (average weight, 0.5 g) were sedated in 100 mg/liter tricaine methanesulfonate (MS-222; Sigma) and injected intramuscularly (i.m.) with 10 μl of the bacterial suspension per fish. A 3/10-ml U-100 ultrafine insulin syringe with a 0.5-in.-long (ca. 1-cm-long) 29-gauge needle (catalog no. BD-309301; VWR) was used to inject the fish. Two sets of controls were used: fish that were not injected and fish that were injected with 10 μl of BSG (12). Moribund fish demonstrating clinical signs were euthanized, necropsied, and plated for enumeration of bacterial loads in various organs (47). Survivors of each dose at 4 weeks after i.m. inoculation were challenged with 10^5 CFU of *E. ictaluri* (100 50% lethal doses [LD_{50}]). The fish were fed twice daily with TetraMin tropical fish flake feed. During the experiments, the fish were observed daily, and every other day the water quality was monitored for pH, NO_2, and NO_3 with standard kits (Lifegard Aquatics water testing strips). The LD_{50} for the infection trials was calculated by the method of Reed and Muench (1). Fish care and use were in accordance with the requirements of the Arizona State University Institutional Animal Care and Use Committee.

i.c. infection and immunization of catfish (*I. punctatus*). Specific-pathogen-free channel catfish fingerlings with a mean weight of 18.5 ± 1.3 g were used. The animals were randomly assigned to treatment groups of 10 to 25 fish each in 100-liter tanks. Each tank was equipped with a recirculating, biofiltered, mechanically filtered, and UV-treated water system with 12-h light cycle per day. The water temperature was set at 28 ± 1°C during the first 2 weeks of acclimatization and during the course of the experiments. The fish were fed daily with commercial Aquamax grower 400 (Purina Mills Inc., St. Louis, MO). During the experiments, the fish were observed daily, and every other day the water quality was monitored for pH, NO_2, and NO_3 with standard kits (Lifegard Aquatics water testing strips). Catfish were infected by the intracelomic (i.c.) route with 10^5 to 10^8 CFU of *E. ictaluri* strains (fish were not fed until 1 h after infection). The fish were anesthetized with buffered tricaine methanesulfonate (pH 7.5) (MS-222; Sigma) (100 mg/liter of water) prior to handling. The LD_{50} was calculated by the method of Reed and Muench (1). Moribund animals were euthanized and then necropsied to evaluate the presence of *E. ictaluri* in kidney, spleen, and liver.

Immersion immunization of catfish. Catfish fingerlings were immersed in a solution of *E. ictaluri* Δ*fur-35* containing 10^7 CFU/ml for 30 min. At 6 weeks postimmunization, fish were challenged by immersion with 10^7 CFU/ml of wild-type *E. ictaluri* (10 LD_{50}) for 30 min. The animals were fasted for 24 h prior to oral inoculation and for 1 h postinoculation. Nonimmunized animals were used as a control. During the experiments, the fish were observed daily.

Oral immunization of catfish. Catfish were fasted for 24 h before oral inoculation with the *E. ictaluri* strains. Fish were anesthetized with buffered tricaine methanesulfonate (pH 7.5) (100 mg/liter of water) prior to handling. The animals were orally inoculated with 100 μl of the corresponding bacterial suspension (see below). BSG (12) was used as a control. The fish were not fed until 1 h after inoculation.

Determination of IgM titers in serum and skin mucus. Cutaneous mucus and blood samples were collected at both 2 weeks prior to immunization and 4 weeks postimmunization as described previously (55). Ten i.c. immunized fish and 10 immersion-immunized fish were sampled individually. The experiment was repeated twice. *E. ictaluri* outer membrane proteins diluted in 20 mM Tris-OH (pH 8.0) and purified *E. ictaluri* lipopolysaccharide (LPS) were independently applied to polyvinyl chloride 96-well plates, both at 100 ng per well. The plates were incubated overnight at 4°C, washed once with 200 μl of PBS–0.05% Tween (PBS-T) per well, and blocked with 1% sea buffer (Thermo, Rockford, IL) diluted in PBS (1 h at room temperature). Catfish serum samples diluted in PBS (1:2) and undiluted mucus samples were plated in triplicate wells at 100 μl/well. Triplicate control wells on each plate contained diluted sera from immunized and nonimmunized catfish. Mucus assays also included wells of pooled mucus from nonexposed fish. The plates were incubated overnight at 4°C and washed five times with PBS-T. The biotinylated rabbit anti-catfish Ig antibody was applied at 0.25 to 0.50 μg/well. The plates were incubated for 1 h at room temperature and were washed five times with PBS-T. Application of the primary antibody was followed by application of streptavidin-alkaline phosphatase conjugate (Southern Biotech, Birmingham, AL) diluted in PBS (1:50,000). The enzyme substrate *p*-nitrophenyl phosphate diethanolamine (100 μl; Sigma) was added and incubated for 30 min at room temperature. The reaction was stopped with 50 μl of 3 M NaOH. The absorbance (A_{405}) values were determined on a kinetic microplate reader (model V-max; Molecular Devices Corp., Sunnyvale, CA) at 30 min and 1 h. The immunized fish were challenged at 6 weeks postimmunization as described previously.

Statistics. Analysis of variance (ANOVA) (SPSS software), followed by least-significant-difference (LSD) analysis, was used to evaluate differences in antibody titers, using 95% confidence intervals. The Kaplan-Meier method (SPSS software) was applied to obtain the survival fractions following challenges. *P* values of <0.05 were considered statistically significant.

RESULTS

Sequence analysis of *fur*. An analysis of DNA and protein sequences, structural alignment, predicted 3D structure, and predicted binding residues revealed that the *E. ictaluri* and *E. tarda* Fur proteins have several key differences from other bacterial iron regulator protein families (Fig. 1 to 3). This provides evidence that these Fur proteins are members of a distinctive bacterial iron regulator protein family, shaped by gene reduction. The *E. ictaluri fur* gene sequence from *E. ictaluri* wild-type J100 is identical to the sequence from the *E. ictaluri* 93-146 strain published at the NCBI. In comparison to *E. coli* and *Salmonella fur* genes, the *E. ictaluri fur* gene has a smaller open reading frame (ORF), and has a guanine

