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Construction and evaluation of an *Edwardsiella ictaluri fhuC* mutant

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ABSTRACT

Edwardsiella ictaluri is a Gram-negative facultative intracellular pathogen causing enteric septicemia in channel catfish. Iron is an essential micronutrient needed for bacterial virulence, and to acquire iron, many Gram-negative bacteria secrete ferric iron chelating siderophores. The ferric hydroxamate uptake (Fhu) system consists of four genes (*fhuC*, *fhuD*, *fhuB*, and *fhuA*), and is involved in the uptake of hydroxamate type siderophores across bacterial membranes. However, the Fhu system and its importance in *E. ictaluri* virulence have been uninvestigated. Here, we present construction and evaluation of an *E. ictaluri* Δ*fhuC* mutant. The *E. ictaluri fhuC* gene was deleted in-frame by allelic exchange, and the mutant's growth in media and virulence in catfish were determined. Our results indicated that deletion of the *E. ictaluri fhuC* gene did not affect the growth of *E. ictaluri* largely in both iron-replete and iron-depleted media. Addition of ferric iron sources into the iron-depleted medium improved the growth of both *E. ictaluri* Δ*fhuC* and wild type (WT). Catfish mortalities indicated that *E. ictaluri* Δ*fhuC* mutant was attenuated 2.05-fold compared with the parent strain. The catfish immunized with the *E. ictaluri* Δ*fhuC* mutant showed a high relative percent survival rate (97.50%) after re-challenge with the WT *E. ictaluri* strain. Taken together, our data indicates that the *fhuC* gene contributes to *E. ictaluri* virulence.

1. Introduction

Channel catfish is one of the most important cultured fish species in the United States. Diseases pose a main problem for the catfish industry, and especially *Edwardsiella ictaluri* is one of the most prevalent pathogens of catfish, causing enteric septicemia of catfish (ESC). Vaccination is an alternative disease prevention strategy for ESC, reducing the need for antibiotic treatments. In general, live attenuated vaccines offer the best prospect for a vaccine, giving similar protection to that seen with the natural infection. Therefore, considerable efforts have been made to develop live attenuated *E. ictaluri* strains as potential vaccines against ESC (Karsi et al., 2009; Klesius and Shoemaker, 1999; Lawrence and Banes, 2005; Santander et al., 2011; Thune et al., 1999).

Iron is essential for bacterial growth within the host. Thus, bacteria use siderophore mediated iron acquisition or direct iron binding mechanisms to obtain iron from the host (Ratledge and Dover, 2000). Siderophores are excreted from the bacterial cell, bind iron with high-affinity, and are then taken up into the bacterial cell via specific transport systems. In many pathogenic bacteria, the ferric hydroxamate uptake (Fhu) system is involved in the uptake of hydroxamate siderophores across the outer and cytoplasmic membranes (del Rio et al., 2006; Mikael et al., 2002). The *Escherichia coli* Fhu region composes four consecutive genes in the order *fhuA*, *fhuC*, *fhuD*, and *fhuB* (Fecker and Braun, 1983). FhuA is a multifunctional receptor protein transporting ferrichrome across the outer membrane while FhuC, FhuD, and FhuB participate in the uptake of ferrichrome and other hydroxamate compounds from the

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Table 1
Bacterial strains and plasmids.

Strain	Relevant characteristics	References
Edwardsiella ictaluri		
93–146	Wild type; pEI1$^+$; pEI2$^+$; Col^r	Lawrence et al. (1997)
*Ei*Δ*fhuC*	93–146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*fhuC*	This study
Escherichia coli		
CC118λ*pir*	Δ(*ara-leu*); *araD*; Δ*lacX74*; *galE*; *galK*; *phoA20*; *thi-1*; *rpsE*; *rpoB*; *argE*(Am); *recAl*; λ*pir*R6K	Herrero et al. (1990)
SM10λ*pir*	*thi*; *thr*; *leu*; *tonA*; *lacY*; *supE*; *recA*;::RP4-2-Tc::Mu; Km^r; λ*pir*R6K	Miller and Mekalanos (1988)
Plasmid		
pMEG-375	8142 bp, Amp^r, Cm^r, *lacZ*, R6K *ori*, *mob incP*, *sacR sacB*	Dozois et al. (2003)
p*Ei*Δ*fhuC*	10,326 bp, Δ*fhuC*, pMEG-375	This study

