Ferric hydroxamate uptake system contributes to *Edwardsiella ictaluri* virulence

Hossam Abdelhamed, Jingjun Lu, Mark L. Lawrence, Attila Karsi*

*Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University,
Mississippi State, MS 39762, USA*

* Corresponding author.

E-mail address: karsi@cvm.msstate.edu (A. Karsi).

ABSTRACT

Edwardsiella ictaluri is a Gram-negative facultative intracellular pathogen causing enteric septicemia in fish, particularly in channel catfish. Ferric iron is an essential micronutrient for bacterial survival, and some bacterial pathogens use secreted hydroxamate-type siderophores to chelate iron in host tissues. Siderophore-iron complexes are taken up by these bacteria via the ferric hydroxamate uptake (Fhu) system. In *E. ictaluri*, the Fhu system consists of *fhuC*, *fhuD*, *fhuB*, and *fhuA* genes. However, the importance of the Fhu system in *E. ictaluri* virulence has not been investigated completely. Here, we present construction of *E. ictaluri fhuD* and *fhuB* mutants (*EiΔfhuD* and *EiΔfhuB*) by in-frame gene deletion and evaluation of the mutants' virulence and immunogenicity in channel catfish fingerlings and fry. Immersion challenges showed that *EiΔfhuD* was not significantly attenuated ($p < 0.05$) in catfish fingerlings, whereas *EiΔfhuB* was significantly attenuated ($p < 0.01$). Catfish fingerlings immunized with *EiΔfhuD* and *EiΔfhuB* showed 100% and 97.62% survival, respectively. Fry immersion challenges indicated *EiΔfhuB* was also significantly attenuated ($p < 0.05$) in two-week old fry compared to the wild-type (48.96% vs. 82.14% mortalities). The survival rate in the fry vaccinated with *EiΔfhuB* was significantly higher ($p < 0.05$) than that of non-vaccinated fry (96.77% vs. 21.42% survival). Our data indicates that the *fhuB* gene, but not the *fhuD* gene, contributes to *E. ictaluri* virulence.

Keywords: Fhu system, *fhuD*, *fhuB*, Vaccine, Channel catfish.

1. Introduction

Channel catfish is a significant agricultural commodity in the United States. *Edwardsiella ictaluri* causes enteric septicemia in farmed catfish. The disease presents as either acute septicemia or chronic meningoencephalitis [1, 2]. Although oxytetracycline, sulfadimethoxine-orometoprim, and florfenicol are approved antimicrobials for treating diseases in aquaculture, use of antimicrobials is not considered a best treatment practice for several reasons, including the early development of anorexia in affected catfish, potential development of antimicrobial-resistant strains, and the withdrawal times before consumption [3-5]. Vaccination is an alternative prevention strategy, reducing the need for antimicrobials. Previously, it has been shown that formalin killed *E. ictaluri* vaccine is ineffective in producing adequate acquired immunity against *E. ictaluri* infection [6, 7]. In general, live attenuated vaccines are relatively inexpensive to produce and easy to apply, and they are potentially efficacious, inducing both local and systemic immune responses. Therefore, several live attenuated *E. ictaluri* strains have been developed [8-13]. Currently, a commercial live attenuated *E. ictaluri* vaccine exists [14], however enteric septicemia remains the most prevalent disease in the commercial catfish industry [15]. The commercial vaccine has failed to achieve widespread industry acceptance because of increased cost of catfish production as well as lack of on farm evaluations of the vaccine's performance [16]. Moreover, all fingerling operations that vaccinated fry against ESC experienced outbreaks of ESC in ponds with vaccinated fingerlings [17].

Because iron is crucial for bacterial growth within the host, bacteria can acquire iron through direct binding to various iron sources such as lactoferrin, transferrin, ferritin, and heme [18]. Alternatively, iron is captured through siderophore-mediated iron uptake mechanism [19]. The ferric hydroxamate uptake (Fhu) system binds and transports siderophore-iron complexes in

many pathogenic bacteria [20-22]. The *Escherichia coli* Fhu region is composed of four consecutive genes (*fhuA* → *fhuC* → *fhuD* → *fhuB*) [23]. FhuA receptor transports ferrichrome-iron across the outer membrane, and periplasmic FhuD and cytoplasmic membrane FhuB transport ferrichrome and other ferric hydroxamates from the periplasm across the cytoplasmic membrane into the cytoplasm. Periplasm to cytoplasm translocation is energized by FhuC-catalyzed ATP hydrolysis [24, 25].