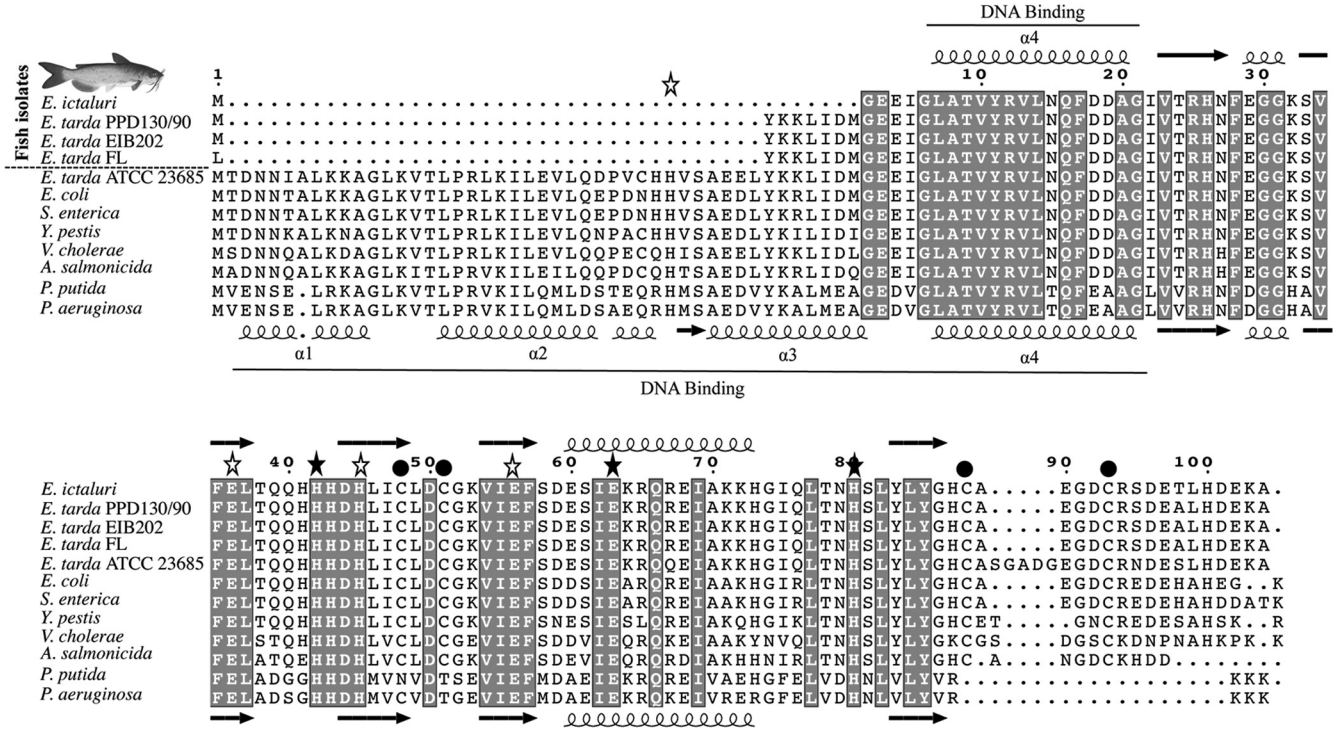


FIG 1 *E. ictaluri* Fur alignment and secondary structure. The white stars indicate the residues related to the Zn^{2+} binding pocket. The black stars indicate the residues related to the Fe^{2+} binding pocket. The black circles indicate the cysteine residues related to *E. coli* Fe^{2+} and Zn^{2+} binding pockets. The secondary structure at the top of the alignment corresponds to the *E. ictaluri* Fur protein. The secondary structure at the bottom of the alignment corresponds to the *Escherichia coli* Fur protein. Spirals represent α-helices, and arrows represent β-sheets.

plus cytosine (G+C) content of 57%, which is 10% higher than those of the *Escherichia coli* (48.5%) and *Salmonella* (47.7%) *fur* genes (see Fig. S1 in the supplemental material). The *fur* gene sequences from the fish-isolated *E. tarda* PPD130/91 and *E. tarda* EIB202 (71) contain 333 identical base pairs, in contrast with the human-isolated *E. tarda* ATCC 23685, which contains a longer *fur* gene (435 bp) similar to those of the rest of the enteric bacteria (see Fig. S1 in the supplemental material). The *E. ictaluri fur* promoter region contains a Crp binding site, indicating that Crp might participate in Fur regulation (see Fig. S2 in the supplemental material).

The Fur protein from fish-isolated *E. tarda* PPD130/91 and *E. tarda* EIB202 (71) contains 111 identical residues, in contrast with the longer Fur proteins in *E. coli* (145 residues), *Salmonella* Typhimurium (150 residues), *Yersinia pestis* (148 residues), and other enteric bacteria (Fig. 1). Interestingly, the human-isolated strain *E. tarda* ATCC 23685 contains a longer Fur protein (145 residues) similar to those of the rest of the enteric bacteria, indicating that it may be more adapted to humans than to fish. *E. ictaluri* 93-146 and *E. ictaluri* J100 contain a shorter Fur with 104 identical residues (Fig. 1). Alignment of these sequences showed that the N-terminal helices, α1, α2, and α3, at the DNA binding domain are missing in *E. ictaluri*, as well as in the *E. tarda* fish-isolated Fur proteins (Fig. 1). The α4 helix at the Fur DNA binding domain is still intact in *E. ictaluri* and *E. tarda* fish isolates, sharing about 90% identity with other bacterial Fur proteins (Fig. 1). Structural protein alignment between functional representative bacterial Fur proteins revealed that 37 amino acid residues out of 104 residues (~36%) are strictly conserved in *E. ictaluri* Fur (Fig. 1). *E. ictaluri* Fur has 29%, 53%, 54%, 57%, and 93% amino acid similarity to the Fur proteins of *Pseudomonas*, *Escherichia coli*, *S. enterica*, *Y. pestis*, and *E. tarda*, respectively.

In terms of phylogeny, the fish-isolated *Edwardsiella* Fur proteins belong to a distinctive group compared to those from the rest of the *Enterobacteriaceae* family (Fig. 2). Although the *Enterobacteriaceae* share a Fur ancestor, the only phylogenic group with a short Fur protein among enteric bacteria is *Edwardsiella* (Fig. 2), indicating that Fur might have undergone host bacterial pathogen specialization.

Structural protein analysis showed that *Edwardsiella* Fur contains two domains, like other Fur proteins (32, 48). The DNA binding domain harbors the Zn^{2+} bond residues, and the dimerization domain harbors the Fe^{2+} bond residues (Fig. 1 and 3A and B). We observed that *E. ictaluri* Fur, as well as the *E. tarda* Fur (only fish-isolated strains), do not contain the α-helices α1, α2, and α3 (Fig. 1). Analysis of the residues related to the Zn^{2+} and Fe^{2+} bonds showed they are located in positions similar to those for their corresponding residues in the *Pseudomonas* Fur protein but misplaced with respect to their corresponding residues in *E. coli* Fur. Specifically, the *E. coli* Fur-related residues Cys92 and Cys95 required for the Zn^{2+} bond (17, 32) (in *E. ictaluri* Fur, Cys48 and Cys51 [Fig. 3A and C]) and the residues Cys132 and Cys88 required for the Fe^{2+} bond (17, 32) (in *E. ictaluri* Fur, Cys93 and Cys88 [Fig. 3A and C]) are located at the loop region in *E. ictaluri* Fur (Fig. 3A and C). The *Pseudomonas* Fur-related residues His24, His86, Asp88, and Glu107 required for the Zn^{2+} bond (48) (in *E. ictaluri* Fur, Glu36, His45, and Glu56 [Fig. 3B and D]) and the residues His32, Glu80, His89, and Glu100