periplasm into the cytoplasm across the cytoplasmic membrane (Braun and Braun, 2002). The iron hydroxamates are transferred from FhuD to FhuB, and the translocation is energized by FhuC-catalyzed ATP hydrolysis, inducing a conformational change in FhuB (Koster, 1991).

The importance of siderophore synthesis for the virulence of pathogenic bacteria has been reported, including *E. tarda* (Mathew et al., 2001), *Neisseria gonorrhoeae* (Yancey and Finkelstein, 1981), *Aeromonas salmonicida* (Hirst et al., 1991), *Vibrio anguillarum* (Wertheimer et al., 1999), and *E. coli* (Williams, 1979). In *Staphylococcus aureus*, deletion of the Fhu system resulted in a strain that was incapable of growth using iron hydroxamates as their sole source of iron, had a growth defect in iron-restricted media, and had decreased virulence in a mouse kidney abscess model (Speziali et al., 2006). Although it is reported that *E. ictaluri* has no detectable siderophore secretion (Santander et al., 2012), the bacterium contains an Fhu operon on its chromosome. However, the importance of the Fhu system in *E. ictaluri* virulence is not known. Therefore, the objective of this study was to knock out the *E. ictaluri fhuC* gene function and determine the virulence of the *E. ictaluri* Δ*fhuC* mutant in the catfish host.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and the plasmid used in this work are listed in Table 1. *E. ictaluri* 93–146 wild type (WT) strain was cultured in brain heart infusion (BHI) agar or broth (Difco, Sparks, MD) and incubated at 30 °C throughout the study. *E. coli* CC118λ*pir* and SM10λ*pir* strains were cultured on Luria–Bertani (LB) agar or broth (Difco) and incubated at 37 °C throughout the study. When required, the following antibiotics and reagents (Sigma–Aldrich, Saint Louis, MN) were added to the 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 (*sacRB mobRP4* R6K *ori* Cm^r Amp^r) was used to construct in-frame gene deletion by allelic exchange.

2.2. Sequence analysis

The nucleotide sequences of the *E. ictaluri fhu* genes were obtained from the *E. ictaluri* 93–146 genome (GenBank accession: CP001600) (Williams et al., 2012). For similarity analyses, the orthologous *fhu* genes were obtained from the *E. tarda* FL6-60, and *E. coli* K-12 MG165 genomes (GenBank accession: CP002154 and U00096, respectively). Similarity analysis of the *fhu* genes from the three bacteria was conducted by using the DNASTAR's Megalign program (DNASTAR Inc., Madison, WI). Sequences were aligned using the Clustal V command.

2.3. In-frame deletion of the E. ictaluri fhuC gene in vitro

For in-frame deletion of the *E. ictaluri fhuC* gene, we designed four primers (A–D) (Table 2) using the Primer3 software (http://frodo.wi.mit.edu). Each of the two flanking primers (A and D) included a different restriction enzyme site for cloning, while the reverse complement of primer B was added to the 5′-end of primer C to create an overlap region to initiate in vitro in-frame deletion by PCR.

Table 2
Primers used to generate and verify in-frame deletion of the *E. ictaluri fhuC* gene.