Siderophore synthesis is associated with virulence in *Edwardsiella tarda* [26], *Neisseria gonorrhoeae* [27], *Aeromonas salmonicida* [28], *Vibrio anguillarum* [29], and *E. coli* [30]. Although *E. ictaluri* has no detectable siderophore secretion [31, 32], the *E. ictaluri* chromosome contains an Fhu operon (*fhuC* → *fhuD* → *fhuB* → *fhuA*). Previously, we have shown that the deletion of *fhuC* gene causes reduction of *E. ictaluri* virulence in catfish [33]. However, the role of other genes in the Fhu operon in *E. ictaluri* virulence is unknown. To attain a better understanding of the significance of the Fhu system in *E. ictaluri* virulence, we developed *E. ictaluri* Δ *fhuD* and Δ *fhuB* and determined their virulence and protective properties in the channel catfish host.

2. Materials and Methods

2.1. Bacterial strains, growth conditions, and plasmids

Bacterial strains and the plasmid used in this work are listed in Table 1. *Edwardsiella ictaluri* 93-146 strain was cultured in brain heart infusion (BHI) agar or broth (Difco, Sparks, MD) at 30 °C. *E. coli* CC118 λ *pir* and SM10 λ *pir* strains were cultured on Luria–Bertani (LB) agar or broth (Difco) at 37 °C. When required, the following antibiotics and reagents (Sigma-Aldrich, Saint Louis, MN) were added to culture medium at the following concentrations:

ampicillin (Amp: 100 µg/ml), colistin (Col: 12.5 µg/ml), sucrose (5%), and mannitol (0.35%). Suicide vector pMEG-375 was used to construct in-frame gene deletions by allelic exchange.

2.2. In-frame deletion of the *E. ictaluri* *fhuD* and *fhuB* genes

The nucleotide sequences of *E. ictaluri* *fhuD* (NT01EI_RS03170) and *fhuB* (NT01EI_RS03175) were obtained from the *E. ictaluri* 93-146 genome (GenBank accession: CP001600) [34]. Four primers (A, B, C, D) were designed for each gene using Primer3 [35] (Table 2). Restriction enzyme sequences were added to A and D primers for cloning, and the reverse complement of primer B was added to the 5' end of primer C to enable gene deletion construction by overlap extension PCR [36]. Recombinant DNA and mutant construction procedures were completed as described [33]. Colony PCR confirmed single and double crossovers using A and D primers, and final mutant validation was done by sequencing of A-D fragments amplified from ampicillin-sensitive mutants, using *EifhuDF01S* and *EifhuBF01S* primers (Table 2). The *E. ictaluri* $\Delta fhuD$ and $\Delta fhuB$ were designated as *Ei* $\Delta fhuD$ and *Ei* $\Delta fhuB$.

2.3. Assessment of virulence and efficacy of the *Ei* $\Delta fhuD$ and *Ei* $\Delta fhuB$ in catfish fingerlings

All fish experiments were conducted by a protocol approved by the Institutional Animal Care and Use Committee at Mississippi State University. Virulence and vaccine efficacy trials were conducted as described [12, 33]. Briefly, 320 specific-pathogen-free (SPF) channel catfish fingerlings (13.88 ± 0.27 cm, 27.77 ± 1.04 g) were stocked into 16 40-liter flow-through tanks (flow rate: 1-liter/min) at a rate of 20 fish/tank and acclimated for one week. Water temperature was maintained at 25°C (± 2) throughout the experiments. Dissolve oxygen (DO) was within the permissible limits for catfish production (4.0 -7.0 mg/l) [37]. Fish were fed twice a day with a

commercial catfish feed (Rangen, Inc., Buhl, ID). Tanks were assigned randomly to four groups: *Ei* Δ *fhuD*, *Ei* Δ *fhuB*, *Ei*WT (wild type strain 93-146), and BHI (negative control/sham control). Each group included four tanks. Virulence/vaccination was conducted by immersion. Briefly, OD₆₀₀ of each bacterial culture was measured. Water level in tanks was lowered to 10 L, and then 100 ml bacterial culture from the group with the lowest OD was added to each tank in the corresponding group. Other bacterial cultures were diluted to give equivalent ODs. During immersion, water was well aerated. After one h, water flow was restored, and fish were maintained as usual. Final bacterial dose in immersion experiment (3.32×10^7 CFU/ml water) was calculated from the original cultures by serial dilutions of two different cultures and calculating the average colony numbers on agar plates. Fish mortalities were recorded daily for a total of 21 days, and percent mortalities was calculated for each group.