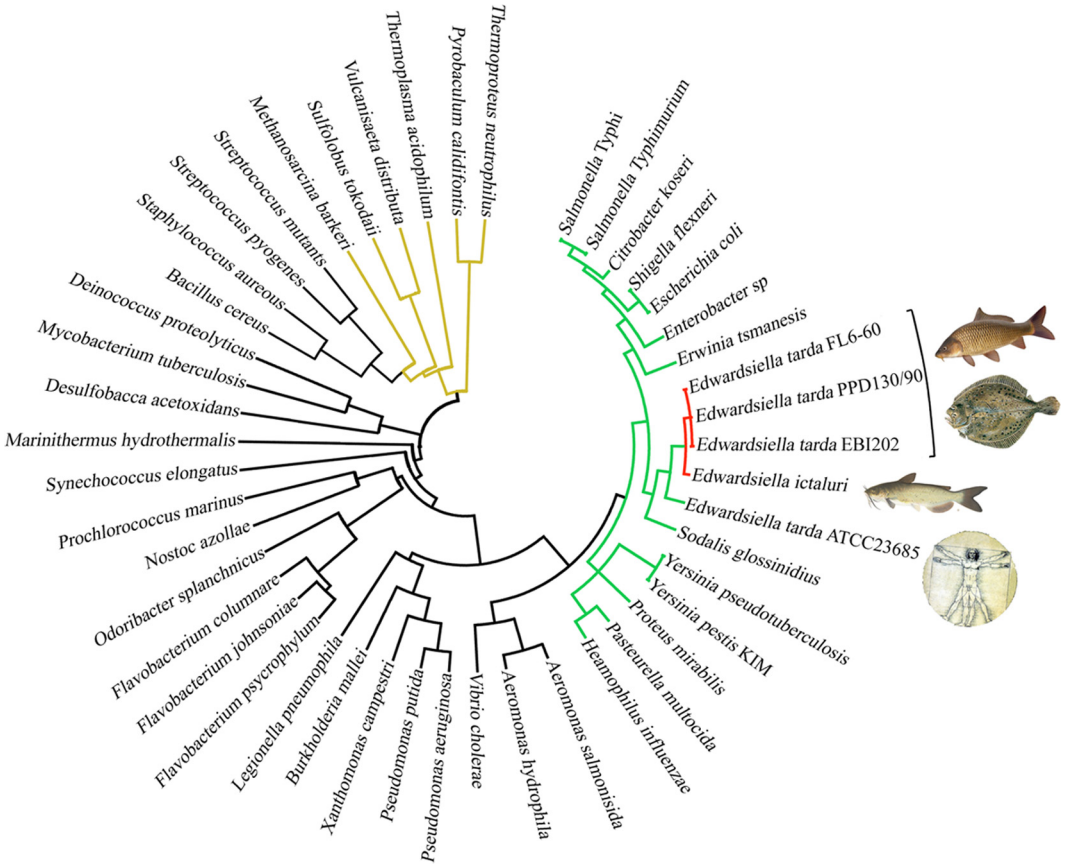


FIG 2 Molecular phylogenetic analysis of *fur*. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model (65). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed (15). Branches corresponding to partitions reproduced in fewer than 50% bootstrap replicates are collapsed. Initial trees for the heuristic search were obtained automatically as follows. When the number of common sites was less than 100 or less than one-fourth of the total number of sites, the maximum-parsimony method was used; otherwise, the BIONJ method with MCL distance matrix was used. The analysis involved 48 nucleotide sequences. Codon positions included were first, second, third, and noncoding. There were a total of 546 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (64). The gold lines indicate a representative archaea *fur* evolutionary path, the green lines indicate the *Enterobacteriaceae fur* family evolution path, and the red lines indicate the fish-isolated *Edwardsiella fur* evolution path.

required for the Fe^{2+} bond (48) (in *E. ictaluri* Fur, His42, Glu63, His80, and Glu90 [Fig. 3D and E]) are located in their respective domains within *E. ictaluri* Fur (Fig. 3B, D, and E). In *P. aeruginosa*, the Fur residue His32 is required for the Fe^{2+} bond and is essential for its activity (4). However, the N-terminal region, where His32 would normally be located, is absent in the *E. ictaluri* Fur protein (Fig. 1). Spectroscopic and biochemical data have shown that the structural zinc sites in the *P. aeruginosa* and *E. coli* Fur proteins are different, which might be related to their specific functions in the different organisms. However, in both the *E. coli* and *P. aeruginosa* Fur proteins, the N-terminal region is essential for Fur activity (4, 9). On the other hand, the importance of the cysteines is different in *E. coli* Fur and in *P. aeruginosa* Fur, because the Cys92 and Cys95 bound to Zn^{2+} in *E. coli* Fur are essential for its activity (8). The single cysteine residue in the *P. aeruginosa* Fur is dispensable for its *in vivo* activity (33), and it is absent in other *Pseudomonas* Fur proteins, such as *P. putida* Fur (Fig. 1). Based on the above analysis, we believe that *E. ictaluri* Fur is a distinct Fur protein among the Fur family. Further structural information on the *E. ictaluri* Fur protein is necessary for a better understanding of the specific structure-function relationship within the Fur family.

Complementation of the *fur* gene. The structural analysis of *E. ictaluri* Fur indicated that the overall domain organization is different from that of other Fur family members, but it has the same set of key residue groups and likely a similar mechanism as other Fur proteins (Fig. 1 to 3). To further evaluate the functionality of *E. ictaluri* Fur, we complemented Δ*fur* mutants of *Salmonella enterica* with the *E. ictaluri* P_{fur}-*fur* gene cloned into the low-copy-number plasmid pEZ136 (Table 1). *S. enterica* serovar Typhimurium Δ*fur-44* was utilized for complementation assays (Table 1). *S. enterica* Δ*fur* mutants present a constitutive synthesis of IROMPs and secretion of siderophores (Fig. 4A to C). *E. ictaluri* P_{fur}-*fur* complements *S. enterica* Δ*fur-44* mutants, repressing IROMP and siderophore synthesis in an iron-dependent fashion (Fig. 4). This indicates that the missing N-terminal region, (containing the α1, α2, and α3 helices) is not essential for the Fur function (59).

Siderophore synthesis in *E. ictaluri*. Results from CAS liquid and plate assays showed that *E. ictaluri* does not synthesize detectable siderophores (Fig. 5A). Although the *E. ictaluri* chromosome contains a ferric enterobactin transport protein (FepE, siderophore receptor; NC_012779.1) and a TonB-dependent fer-

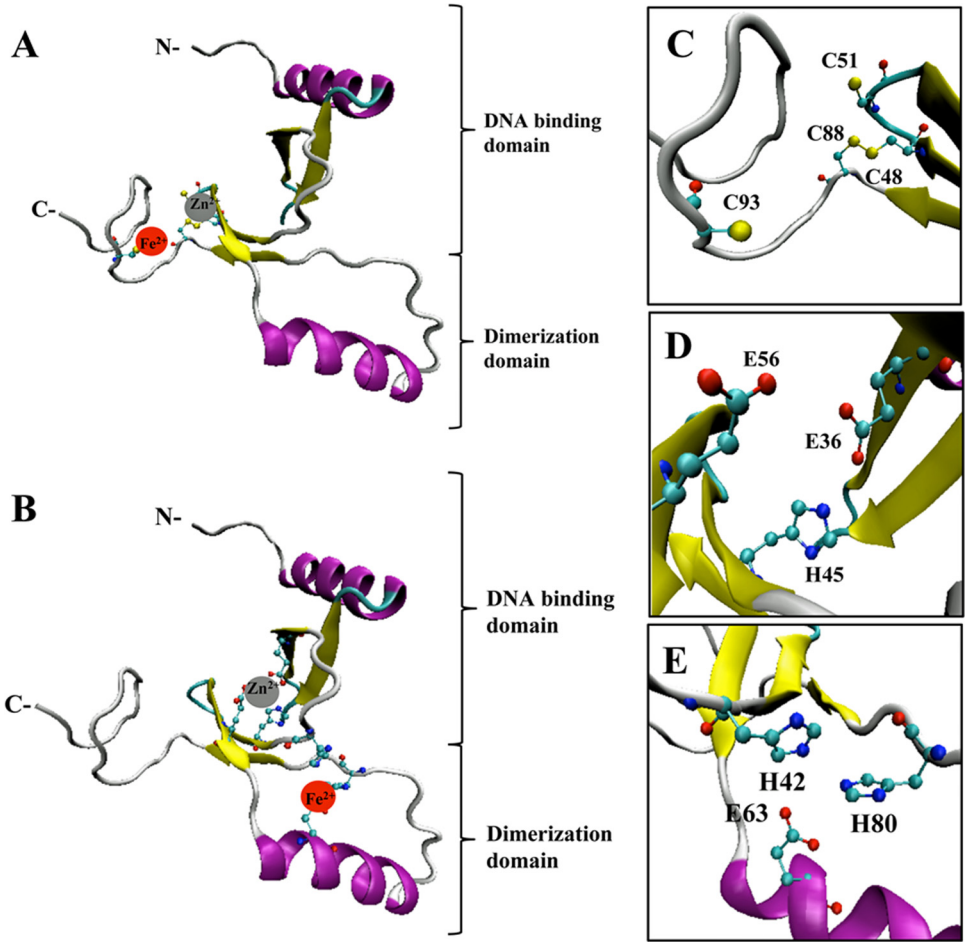


FIG 3 *E. ictaluri* Fur protein functional analysis. (A) Predicted *E. ictaluri* Fur protein displaying the (C51; C48) Zn^{2+} and (C88; C93) Fe^{2+} binding pockets; (B) predicted *E. ictaluri* Fur protein displaying the (E36; H45; E56) Zn^{2+} and (H42; E63; H80) Fe^{2+} binding pockets; (C) residues C51 and C48 related to Zn^{2+} binding pocket and residues C88 and C93 related to Fe^{2+} binding pocket; (D) residues E36, H45, and E56 related to Zn^{2+} binding pocket; (E) residues H42, E63, and H80 related to Fe^{2+} binding pocket. Residues E36, H45, and E56, related to the Zn^{2+} binding pocket, and residues H42, E63, and H80, related to the Fe^{2+} binding pocket, are related to the *Pseudomonas* Fur protein. Residues C51 and C48, related to the Zn^{2+} binding pocket, and residues C88 and C93, related to the Fe^{2+} binding pocket, are related to the *Escherichia coli* Fur protein and are misplaced in the overall *E. ictaluri* Fur protein structure.

richrome receptor protein (FcuA; NC_012779.1), thin-layer chromatography (TLC) analysis showed that *E. ictaluri* does not secrete either catechol- or hydroxylamine-related siderophores or heme binding molecules (data not shown), regardless of the presence of iron in the growth medium or Fur protein (Fig. 5B). Our results correlate with previous observations (67) and with the fact that siderophore biosynthesis genes are not present in the *E. ictaluri* chromosome (72).