Primers		Sequence (5′ → 3′)[b]	RE[a]
*Eifhu*CF01	A	AA**GAGCTC**ACTTGGACATGCCCTGTAGAC	*Sac*I
*Eifhu*CR24	B	CTCTAGGTTGGTTAGCGAAAACA	
*Eifhu*CF741	C	TGTTTTCGCTAACCAACCTAGAGCCGCCTCATAAAGTTGCTATC	
*Eifhu*CR01	D	AA**CCCGGG**CGAGTACAGCCACAGAATGTC	*Sma*I
*Eifhu*CF01S		GCCAAGTGGAAAAGGTGAATA	

[a] RE stands for restriction enzyme added to the 5′-end of the primer sequence.
[b] Bold 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.

Genomic DNA was isolated from *E. ictaluri* using a Wizard Genomic DNA Kit (Promega, Madison, WI). To delete the functional *fhuC* gene of *E. ictaluri* WT, we used gene splicing by overlap extension method (Horton et al., 1989). Briefly, the upstream (AB fragment) and downstream (CD fragment) regions of the *fhuC* gene were amplified using 50–100 ng *E. ictaluri* WT genomic DNA as template in 50 μl PCR, containing 1.25 U *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 mM primers, and 0.2 mM dNTP mix. The thermocycler conditions included: an initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Upstream and downstream fragments were mixed equally, and 4 μl was used as a template in the subsequent overlap extension PCR, which included the outside flanking primers (A and D) to generate the overlapped product (AD fragment). The conditions of the overlap extension PCR were as following: an initial denaturation at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 55 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 10 min.

After confirming the size of the fusion product on agarose gel, the remaining PCR was separated on an agarose gel, and the fusion fragment was purified by using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified fusion fragment was digested with *Sac*I and *Sma*I restriction enzymes (Promega), and cleaned using a Wizard SV Gel and PCR Clean-Up Kit (Promega). The suicide plasmid pMEG-375 was purified from an overnight *E. coli* culture by a QIAprep Spin Miniprep Kit (Qiagen) and cut with *Sac*I and *Sma*I restriction enzymes, producing compatible ends. The linearized plasmid was run on an agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen). After quantification using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE), fusion product containing the deleted *fhuC* gene was ligated into the linear pMEG-375 by T4 DNA Ligase (Promega) at 4 °C overnight.

Competent *E. coli* CC118λ*pir* cells were transformed using 1–2 μl ligation reaction using a Gene Pulser II system (Bio-Rad, Hercules, CA) set to 1.8 kV, 25 μF, and 400 Ω. Then, cells were recovered in SOC medium (Hanahan, 1983) for 1 h at 37 °C and spread on selective BHI agar plates with ampicillin. After overnight growth on agar plates, Amp^r colonies were picked and inoculated into BHI broth with ampicillin. The recombinant plasmids were isolated from the selected positive colonies and run on an agarose gel alongside the empty pMEG-375 plasmid for insert verification. Plasmids that were larger than the empty pMEG-375 were chosen for further confirmation of successful cloning by restriction enzyme digestion.

2.4. Mutation of the E. ictaluri fhuC gene in vivo

The recombinant plasmid p*Ei*Δ*fhuC* was electroporated into the donor *E. coli* SM10λ*pir*. and then introduced into *E. ictaluri* WT by conjugation to allow homologous recombination and allelic exchange to occur between the cloned fragment with in-frame deleted *fhuC* gene and the *E. ictaluri* chromosome. The selection of the *E. ictaluri* Δ*fhuC* mutant was conducted in two steps: in the first step, the colonies with the entire plasmid insertion by a single crossover were selected on BHI agar plates with ampicillin and colistin. In the second step, the single crossover mutants were propagated on LB agar with 5% sucrose and 0.35% mannitol to allow the loss of the suicide vector by the second homologous recombination. After this, mutant colonies were picked and grown in BHI broth with colistin. At each step, a colony PCR was conducted to check for the correct single and double crossover mutant bands using the A and D primers. The mutants with expected Δ*fhuC* phenotype were tested for ampicillin sensitivity to ensure the loss of the plasmid from the *E. ictaluri* chromosome. Final sequence verification was done by sequencing of the amplified and ExoSAP-IT (Affymetrix, Santa Clara, CA) treated mutant band using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and gene specific sequencing primer *Eifhu*CF01S (Table 2) in an Applied Biosystems 310 genetic Analyzer (Applied Biosystems). The *E. ictaluri* Δ*fhuC* mutant was designated as *Ei*Δ*fhuC*.