Protection against wild-type *E. ictaluri* challenge was determined in survived fingerlings vaccinated by the Δ *fhuD* and Δ *fhuB* mutants. Briefly, after 21 days post-vaccination, catfish fingerling were re-challenged by *Ei*WT by immersion (3.83×10^7 CFU/ml water), and mortalities were recorded daily for 14 days (until no mortalities were observed in three consecutive days). At the end of the experiments, percent survival was calculated.

2. 4. Assessment of virulence and efficacy of the *Ei* Δ *fhuB* in catfish fry

The most practical vaccination under commercial production settings is applied before moving fish from hatchery to nursery ponds when fry are one- or two-week-old. Thus, we conducted virulence/vaccination on two-week-old channel catfish fry as described above. Briefly, 450 fry were stocked into nine tanks at a rate of 50 fry per tank, and tanks were divided randomly into three treatment groups with three replicates in each group. Treatment groups

consisted of *Ei* Δ *fhuB*, *Ei*WT, and BHI (negative/sham control). After OD adjustment, overnight bacterial cultures were added to tanks with 10 L water. The final immersion dose of 2.7×10^7 CFU/ml water was applied. During immersion, water was well aerated. After one h, water flow was resumed. Mortalities were recorded daily for a total of 21 days post-immersion, and the percent mortalities calculated.

Protection against wild-type *E. ictaluri* challenge was determined in surviving fry vaccinated by the *Ei* Δ *fhuB* mutant. Briefly, after 21 days post-vaccination, fry catfish were re-challenged by *Ei*WT by immersion (3.10×10^7 CFU/ml water), and mortalities were recorded daily for 14 days (until no mortalities were observed in three consecutive days), and percent mortalities was calculated.

2.5. Statistical analyses

The percent mortality data were arcsine transformed, and analysis of variance (ANOVA) was applied using PROC GLM in SAS for Windows v9.4 (SAS Institute, Inc., Cary, NC) to assess significance. Dunnett's Post-hoc test was used to resolve differences between groups. An alpha level of 0.05 was used in all analyses.

3. Results

3.1. Construction of *Ei* Δ *fhuD* and *Ei* Δ *fhuB*

The *E. ictaluri* *fhu* operon has four genes in the following order: *fhuC* $\rightarrow$ *fhuD* $\rightarrow$ *fhuB* $\rightarrow$ *fhuA*. The end of *fhuC* and the beginning of *fhuD* overlap ten bases. Similarly, the end of *fhuD* and the beginning of *fhuB* overlap four bases. The distance between the stop codon of *fhuB* and the start codon of *fhuA* is 186 bases. In the present study, we successfully introduced in-

frame deletions to the *E. ictaluri fhuD* and *fhuB* genes (Table 3). The $\Delta fhuD$ has a deletion of 822 bp out of 930 bp (88.38%), and the $\Delta fhuB$ has a deletion of 1,881 bp out of 1,992 bp (94.43%).

3.2. Virulence and protection of *Ei* $\Delta fhuD$ and *Ei* $\Delta fhuB$ in catfish fingerlings

Results of the immersion challenge in catfish fingerlings indicated that mortality rate was not significantly different ($p < 0.05$) between *Ei* $\Delta fhuD$ and *Ei*WT (44.44% vs. 46.91% mortality). By contrast, the mortality rate in *Ei* $\Delta fhuB$ challenged fish was significantly lower ($p < 0.01$) compared to *Ei*WT (6.67 % vs. 46.91% mortality) (Fig. 1A).

Experiments determining the protection of *Ei* $\Delta fhuD$ and *Ei* $\Delta fhuB$ vaccinated fingerlings against wild-type *E. ictaluri* challenge indicated that vaccination of catfish fingerlings with the *Ei* $\Delta fhuD$ and *Ei* $\Delta fhuB$ provided significant protection against *Ei*WT challenge at 21 days post-vaccination. Fingerlings immunized with *Ei* $\Delta fhuD$ had a 100% survival rate, and with *Ei* $\Delta fhuB$ had a 97.62% survival rate compared to 40.74% survival rate in the non-vaccinated sham control group (Fig. 1B).

3.3. Virulence and protection of *Ei* $\Delta fhuB$ in catfish fry

The *Ei* $\Delta fhuB$ was significantly less ($p < 0.05$) virulent than *Ei*WT (48.96% vs. 82.14%) in two-week old catfish fry (Fig. 2A). Experiments determining the protection of *Ei* $\Delta fhuB$ vaccinated fry against wild-type *E. ictaluri* challenge showed that the survival rate in vaccinated fry was significantly higher ($p < 0.05$) than that of the sham-vaccinated control group, following challenge with *Ei*WT (96.77% vs. 21.42% survival) (Fig. 2B).