Fur iron-regulated outer membrane proteins and heme uptake machinery. Wild-type *E. ictaluri* grown under iron limiting conditions and *E. ictaluri* Δ*fur-35* mutants both upregulate an IROMP of ~72 kDa, indicating that this protein is iron-Fur dependent and related to iron acquisition (Fig. 5C to E). Protein identification indicated that this IROMP corresponds to a TonB-dependent heme receptor protein (HemR) (Fig. 6). The gene encoding HemR is part of the *hemPRSTUV* operon, which contains the genes required for the synthesis of the heme uptake machinery (Fig. 6).

Structural analysis of the iron uptake proteins reveled that these are similar to the putative *E. tarda* heme uptake system and to the *Yersinia* heme uptake system (Fig. 6). The outer membrane receptor HemR contains a β-barrel structure with a cork required to transfer the heme into the cell (Fig. 7). Structural analysis of HemR predicts a signal sequence peptide in the N-terminal region (see Fig. S3 in the supplemental material), which was observed in 8% SDS-PAGE gel analysis (data not shown). The predicted model of heme transport is described in Fig. 7 and consists of the following steps. First, heme is transferred into the cell, mediated by HemR, where it is captured by HemT, a periplasmic hemin binding protein. It is then transported through the cytoplasmic membrane by the ATP-dependent HemU-HemV cytoplasmic membrane ABC transport complex. Once heme is transferred to the cytoplasm, it is captured by HemS, a predicted heme degradation-storage protein (Fig. 7).

***E. ictaluri* uses heme and hemoglobin as iron sources, mediated by HemR.** To determine whether *E. ictaluri* has a functional heme-hemoglobin uptake system, we constructed an *E. ictaluri* Δ*hemR* mutant (thereby disrupting heme-hemoglobin receptor synthesis) (Fig. 8A to C). *E. ictaluri* iron-depleted cells grow poorly in iron-depleted medium. Wild-type *E. ictaluri* iron-de-

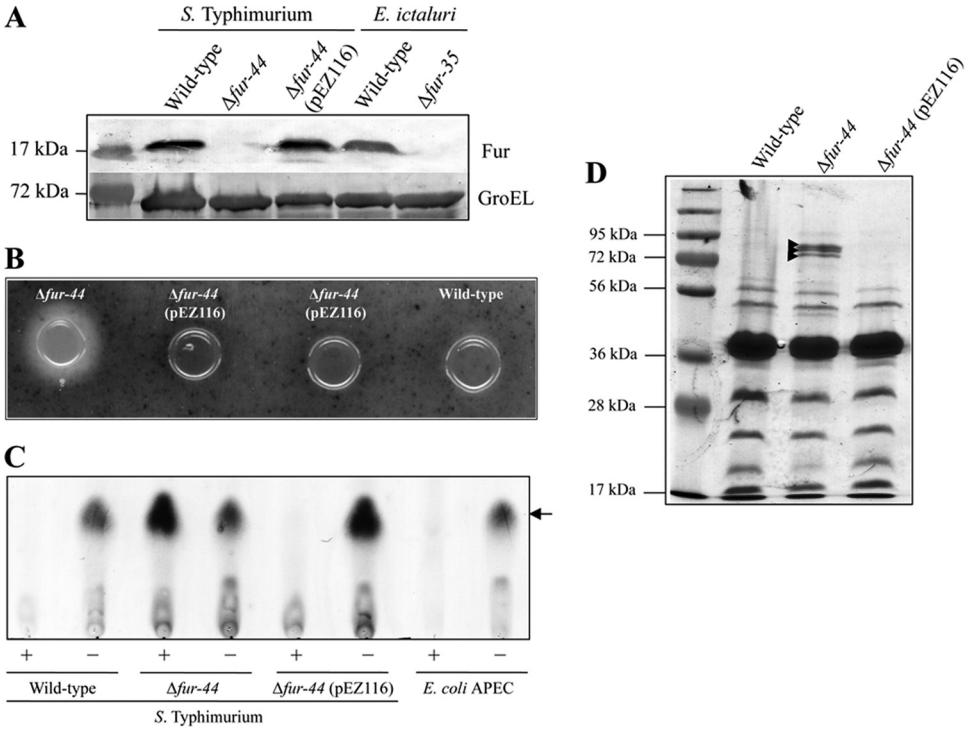


FIG 4 Complementation of *Salmonella* Typhimurium Δ*fur* mutants with the *E. ictaluri fur* gene cloned in pEZ116. (A) Synthesis of Fur verified by Western blot analysis. GroEL was used as a control. (B) Detection of siderophores in CAS indicator agar plates. (C) Detection of secreted siderophores in *Salmonella* Typhimurium strains grown under iron-rich (+) and iron-limited (−) conditions by TLC. (D) Outer membrane protein profile of *S.* Typhimurium Δ*fur* complemented with the *E. ictaluri fur* gene cloned in pEZ136. The arrowheads indicate the Fur-regulated *S.* Typhimurium IROMP proteins.

pleted cells were able to grow in iron-depleted medium when it was supplemented with heme or hemoglobin as an iron source (Fig. 8D and G). In contrast, *E. ictaluri* Δ*hemR* iron-depleted cells were not able to grow in iron-depleted medium supplemented with heme or hemoglobin (Fig. 8E and G). Δ*hemR* and Δ*fur* Δ*hemR* mutants complemented with the *hemR* gene in *trans* (Fig. 8B and C) recovered their ability to utilize heme and hemoglobin (Fig. 8F and G).

Fur-regulated genes. Fur binds to promoter DNA containing a 19-bp putative Fur box with the consensus sequence 5′-GATA

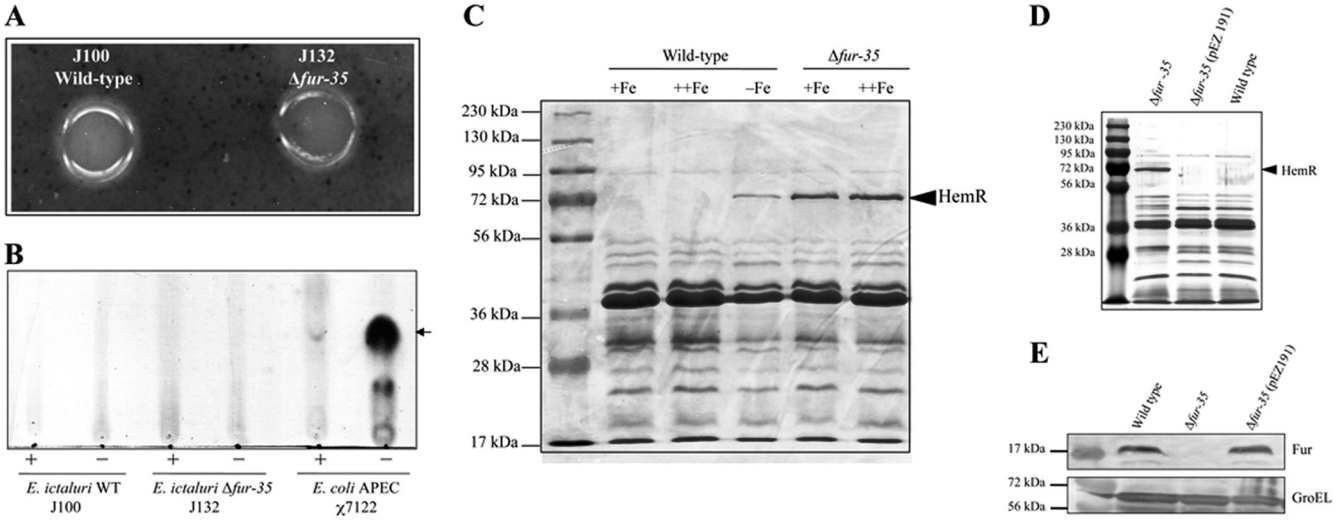
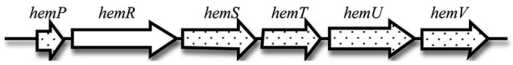