2.5. Growth of the E. ictaluri ΔfhuC mutant

Growth kinetics of the *E. ictaluri* Δ*fhuC* mutant and *E. ictaluri* WT were compared in iron-replete and iron-restricted medium. Iron chelator 2,2′-dipyridyl (Sigma–Aldrich) was prepared as 10 mM stock solution and filter-sterilized. Dipyridyl concentration inhibiting the growth of the *E. ictaluri* WT was determined in 5 ml BHI broth containing 0, 25, 50, 100, 150, 200, and 400 μM dipyridyl. The experiment was conducted using five replica cultures grown in a shaking incubator at 30 °C for 18 h. The optical densities were measured at 600 nm (OD_{600}) using a Spectronic GENESYS 20 spectrophotometer (Thermo Electron, Waltman, MA). The mutant's ability to grow in iron-replete and iron-depleted media was determined by inoculating the *E. ictaluri* Δ*fhuC* mutant and *E. ictaluri* WT into 15 ml BHI without or with 100 μM dipyridyl. Cultures were grown as described above for 24 h and the growth kinetics studied by measuring the bacterial growth at 0, 6, 12, 18, and 24 h. In addition, effects of ferric chloride ($FeCl_3$) and ferric nitrate $Fe(NO_3)_3$ (Sigma–Aldrich) on the growth of the *E. ictaluri* Δ*fhuC* mutant and *E. ictaluri* WT were determined under iron-depleted conditions. Six replica samples of 5 ml BHI broth containing 100 μM dipyridyl, 10 μM $FeCl_3$, and 10 μM $Fe(NO_3)_3$ were inoculated with the *E. ictaluri* Δ*fhuC* mutant and *E. ictaluri* WT. Cultures were grown in a shaking incubator at 30 °C for 18 h and OD_{600} readings were recorded.

2.6. Assessment of virulence of the E. ictaluri ΔfhuC mutant in catfish

All fish experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Mississippi State University. Vaccination and efficacy was conducted as described in our earlier work (Karsi et al., 2009). Briefly, 240 specific-pathogen-free (SPF) channel catfish fingerlings (13.88 ± 0.27 cm, 27.77 ± 1.04 g) were stocked into 12 40-l flow-through tanks (flow rate: 1-l/min) at a rate of 20 fish/tank and acclimated for one week.

Chlorine, dissolved oxygen, and temperature were monitored daily. Tanks were randomly assigned to *Ei*Δ*fhuC* (vaccination), *Ei*WT (positive control), and BHI (negative control) groups, with four tanks per group. Immersion vaccination was applied by lowering water in each tank to 10-l, and by adding 100 ml of bacterial culture (final dose of 3.32×10^7 CFU/ml water). After 1 h water flow (1-l/min) was restored to each tank. Mortalities were recorded daily for a total of 21 days, and the percent mortalities calculated for each group.

To assess the *E. ictaluri* Δ*fhuC* mutant as a possible vaccine candidate, all fish that survived the *Ei*Δ*fhuC* vaccination were re-challenged with *E. ictaluri* WT (3.83×10^7 CFU/ml) 21 days post-vaccination as described above. Fish mortalities were recorded daily, and relative percent survival (RPS) was calculated according to the following formula: RPS = [1 − (% mortality of vaccinated fish/% mortality of non-vaccinated fish)] × 100 (Amend, 1981).