4. Discussion

The primary objectives of this study were to elucidate the importance of the *fhuD* and *fhuB* genes in *E. ictaluri* virulence in catfish and to determine the protection potential of the *EiΔfhuD* and *EiΔfhuB* against the wild-type *E. ictaluri* infection in catfish. To accomplish this, we constructed two in-frame deletion mutants, *EiΔfhuD* and the *EiΔfhuB*. All our attempts to delete the *fhuA* gene were not successful.

The *EiΔfhuD* and the *EiΔfhuB* do not contain any extraneous antibiotic resistance gene as the mutations were conducted by precise in-frame deletions. Because resistance genes could spread to aquatic environment through vaccine strains, presence of additional resistance genes is not desirable [38, 39].

Our virulence assessment demonstrated that deletion of the *fhuD* gene did not affect *E. ictaluri* virulence significantly, whereas *fhuB* was required for *E. ictaluri* virulence (7.03-fold reduction in virulence in catfish fingerlings compared to the parent strain). Although neither strain is completely safe, immersion vaccination of catfish fingerlings with *EiΔfhuD* and *EiΔfhuB* provided good protection against experimental *Ei*WT infections, resulting in 100% and 97.62% survival, respectively. The difference in virulence between the *EiΔfhuD* and *EiΔfhuB* in catfish fingerlings could be due to location or function of these genes. The *fhuD* gene encodes periplasmic binding protein while the *fhuB* gene encodes a membrane protein, forming a passage across the cytoplasmic membrane [40, 41].

In commercial channel catfish production, the most practical vaccination method is immersion vaccination of fry before they are transferred to nursery ponds. Thus, we evaluated *EiΔfhuB*, providing good protection in fingerlings, in two-week old fry using immersion exposure. *EiΔfhuB* causes approximately a 1.67-fold attenuation in catfish fry compared to

*Ei*WT (48.96% vs. 82.14% mortality). The reason for the higher virulence of *Ei* Δ *fhuB* in fry (48.96% mortality) compared to fingerling catfish (6.67 % mortality) is likely due to lack of a fully developed immune system in catfish fry compared to catfish fingerlings [42]. Surviving catfish fry vaccinated with *Ei* Δ *fhuB* were significantly protected against subsequent challenge with *Ei*WT at 21 days post-vaccination. The survival rate in fry vaccinated with *Ei* Δ *fhuB* was an average of 4.51-fold higher than that of the sham-vaccinated group following challenge with the *E. ictaluri* WT (96.77% vs. 21.42% survival).

A practical live attenuated bacterial vaccine should achieve a balance between lack of pathogenicity and adequate immunogenicity to provide high levels of protection especially in catfish fry. Our results demonstrated that the *Ei* Δ *fhuB* was 1.67-fold attenuated compared to the parent wild-type in catfish fry and induced excellent protection. In previous studies, the relative percent survival in fry (as young as seven days post-hatch) vaccinated with the Aquavac-ESC ranged from 58.4 to 77.5% following *E. ictaluri* WT challenge [43]. *Ei* Δ *fhuB* showed relatively stronger protection (96.77%) than that of Aquavac-ESC in fry, but it is not as safe as Aquavac-ESC. However direct comparison is difficult between the two studies because there are several variables one study to another, including strains used, fry age at the time of vaccination (1 week vs. 2 weeks old), catfish stocks used, challenge methods used, challenge dose applied, and water parameters. Nevertheless, the safety of *Ei* Δ *fhuB* would need to be improved by introducing new gene deletions before it could be developed as a safe vaccine.

The Fhu system is highly conserved among different bacterial strains, and *E. ictaluri* is no exception. The *E. ictaluri* genome contains four *fhu* genes organized slightly different than the *E. coli* *fhu* operon (*fhuC* $\rightarrow$ *fhuD* $\rightarrow$ *fhuB* $\rightarrow$ *fhuA* compared to *fhuA* $\rightarrow$ *fhuC* $\rightarrow$ *fhuD* $\rightarrow$ *fhuB*). Iron is an essential nutrient for bacterial growth, and acquisition of iron is essential for

virulence in bacterial pathogens. Thus, inactivation of iron acquisition systems correlates with reduced virulence in animal models [44-47]. FhuD and FhuB participate in iron uptake through ferric hydroxamate [24, 48]. As mentioned previously, no siderophore production has been detected in *E. ictaluri* in two earlier studies [31, 32].