FIG 5 Siderophore- and Fur-regulated IROMPs in *E. ictaluri*. (A) Detection of siderophores in CAS indicator agar plates. (B) Detection of secreted siderophores in *E. ictaluri* strains grown under iron-rich (+) and iron-limited (−) conditions by TLC. (C) Outer membrane profiles of *E. ictaluri* strains grown under iron-rich (+Fe, BHI broth; ++Fe, BHI broth plus 150 μM $FeSO_4$) and iron-limited (−Fe) conditions. (D) Outer membrane profiles of *E. ictaluri* Δ*fur-35* and *E. ictaluri* Δ*fur-35*(pEZ191) (complemented with *fur* gene in *trans*). (E) Western blot analysis of Fur in *E. ictaluri* Δ*fur-35* and *E. ictaluri* Δ*fur-35* complemented in *trans*. GroEL was used as control.



Protein	Hypothetical function	BLAST (ID%:Positives%)	pI	Mass (Da)
HemP	Hemin uptake	*E. tarda* (86:94) *P. stuartii* (68:76)	10.29	8345.85
HemR	TonB dependent Hemin receptor	*E. tarda* (94:97) *Y. mollaretii* (61:75)	6.13	72904.86
HemS	Hemin degradation/storage	*E. tarda* (92:93) *Y. pestis* (62:76)	6.19	38018.77
HemT	Periplasmatic hemin-binding protein	*E. tarda* (91:94) *Y. pestis* (62:77)	9.56	28631.10
HemU	Hemin ABC transporter, permease	*E. tarda* (91:94) *Y. pestis* (62:77)	9.40	35576.78
HemV	Hemin ABC transporter, ATPase	*E. tarda* (96:96) *Y. intermedia* (67:77)	9.26	28914.41

FIG 6 *E. ictaluri* heme uptake operon gene map and protein descriptions.

ATGATAATCATTATC-3′; this sequence can be described as three adjacent hexamers of the sequence 5′-GATAAT-3′, with the third being in a reverse orientation, or symbolically "F-F-X-R," where "F" stands for the forward sequence "GATAAT," "R" stands for the reverse sequence "ATTATC," and "X" stands for any nucleotide (30). We identified a Fur box with an F-F-X-R orientation at the *hem* operon, between the −4 and +14 positions in the *hemP* gene (Fig. 9A). The Fur binding box in the F-F-X-R conformation found in *E. ictaluri* is similar to the *Pseudomonas aeruginosa* Fur binding box (40) (Fig. 9A).

Using the consensus hexamers for the *E. ictaluri* Fur box, we identified six different promoters not related to the *hemPRSTUV* operon, which could be regulated by the *E. ictaluri* Fur protein (Fig. 9B). These are promoters for Mg^{2+} transport (*mgtB*), manganese transport (*mntH*), fatty acid degradation (*fadR*), heme synthesis (*hemF*), and glycolysis (*gmpA*). The expression of the *hemR*, *mgtB*, *mntH*, *hemF*, and *gmpA* genes in the absence of iron in wild-type *E. ictaluri*, as well as in our Δ*fur* mutant, was confirmed by RT-PCR (Fig. 9C). Although we predicted a Fur binding box at the *fadR* promoter, its expression does not depend on either iron or the Fur protein (Fig. 9C).

Virulence of *E. ictaluri* Δ*fur-35* mutants in the fish host. Zebrafish (*Danio rerio*) is not the natural host of *E. ictaluri* but has been established as a reliable model system to evaluate *E. ictaluri* virulence (47, 55, 57). We found that *E. ictaluri* Δ*fur* was not fully

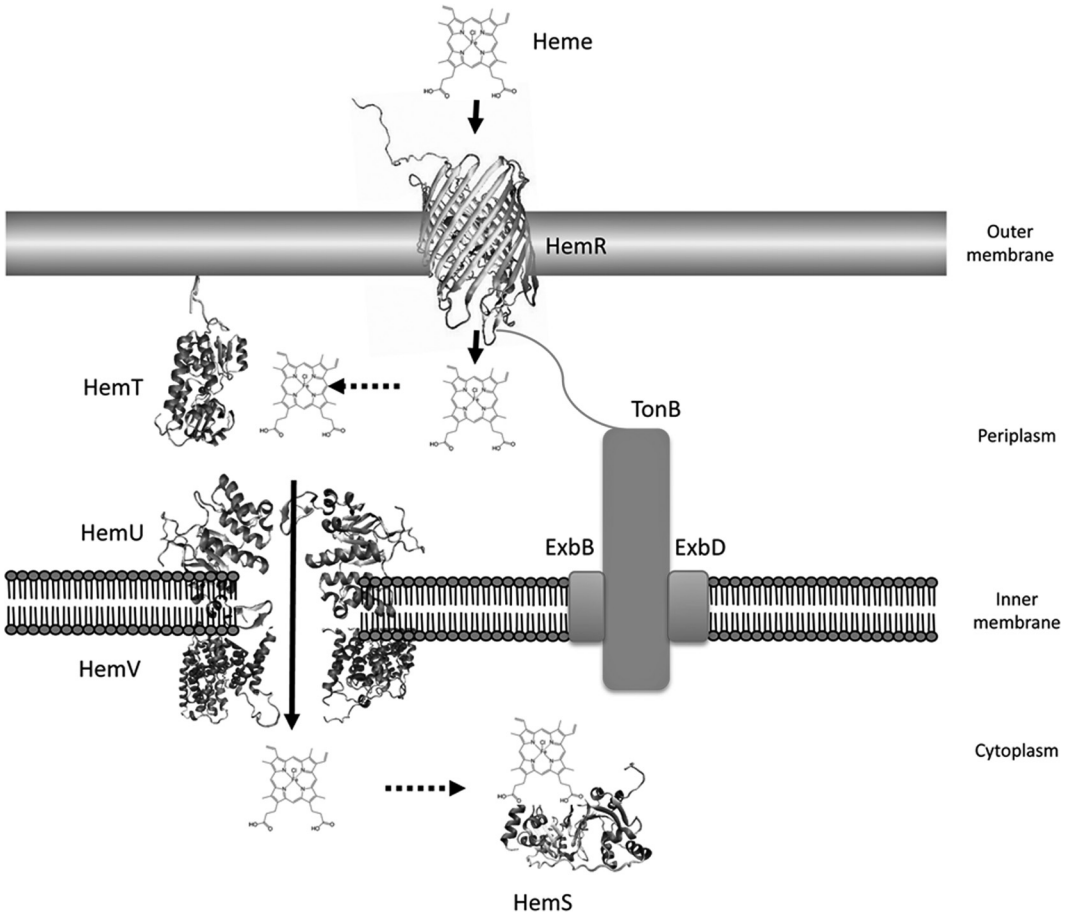


FIG 7 Proposed model for *E. ictaluri* heme transport system.