2.7. Statistical analyses

We used SPSS V19 (IBM Corp., Armonk, NY) to conduct statistical analysis. When analyzing the sensitivity data of *E. ictaluri* WT to dipyridyl, the exposure concentration was the independent and OD was the dependent variable. Normality of data was checked using normal Q–Q Plots and Shapiro–Wilk test of normality. Homogeneity of variances was checked using Levene's test of homogeneity of variance. One-way ANOVA was used to determine mean differences among different dipyridyl doses. ANOVA or Robust test of Equality of Means tables were used to determine presence of significant differences among means ($p < 0.05$). Post hoc tests were conducted using Tukey or Games–Howell tests. Differences between the growth of *E. ictaluri* Δ*fhuC* mutant and *E. ictaluri* WT in medium without and with dipyridyl as well as with ferric iron sources under iron-depleted conditions were analyzed with Student's *t*-test ($p < 0.05$).

3. Results

3.1. The E. ictaluri fhu operon

Analysis of the *E. ictaluri* genome revealed the presence of four consecutive *fhu* genes in the following order: *fhuC* → *fhuD* → *fhuB* → *fhuA* (Fig. 1A). The end of *fhuC* and the beginning of *fhuD* open reading frames (ORF) overlap 10 bases. Similarly, the end of *fhuD* and the beginning of *fhuB* ORFs overlap 4 bases. The distance between the stop codon of *fhuB* and the start codon of *fhuA* is 186 bases. Alignment of the *E. ictaluri*, *E. tarda* FL6-60, and *E. coli* K-12 MG165 *fhu* genes indicated that all four *fhu* genes were highly similar between *E. ictaluri* and *E. tarda* (average 94.53%). In contrast, low level similarity was observed between the *E. ictaluri* and *E. coli fhu* genes (average 33.35%) (Table 3).

3.2. Construction of the E. ictaluri ΔfhuC mutant

In the present study, we introduced successfully in vitro in-frame deletion to the *E. ictaluri fhuC* gene and cloned it into the pMEG-375 suicide plasmid. The 1073 bp upstream (AB) and the 1111 bp downstream (CD) regions of the *E. ictaluri fhuC* gene were amplified successfully (Fig. 1B1 and B2). A 2184 bp overlap extension product (AD) containing the in-frame deletion of the *fhuC* gene was produced (Fig. 1B3) and then cloned into the pMEG-375 suicide plasmid, yielding the p*Ei*Δ*fhuC* plasmid (Fig. 1C3). *E. ictaluri* Δ*fhuC* mutant, designated as *Ei*Δ*fhuC*, was obtained