Accumulated evidence indicate that the Fhu system is involved in bacterial virulence and it is a potential target for vaccine development. For example, deletion of the *fhuC* gene in the Fhu operon caused 2.05-fold attenuation of virulence of *E. ictaluri* in catfish [33]. In *Staphylococcus aureus*, FhuD2 is a possible staphylococcal vaccine antigen, and FhuD2 vaccination generated protective immunity against diverse clinical *S. aureus* isolates in murine infection models [49]. In *S. aureus*, FhuD2 and FhuD1 proteins are paralogues arising from gene duplication [50]. FhuD2 binds exclusively to hydroxamate siderophores and then FhuCBG complex mediates ATP-dependent transport across the staphylococcal membrane [51]. Interestingly, deletion of the Fhu system (*fhuCBG*) in *S. aureus* resulted in a strain with moderately decreased virulence in a mouse kidney abscess model [52]. In *Listeria monocytogenes*, the deletion of *fhuD* (*lmo1959*) had no impact on virulence in mice [53]. On the other hand, *fhuA* deletion in *Actinobacillus pleuropneumoniae* did not result in significant differences in virulence in swine [54].

In summary, *fhu* genes show different levels of contribution to *E. ictaluri* virulence in catfish. The *E. ictaluri* Δ *fhuB* is attenuated and provided protection in catfish fry and fingerlings.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

This project was supported by Agriculture and Food Research Initiative competitive grant no. 2014-70007-22359 from the USDA National Institute of Food and Agriculture. We thank Dr. Robert Wills at Mississippi State University for his assistance with statistical analysis. We thank the Laboratory Animal Resources and Care at the College of Veterinary Medicine for providing the SPF channel catfish.

References

- [1] Miyazaki T, Plumb JA. Histopathology of *Edwardsiella ictaluri* in channel catfish, *Ictalurus punctatus* (Rafinesque). J Fish Dis. 1985;8:389-92.
- [2] Shotts EB, Blazer VS, Waltman WD. Pathogenesis of experimental *Edwardsiella ictaluri* infections in channel catfish (*Ictalurus punctatus*). Can J Fish Aquat Sci. 1986;43:36-42.
- [3] Johnson MJ. Bacterial resistance to antibiotics: a growing problem in the channel catfish industry. In: Proceedings of Louisiana Aquaculture Conference, pp 22-23 Louisiana State University Agricultural Center, Baton Rouge, LA. 1991.
- [4] Taylor PW, Johnson MR. Antibiotic resistance in *Edwardsiella ictaluri*. American Fisheries Society, Fish Health Section Newsletter 1991;19:3-4.
- [5] Hawke JP, Durborow RM, Thune RL, Camus AC. ESC-enteric septicemia of catfish. Publication No. 477, Southern Regional Aquaculture Center, Stoneville, Mississippi. 1998.
- [6] Thune RL, Hawke JP, Johnson MC. Studies on vaccination of channel catfish, *Ictalurus punctatus*, against *Edwardsiella ictaluri*. J Appl Aquac. 1994;3:11-24.
- [7] Thune RL, Collins LA, Penta MP. A comparison of immersion, immersion/oral combination and injection methods for the vaccination of channel catfish *Ictalurus punctatus* against *Edwardsiella ictaluri*. J World Aquac Soc. 1997;28:193-201.
- [8] Thune RL, Fernandez DH, Battista JR. An *aroA* mutant of *Edwardsiella ictaluri* is safe and efficacious as a live attenuated vaccine. J Aquat Anim Health. 1999;11:358-72.
- [9] Lawrence ML, Cooper RK, Thune RL. Attenuation, persistence, and vaccine potential of an *Edwardsiella ictaluri purA* mutant. Infect Immun. 1997;65:4642-51.
- [10] Lawrence ML, Banes MM. Tissue persistence and vaccine efficacy of an o polysaccharide mutant strain of *Edwardsiella ictaluri*. J Aquat Anim Health. 2005;17:228-32.

- [11] Santander J, Mitra A, Curtiss R, 3rd. Phenotype, virulence and immunogenicity of *Edwardsiella ictaluri* cyclic adenosine 3',5'-monophosphate receptor protein (Crp) mutants in catfish host. *Fish Shellfish Immunol.* 2011;31:1142-53.
- [12] Karsi A, Gülsoy N, Corb E, Dumpala PR, Lawrence ML. High-throughput bioluminescence-based mutant screening strategy for identification of bacterial virulence genes. *App Environ Microbiol.* 2009;75:2166-75.
- [13] Dahal N, Abdelhamed H, Lu J, Karsi A, Lawrence ML. Tricarboxylic acid cycle and one-carbon metabolism pathways are important in *Edwardsiella ictaluri* virulence. *PloS one.* 2013;8:e65973.
- [14] Klesius PH, Shoemaker CA. Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. In: Roland DS, editor. *Adv Vet Med: Academic Press*; 1999. p. 523-37.
- [15] Anonymous. Case summary report aquatic diagnostic laboratory. Mississippi State University College of Veterinary Medicine. 2006.
- [16] Bebak J, and Wagner, B.,. Use of vaccination against enteric septicemia of catfish and columnaris disease by the U.S. catfish industry. *J Aquat Anim Health.* 2012;24:30-6.
- [17] USDA. Catfish 2010 Part I: Reference of Catfish Health and Production Practices in the United States, 2009. USDA–APHIS–VS, CEAH. Fort Collins, CO.
- [18] Wandersman C, Delepelaire P. Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol.* 2004;58:611-47.
- [19] Ratledge C, Dover LG. Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol.* 2000;54:881-941.