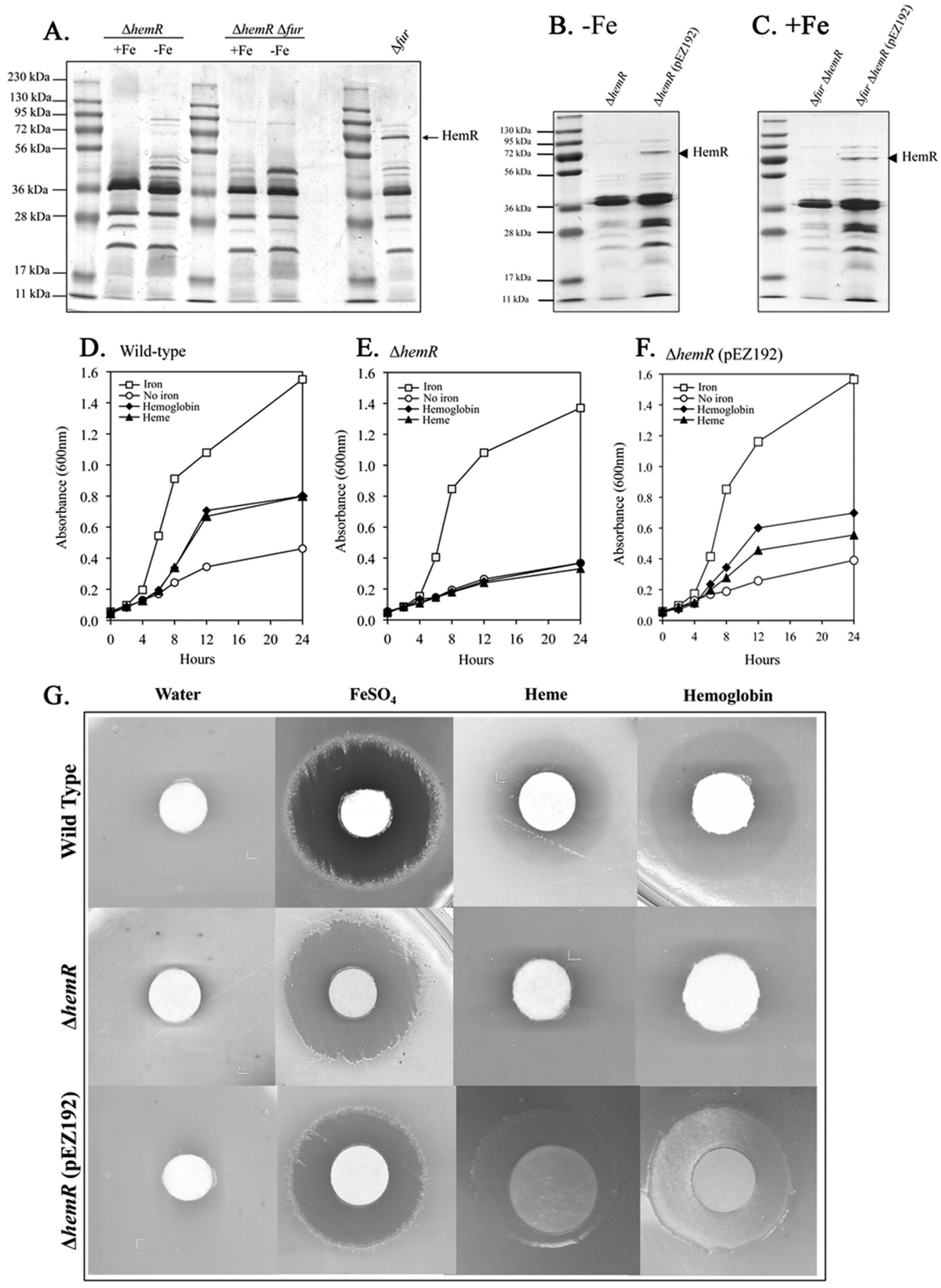


FIG 8 Heme and hemoglobin uptake is mediated by HemR. (A) Outer membrane protein profiles of *E. ictaluri* Δ*hemR* and *E. ictaluri* Δ*fur* Δ*hemR* mutants grown under iron-rich (+Fe) and iron-limited (−Fe) conditions. (B) Outer membrane protein profiles of *E. ictaluri* Δ*hemR* and Δ*hemR*(pEZ192) (in *trans* complemented) mutants grown under iron-limited conditions (−Fe). (C) Outer membrane profiles of *E. ictaluri* Δ*fur* Δ*hemR* and Δ*fur* Δ*hemR*(pEZ192) (in *trans* complemented) mutants grown under iron-rich conditions. (D) Growth of wild-type *E. ictaluri* under iron-limited conditions supplemented with heme (50 μg/ml or 833 nM) or hemoglobin (50 μg/ml or ~7.75 μM). (E) Growth of *E. ictaluri* Δ*hemR* mutant under iron-limited conditions supplemented with heme or hemoglobin. (F) Growth of *E. ictaluri* Δ*hemR*(pEZ192) (in *trans* complemented) mutant under iron-limited conditions supplemented with heme (50 μg/ml or 833 nM) or hemoglobin (50 μg/ml or ~7.75 μM). (G) Growth zones of wild-type *E. ictaluri* and the *E. ictaluri* Δ*hemR* mutant around discs soaked in 5 μl of $FeSO_4$ (200 μM), heme (600 μg or 10 μM), or hemoglobin (60 μg or ~9.3 μM) in BHI agar supplemented with 150 μM 2′,2′-dipyridyl.

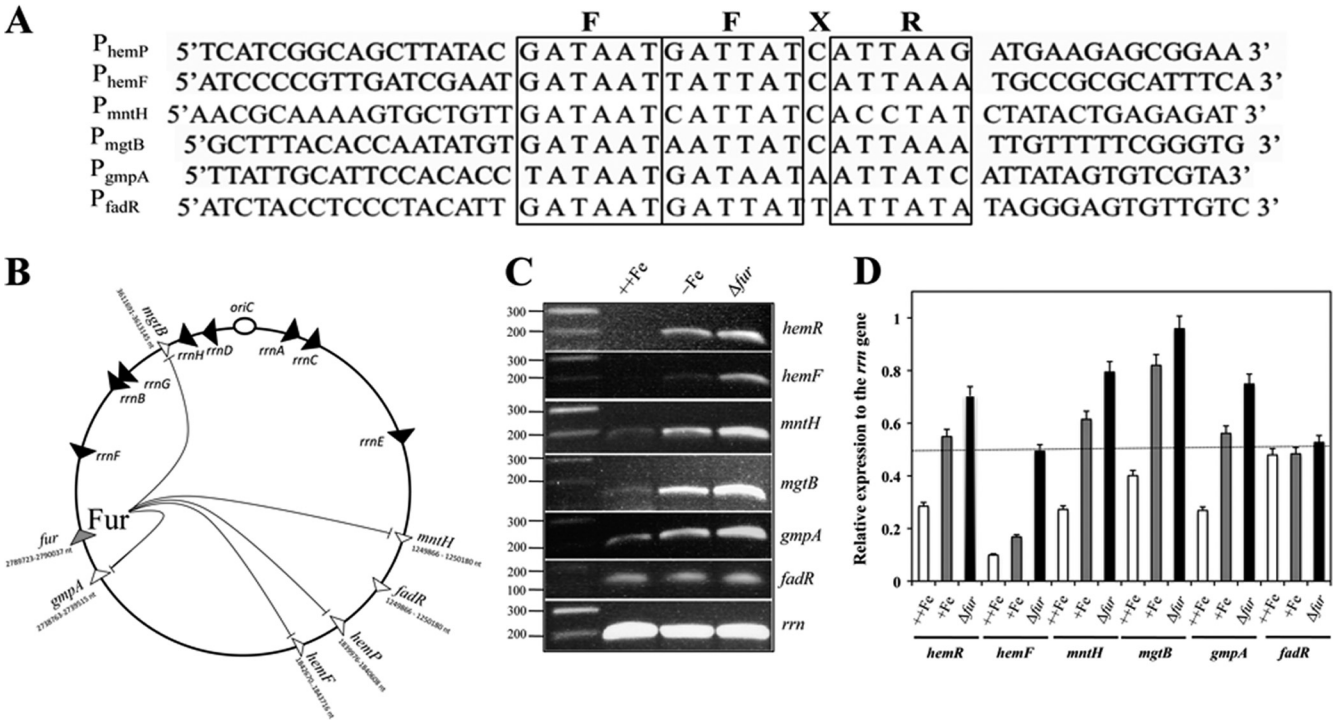


FIG 9 Genes regulated by Fur in *E. ictaluri*. (A) Predicted chromosomal genes/operons regulated by Fur. (B) Maps of operons regulated by Fur. The arrows indicate the direction of the gene. Bold arrows indicate the rRNA operons. (C) Semiquantitative RT-PCR of genes regulated by Fur (27 cycles). The *rrn* gene (16S rRNA gene) was used as a protein synthesis housekeeping gene expression control, and *fadR* was used a single-gene expression control. (D) Semiquantitative RT-PCR values for genes regulated by Fur related to the *rrn* gene. The dashed line indicates the mean of *fadR* expression.

virulent, with an LD_{50} of 1.42×10^5 CFU, a 10-fold increase over that of the wild type in zebrafish (Fig. 10A and B). The *E. ictaluri* Δ*fur* mutant was also evaluated in catfish (*I. punctatus*), the natural host of *E. ictaluri*. We found that *E. ictaluri* Δ*fur* administered i.c. to catfish was attenuated, with around a 1,000-fold LD_{50} increase over that of the wild type (Fig. 10C and D). *E. ictaluri* Δ*fur-35* delivered by immersion immunization was fully attenuated in frys and fingerlings (data not shown). *In vitro* growth of the *E. ictaluri* wild-type, Δ*fur-35*, and Δ*fur-35*(pEZ191) (complemented in *trans*) strains was similar in presence and absence of iron, excluding the possibility that the attenuation is due to slow growth of the Δ*fur-35* mutant (see Fig. S4 in the supplemental material).