- [20] Sebulsky MT, Speziali CD, Shilton BH, Edgell DR, Heinrichs DE. FhuD1, a ferric hydroxamate-binding lipoprotein in *Staphylococcus aureus*: a case of gene duplication and lateral transfer. J Biol Chem. 2004;279:53152-9.
- [21] Mikael LG, Pawelek PD, Labrie J, Sirois M, Coulton JW, Jacques M. Molecular cloning and characterization of the ferric hydroxamate uptake (*fhu*) operon in *Actinobacillus pleuropneumoniae*. Microbiology. 2002;148:2869-82.
- [22] del Rio ML, Navas J, Martin AJ, Gutierrez CB, Rodriguez-Barbosa JI, Rodriguez Ferri EF. Molecular characterization of *Haemophilus parasuis* ferric hydroxamate uptake (*fhu*) genes and constitutive expression of the FhuA receptor. Vet Res. 2006;37:49-59.
- [23] Fecker L, Braun V. Cloning and expression of the *fhu* genes involved in iron (III)-hydroxamate uptake by *Escherichia coli*. J Bacteriol. 1983;156:1301-14.
- [24] Braun V, Braun M. Iron transport and signaling in *Escherichia coli*. FEBS Lett. 2002;529:78-85.
- [25] Koster W. Iron (III) hydroxamate transport across the cytoplasmic membrane of *Escherichia coli*. Biol Met. 1991;4:23-32.
- [26] Mathew JA, Tan YP, Srinivasa Rao PS, Lim TM, Leung KY. *Edwardsiella tarda* mutants defective in siderophore production, motility, serum resistance and catalase activity. Microbiology. 2001;147:449-57.
- [27] Yancey RJ, Finkelstein RA. Siderophore production by pathogenic *Neisseria* spp. Infect Immun. 1981;32:600-8.
- [28] Hirst ID, Hastings TS, Ellis AE. Siderophore production by *Aeromonas salmonicida*. J Gen Microbiol. 1991;137:1185-92.

- [29] Crosa JH. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. *Nature*. 1980;284:566-8.
- [30] Williams PH. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. *Infect Immun*. 1979;26:925-32.
- [31] Santander J, Golden G, Wanda SY, Curtiss R, 3rd. The *Fur* regulated iron uptake system of *Edwardsiella ictaluri* and its influence on pathogenesis and immunogenicity in the catfish host. *Infect Immun*. 2012.
- [32] Thune RL, Fernandez DH, Battista JR. An *aroA* Mutant of *Edwardsiella ictaluri* Is Safe and Efficacious as a Live, Attenuated Vaccine. *Journal of Aquatic Animal Health*. 1999;11:358-72.
- [33] Abdelhamed H, Lu J, Shaheen A, Abbass A, Lawrence ML, Karsi A. Construction and evaluation of an *Edwardsiella ictaluri* *fhuC* mutant. *Veterinary microbiology*. 2013;162:858-65.
- [34] Williams ML, Gillaspay AF, Dyer DW, Thune RL, Waldbieser GC, Schuster SC, et al. Genome sequence of *Edwardsiella ictaluri* 93-146, a strain associated with a natural channel catfish outbreak of enteric septicemia of catfish. *J Bacteriol*. 2012;194:740-1.
- [35] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3--new capabilities and interfaces. *Nucleic acids research*. 2012;40:e115.
- [36] Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*. 1989;77:61-8.
- [37] Andrews JW, Murai T, Gibbons G. The Influence of Dissolved Oxygen on the Growth of Channel Catfish. *Transactions of the American Fisheries Society*. 1973;102:835-8.