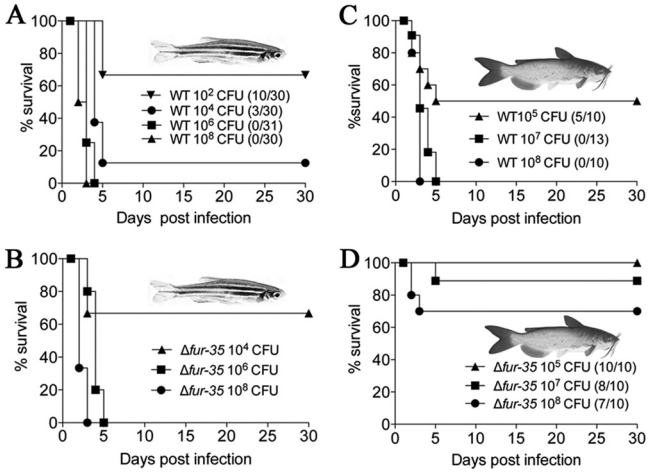


FIG 10 Virulence of *E. ictaluri* Δ*fur* in fish hosts. (A) Zebrafish i.m. infected with wild-type *E. ictaluri* (LD_{50}, 1.1×10^3 CFU); (B) zebrafish i.m. infected with *E. ictaluri* Δ*fur-35* (LD_{50}, 1.42×10^5 CFU); (C) catfish i.c. infected with wild-type *E. ictaluri* (LD_{50}, 1.01×10^5 CFU); (D) catfish i.c. infected with *E. ictaluri* Δ*fur-35* (LD_{50} not calculated).

Virulence of the *E. ictaluri* Δ*hemR* mutant in the catfish host. The *E. ictaluri* Δ*hemR* mutant was evaluated in catfish. We found that immersion-administered *E. ictaluri* Δ*hemR* (10^7 CFU/ml during 30 min) was totally attenuated. *E. ictaluri* Δ*hemR* that was orally administered to the catfish (10^8, 10^7, and 10^6 CFU/dose) was attenuated, with 80% survival at 10^8 CFU/dose and 100% survival at the lower doses. No hemorrhagic symptoms were observed in catfish that were immersion or orally infected with the Δ*hemR* mutant. *E. ictaluri* Δ*hemR* administered i.c. to the catfish was attenuated but still caused significant mortality (Fig. 11) and hemorrhagic symptoms in the fish. *In vitro* growth of the *E. ictaluri* wild-type, Δ*hemR*, and Δ*hemR*(pEZ192) (complemented in *trans*) strains was similar in presence and absence of iron, excluding the possibility that the attenuation is due to slow growth of the Δ*hemR* mutant (Fig. 8D to F). These results suggest that HemR might be required during colonization and during the early stages of the infection.

Immune protection of *E. ictaluri* Δ*fur* mutants in the fish host. The ideal live attenuated bacterial vaccine should be totally attenuated and immunogenic. Synthesis of IROMPs is upregulated inside the host after invasion, and these proteins are not constantly exposed to the immune system. We hypothesized that constant synthesis of IROMPs by *E. ictaluri* could trigger a protec-

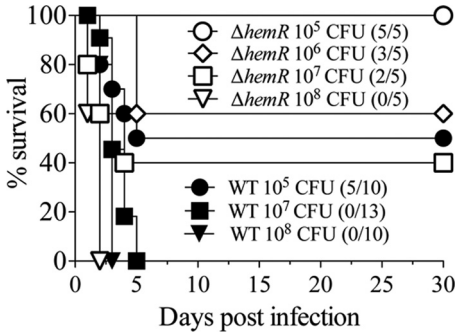


FIG 11 Virulence of *E. ictaluri* Δ*hemR* in catfish i.c. infected with *E. ictaluri* Δ*hemR* (LD_{50}, 2.2×10^6 CFU).

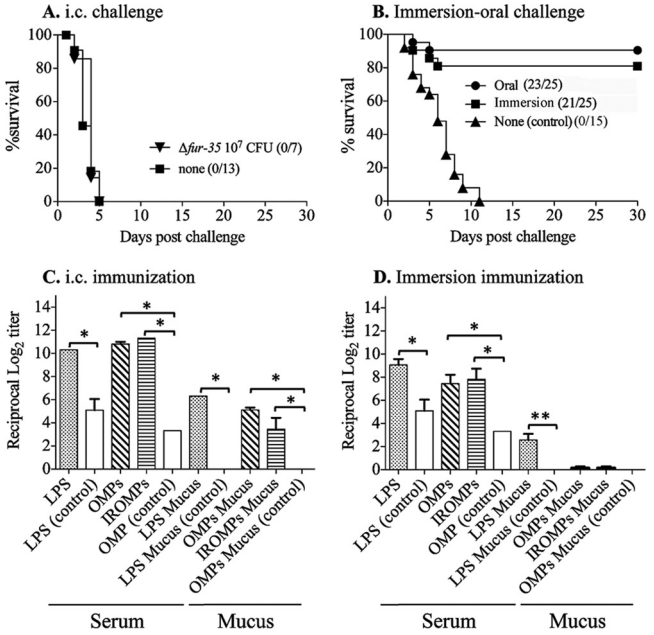


FIG 12 Immune protection of *E. ictaluri* Δ*fur-35* in catfish host. (A) Catfish i.c. challenged with wild-type *E. ictaluri* (10^7 CFU/dose) at 6 weeks after immersion immunization with *E. ictaluri* Δ*fur-35* (10^7 CFU/dose during 30 min). (B) Catfish immersion challenged with wild-type *E. ictaluri* (10^7 CFU/dose during 30 min) at 6 weeks after immersion (10^7 CFU/dose during 30 min) or oral (10^7 CFU/dose) vaccination with *E. ictaluri* Δ*fur-35*. The protection of *E. ictaluri* Δ*fur-35* was significantly different from that for the nonimmunized fish ($P < 0.05$). (C) Catfish IgM titers at 4 weeks after i.c. immunization with *E. ictaluri* Δ*fur-35* (10^7 CFU/dose) ($n = 22$). (D) Catfish IgM titers at 4 weeks after immersion immunization with *E. ictaluri* Δ*fur-35* (10^7 CFU/ml during 30 min) ($n = 24$). The samples correspond to two independent experiments with 10 to 14 animals each. *, $P < 0.001$; **, $P < 0.05$.

tive immune response, and so we evaluated whether fish immunized i.c. with *E. ictaluri* Δ*fur* were protected against a wild-type *E. ictaluri* i.c. challenge. We determined that *E. ictaluri* Δ*fur* does not confer immune protection against i.c. challenge (Fig. 12A) but does confer full protection against immersion challenge (data not shown). Catfish that were immersion immunized or orally immunized with *E. ictaluri* Δ*fur* survived the immersion challenge (Fig. 12B). Catfish i.c. immunized presented significant levels of IgM titers, either in the blood or in the mucus (Fig. 12C). However, these IgM levels were not protective for i.c. challenged fish (Fig. 12A). Immersion-immunized catfish presented significant levels of systemic IgM and low levels of skin IgM titers (Fig. 12D). These levels were sufficient to protect the fish against the immersion challenge (Fig. 12B).