- [38] Heuer OE, Kruse H, Grave K, Collignon P, Karunasagar I, Angulo FJ. Human Health Consequences of Use of Antimicrobial Agents in Aquaculture. *Clinical Infectious Diseases*. 2009;49:1248-53.
- [39] Cantas L, Shah SQ, Cavaco LM, Manaia CM, Walsh F, Popowska M, et al. A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. *Front Microbiol*. 2013;4:96.
- [40] Braun V, Hantke K, Koster W. Bacterial iron transport: mechanisms, genetics, and regulation. *Metal ions in biological systems*. 1998;35:67-145.
- [41] Groeger W, Koster W. Transmembrane topology of the two FhuB domains representing the hydrophobic components of bacterial ABC transporters involved in the uptake of siderophores, haem and vitamin B12. *Microbiology*. 1998;144 (Pt 10):2759-69.
- [42] Petrie-Hanson LaAJA. Humoral immune responses of channel catfish (*Ictalurus punctatus*) fry and fingerlings exposed to *Edwardsiella ictaluri*. *Fish and Shellfish Immunology* 1999;9:579-89.
- [43] Shoemaker. CA, Klesius. PH, Bricker. JM. Efficacy of a modified live *Edwardsiella ictaluri* vaccine in channel catfish as young as seven days post hatch. *Aquaculture* 1999;76:189-93.
- [44] Dale SE, Doherty-Kirby A, Lajoie G, Heinrichs DE. Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: identification and characterization of genes involved in production of a siderophore. *Infect Immun*. 2004;72:29-37.
- [45] Cendrowski S, MacArthur W, Hanna P. *Bacillus anthracis* requires siderophore biosynthesis for growth in macrophages and mouse virulence. *Mol Microbiol*. 2004;51:407-17.

- [46] Henderson DP, Payne SM. *Vibrio cholerae* iron transport systems: roles of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. *Infect Immun*. 1994;62:5120-5.
- [47] Visser MB, Majumdar S, Hani E, Sokol PA. Importance of the ornibactin and pyochelin siderophore transport systems in *Burkholderia cenocepacia* lung infections. *Infect Immun*. 2004;72:2850-7.
- [48] Hantke K. Identification of an iron uptake system specific for coprogen and rhodotorulic acid in *Escherichia coli* K12. *Molecular & general genetics* : MGG. 1983;191:301-6.
- [49] Mishra RPN, Mariotti P, Fiaschi L, Nosari S, Maccari S, Liberatori S, et al. *Staphylococcus aureus* FhuD2 Is Involved in the Early Phase of Staphylococcal Dissemination and Generates Protective Immunity in Mice. *Journal of Infectious Diseases*. 2012;206:1041-9.
- [50] Andrews JW, Murai T, Campbell C. Effects of dietary calcium and phosphorus on growth, food conversion, bone ash and hematocrit levels of catfish. *J Nutr*. 1973;103:766-71.
- [51] Mariotti P, Malito E, Biancucci M, Lo Surdo P, Mishra RP, Nardi-Dei V, et al. Structural and functional characterization of the *Staphylococcus aureus* virulence factor and vaccine candidate FhuD2. *The Biochemical journal*. 2013;449:683-93.
- [52] Speziali CD, Dale SE, Henderson JA, Vines ED, Heinrichs DE. Requirement of *Staphylococcus aureus* ATP-binding cassette-ATPase FhuC for iron-restricted growth and evidence that it functions with more than one iron transporter. *J Bacteriol*. 2006;188:2048-55.
- [53] Jin B, Newton SM, Shao Y, Jiang X, Charbit A, Klebba PE. Iron acquisition systems for ferric hydroxamates, haemin and haemoglobin in *Listeria monocytogenes*. *Molecular microbiology*. 2006;59:1185-98.

- [54] Baltes N, Tonpitak W, Hennig-Pauka I, Gruber AD, Gerlach GF. *Actinobacillus pleuropneumoniae* serotype 7 siderophore receptor FhuA is not required for virulence. FEMS Microbiol Lett. 2003;220:41-8.
- [55] Herrero M, de Lorenzo V, Timmis KN. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol. 1990;172:6557-67.
- [56] Miller VL, Mekalanos JJ. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J Bacteriol. 1988;170:2575-83.
- [57] Dozois CM, Daigle F, Curtiss R, 3rd. Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. Proc Natl Acad Sci U S A. 2003;100:247-52.

Table 1

Bacterial strains and plasmids.

Strain	Relevant Characteristics	References
<i>Edwardsiella ictaluri</i>		
93-146	Wild type; pEI1 ⁺ ; pEI2 ⁺ ; Col ^r	[9]
<i>Ei</i> Δ <i>fhuD</i>	93-146 derivative; pEI1 ⁺ ; pEI2 ⁺ ; Col ^r ; Δ <i>fhuD</i>	This study
<i>Ei</i> Δ <i>fhuB</i>	93-146 derivative; pEI1 ⁺ ; pEI2 ⁺ ; Col ^r ; Δ <i>fhuB</i>	This study
<i>Escherichia coli</i>		
CC118λ <i>pir</i>	Δ(<i>ara-leu</i>); <i>araD</i> ; Δ <i>lacX74</i> ; <i>galE</i> ; <i>galK</i> ; <i>phoA20</i> ; <i>thi-1</i> ; <i>rpsE</i> ; <i>rpoB</i> ; <i>argE</i> (Am); <i>recA</i> l; λ <i>pir</i> R6K	[55]
SM10λ <i>pir</i>	<i>thi</i> ; <i>thr</i> ; <i>leu</i> ; <i>tonA</i> ; <i>lacY</i> ; <i>supE</i> ; <i>recA</i> ; ::RP4-2-Tc::Mu; Km ^r ; λ <i>pir</i> R6K	[56]
Plasmid		
pMEG-375	8,142 bp, Amp ^r , Cm ^r , <i>lacZ</i> , R6K <i>ori</i> , <i>mob incP</i> , <i>sacR sacB</i>	[57]
p <i>Ei</i> Δ <i>fhuD</i>	10,395 bp, Δ <i>fhuD</i> , pMEG-375	This study
p <i>Ei</i> Δ <i>fhuB</i>	10,250 bp, Δ <i>fhuB</i> , pMEG-375	This study

Table 2

Primers used to generate and verify in-frame deletion of the *E. ictaluri fhuD* and *fhuB*.

Primers		Sequence 5'-3' ^a	RE ^b
<i>EifhuDF01</i>	A	AAG AGCT CAACAGTATGACCTCGCAGACG	<i>SacI</i>
<i>EifhuDR72</i>	B	GGGTGAAAACAGGGAGAACAG	
<i>EifhuDF894</i>	C	<u>CTGTTCTCCCTGTTTT</u> CACCCATGGCGGAAAGCCTGATG	
<i>EifhuDR01</i>	D	AAT CTAG ACTCTGGCTGGTAGGTCAGGTA	<i>XbaI</i>
<i>EifhuDF01S</i>		CTGTCCGAGCTGTACCAAACC	
<i>EifhuBF01</i>	A	AAG AGCT CAGTTGCTATCGTATGTGCTTGA	<i>SacI</i>
<i>EifhuBR48</i>	B	CAGCGTCAACAGCAGCAG	
<i>EifhuBF1929</i>	C	<u>CTGCTGCTGTTGACGCT</u> GGCCTATTTCTGCTGCTGCTG	
<i>EifhuBR01</i>	D	AAC CCGGG ACGGGTGTTGTCGTTTCAGTAA	<i>SmaI</i>
<i>EifhuBF01S</i>		CGTTACAGTATGCCGCAGAG	

^aBold letters at the 5' end of the primer sequence represent RE site added. AA nucleotides were added to the end of each primer containing a RE site to increase the efficiency of enzyme cut.

Underlined bases in primer C indicate reverse complemented primer B sequence.

^bRE stands for restriction enzyme added to the 5' end of the primer sequence.

Table 3

The sizes of upstream (AB), downstream (CD), and in-frame fused fragments (AD).

Gene	USF (AB) (bp) ^a	DSF (CD) (bp)	FF (AD) (bp)	DR (bp / aa)	5' UD (bp / aa)	3' UD (bp / aa)
<i>fhuD</i>	1117	1136	2253	822 / 274	72 / 24	39 / 12
<i>fhuB</i>	989	1119	2108	1881 / 627	48 / 16	66 / 21

^aUSF = Upstream fragment; DSF = Downstream fragment; FF = Fusion fragment; DR = Deleted region; UD = Undeleted region; AB = A and B primers; CD = C and D primers; AD = A and D primers; bp = base pair; aa = amino acid.

Figure legends

Fig. 1. Cumulative percent mortalities of channel catfish fingerlings challenged with *Ei* Δ *fhuD*, *Ei* Δ *fhuB*, and *Ei*WT (A). Percent survival of channel catfish fingerlings vaccinated with *Ei* Δ *fhuD* and *Ei* Δ *fhuB*, and challenged with *Ei*WT (B).

Fig. 2. Cumulative mortalities of two-week old catfish fry challenged with *Ei* Δ *fhuB* and *Ei*WT (A). Percent survival of immersion vaccinated fry with *Ei* Δ *fhuB* and challenged with *Ei*WT (B).

Fig. 1

Fig. 2