DISCUSSION

The ferric uptake regulator (Fur) is a global regulatory protein that is involved in diverse aspects of bacterial life. It is a metalloregulatory protein that requires Fe^{2+}, or other divalent transition metal ions such as Zn^{2+}, as a cofactor (3, 40, 74). Fur possesses three functional domains, the helix-turn-helix DNA binding domain, the protein-protein dimerization domain, and the metal ion-responsive domain. The last domain is essential for Fur dimerization to form a functional protein (9, 48, 53). It has already been suggested by Pohl et al. (48) that the N-terminal helix is required for efficient DNA binding at the Fur box. This is supported by mutagenesis studies. For example, *P. aeruginosa* Fur having Ala10 mutated to glycine (a much poorer helix former) was unable to bind to the Fur box at the *pvdS* gene promoter (4). In *E. coli* it was shown that proteolytic cleavage of the 8 or 9 N-terminal residues resulted in a protein with reduced DNA binding affinity and specificity (9). However, as we have shown, the N-terminal region present in most of the Fur proteins is missing in *E. ictaluri* Fur (Fig. 1). The His32 residue, which interacts with Fe^{2+} in *P. aeruginosa* Fur, is also missing (Fig. 1). This indicates that the binding residues for Fe^{2+} and other divalent ions in *E. ictaluri* Fur are different than those used in *E. coli* and *Pseudomonas* Fur proteins.

In terms of phylogeny, the sequenced *E. tarda* isolated from humans contains a *fur* gene longer than *fur* genes in *E. tarda* isolated from fish, which contain a short version and appear to have a common ancestor with the *E. ictaluri fur* gene (Fig. 2). Numerous examples of genome reduction in the transition from free-living bacteria to a parasitic life style have been documented (37). Pathogenic bacteria seem to have embarked on some of the same processes of gene degradation and deletion that have led to extensive genome shrinkage in host-restricted pathogenic groups. For example, large numbers of pseudogenes have been identified in both *Y. pestis* (43) and *S. enterica* serovar Typhi (42). Perhaps the emergence of a shorter *fur* gene in the *Edwardsiella* genus is an adaptation to the fish host. *E. ictaluri* was first described by Hawke in 1979 (22) and then characterized in 1981 (23), during the industrial expansion of the catfish aquaculture industry (70). Thus, an alternate hypothesis is that the reduction of the *fur* gene could be part of a process of *E. ictaluri* host specialization due to intensive fish aquaculture. This observation raises the question of how this shorter *fur* gene increases the fitness of *Edwardsiella* in the fish host and how it appears during the evolution to host adaptation.

Although *E. ictaluri* Fur is missing the N-terminal region of other Fur proteins, it is fully functional in *S. enterica*, complementing the regulation of siderophore and IROMP synthesis in an iron-dependent fashion (Fig. 4). This indicates that a single α-helix in the DNA binding domain is enough for DNA binding and gene repression. Detailed studies are required to evaluate this hypothesis. Nevertheless, the fact that *E. ictaluri fur* complements *S. enterica* indicates a recent divergence from the Fur phylogenetic trunk and supports the idea that *E. ictaluri fur* might be part of an ongoing process of host specialization.

As mentioned above, iron acquisition mechanisms, such as siderophores, are essential for bacterial pathogens to overcome

host defenses. However, we did not detect siderophore synthesis in *E. ictaluri*, regardless of the presence or absence of iron in the growth medium, the volume of culture, or the presence or absence of the *fur* gene (Fig. 5). Our results correlate with previous observations (67) and with the absence of know siderophore biosynthesis genes in the *E. ictaluri* chromosome (72). This observation reinforces the hypothesis of genome degradation during the process of host specialization of *E. ictaluri* to catfish, where siderophore genes might be lost. The siderophore receptor genes *fepA* and *fcuA* were identified in the chromosome of *E. ictaluri* within their respective operons, containing an undefined *fur* binding box (data not shown). This indicates that *E. ictaluri* could utilize siderophores secreted from other bacterial species, likely in a Fur-independent fashion. Further studies are required to evaluate the foreign utilization of siderophores by *E. ictaluri*. Nevertheless, in the absence of iron, *E. ictaluri* upregulates a heme-hemoglobin acquisition system in a Fur-dependent fashion (Fig. 5 to 8). During pathogenesis, clearly there is a battle between *E. ictaluri* and the catfish host for iron (34, 35, 45, 46, 63), where the *E. ictaluri* heme-hemoglobin acquisition system becomes a lethal weapon contributing to the systemic infection of the fish. However, this heme-hemoglobin acquisition system needs to be tightly regulated and synthesized at a precise time and location within the host.

Fur protein is a global regulator that controls genes related to acquisition of iron and other divalent metal ions and genes related to bacterial catabolism, affecting virulence. We determined that *E. ictaluri* Fur regulates genes related to iron acquisition and heme synthesis as well as Mg^{2+} and Mn^{2+} uptake (Fig. 9). Fur also regulates genes related to heme biosynthesis (*hemF*), where, in the absence of iron, the bacterial cell has to preserve the integrity of the electron transport chain parts and respiration by upregulating the synthesis of tetrapyrroles such as heme. On the other hand, Fur regulates catabolism of sugars by regulation of *gmpA*, linking the availability of nutrients to the availability of essential ion metals. The *gmpA* gene encodes a phosphoglycerate mutase that is regulated by Fur in *E. coli* (19) and *Salmonella* (69). Here we have described that Fur contributes to the regulation of *gmpA* in *E. ictaluri* (Fig. 9). This indicates that Fur participates in the general regulation of metabolic pathways, linking bacterial growth to the supply of key nutrients such as iron. We found a cyclic AMP receptor protein (Crp) binding box in the *fur* promoter region (see Fig. S2 in the supplemental material), which is conserved in *E. coli* and *Salmonella fur* promoters (19, 69). Crp may link expression of the Fur regulator to the availability of carbon sources.

The main described virulence factors in *Edwardsiella* are the type III secretion system, required for cell invasion and survival in *Edwardsiella*-containing vesicles in macrophages, and the type VI secretion system, required for full virulence (66, 68, 75). The *Edwardsiella* type III and VI secretion systems, as well as the transcriptional regulators EsrB and EsrC, are very similar in *E. tarda* and *E. ictaluri* (66, 68, 75). Recently the linkage of Fur with virulence in *E. tarda* has been reported (6). Fur regulates the type VI secretion system-mediated repression of *evpP* gene expression (a type VI secretion effector), binding to its promoter region and blocking the binding of the EsrC activator (6). We did not find either an *E. ictaluri evpP* gene ortholog or a Fur binding box at the promoter region of the *evpA* gene or *E. ictaluri* type VI secretion system (data not shown). Protein-protein interaction between Fur and EsrC or between Fur and EsrB response regulators was described for *E. tarda* (6). Although we did not find a Fur binding box in the type III secretion system or in the type VI secretion system of *E. ictaluri*, it is possible that Fur influences the virulence of *E. ictaluri*-mediated protein-protein interaction as was described in *E. tarda*.

Salmonella Δ*fur* mutants are attenuated in mammals when administered orally (51) or intraperitoneally (16) but are not very immunogenic (11). However, Δ*fur* mutants constitutively synthesize their IROMPs, exposing them to the immune system of the host. Although *E. ictaluri* Δ*fur* is not an efficient vaccine compared to the recently described *E. ictaluri* Δ*crp* mutant (55), the *fur* gene is an evolutionarily conserved regulon that controls iron homeostasis and bacterial virulence. Therefore, deletion of *fur* is an effective modification for live attenuated vaccines and can be combined with other deletions, such as that of *crp*, to improve the safety and immunogenicity of the vaccines.

In summary, we conclude that *E. ictaluri* does not secret detectable siderophores under the growth conditions tested, regardless of the presence or absence of Fur and iron. Fur regulates a heme-hemoglobin uptake system in *E. ictaluri*, and deletion of *fur* can be successfully used as a means to attenuate *E. ictaluri* in order to develop effective immersion live attenuated vaccines for the aquaculture industry.

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