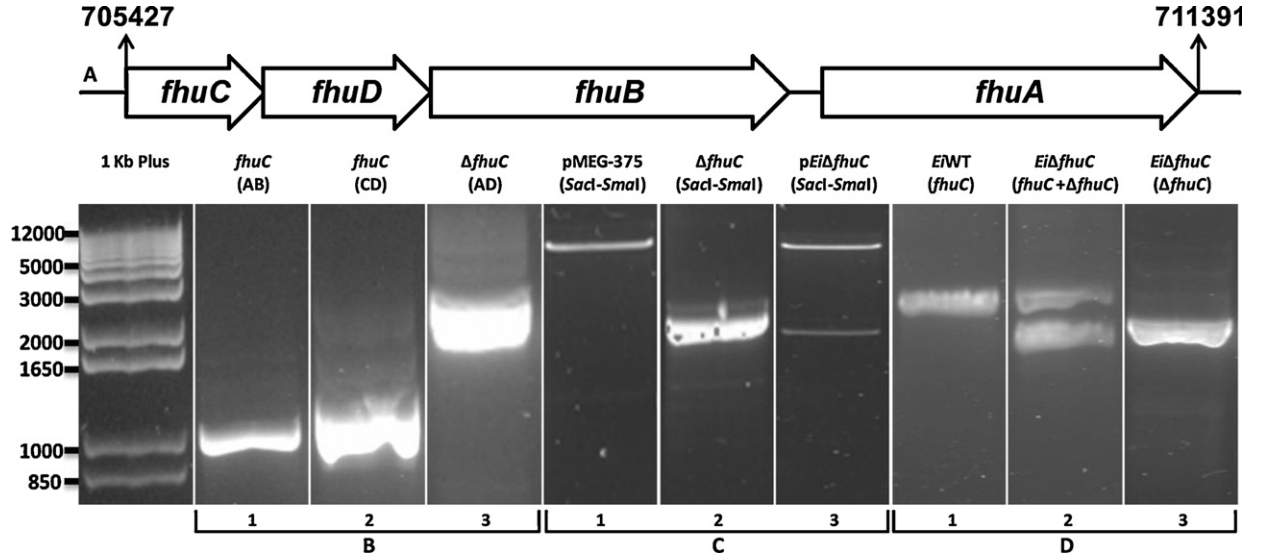

Fig. 1. Organization of the *fhu* operon in the *E. ictaluri* genome and construction and verification of the *Ei*Δ*fhuC* mutant. The arrows indicate the direction of transcription and numbers at the beginning and at the end indicate genomic coordinates of the *fhu* operon (A). Construction of in vitro in-frame gene deletion (B). The upstream (AB) (B1) and downstream (CD) (B2) fragments of the *fhuC* gene amplified from the *E. ictaluri* WT genomic DNA. The in-frame deleted fusion fragment (AD) (B3). Verification of cloning by RE digestion (C), linear pMEG-375 (C1), purified insert containing Δ*fhuC* (C2), the positive recombinant plasmid (C3). Mutant verification by colony PCR (D), products amplified from the wild-type parent strain (D1), the single cross-over mutant (D2), and the double cross-over mutant *Ei*Δ*fhuC* (D3).

Table 3
Properties of the *E. ictaluri fhu* genes and their similarities to *E. coli* and *E. tarda*.

E. ictaluri fhu genes	Locus tag	ORF (bp)	Location in *E. ictaluri* genome (bp)	Similarity to *E. coli fhu* genes	Similarity to *E. tarda fhu* genes
fhuC	NT01EI_0713	774	705427–706200	44.7%	95.6%
fhuD	NT01EI_0714	933	706191–707123	27.9%	94.4%
fhuB	NT01EI_0715	1995	707120–709114	37.6%	94.4%
fhuA	NT01EI_0716	2091	709301–711391	23.2%	93.7%

successfully by allelic exchange and verified by colony PCR after single and double crossover (Fig. 1D2 and D3). Sequencing of the amplified Δ*fhuC* fragment (Fig. 1D3) from the *Ei*Δ*fhuC* mutant confirmed in vivo in-frame deletion of the functional *fhuC* gene. We were able to delete 92.64% (717 bp/239 aa) of the *E. ictaluri fhuC* gene in-frame, leaving 24 bp (8 aa) at the 5′ end and 33 bp (10 aa) at the 3′-end.

3.3. Growth of the E. ictaluri ΔfhuC mutant

The growth of *E. ictaluri* WT in BHI broth with 0–400 μM dipyridyl indicated that dipyridyl concentration of 100 μM slowed down the growth of *E. ictaluri* WT significantly ($p < 0.05$) (Fig. 2 A). The growth of *E. ictaluri* WT declined 19.36% and 63.56% at 100 and at 200 μM dipyridyl, respectively. At the highest dipyridyl dose of 400 μM, bacterial growth declined 77.42% (Fig. 2A). There were significant ($p < 0.05$) differences at the early stages (6 h and 12 h) of growth between the *E. ictaluri* Δ*fhuC* mutant and *E. ictaluri* WT in iron-replete media, while the only significant growth difference was observed at 6 h in iron-depleted media (Fig. 2B). The growth of both the *E. ictaluri* Δ*fhuC* mutant and *E. ictaluri* WT in iron-depleted media declined significantly, but mutant's growth in iron-depleted media was similar to that of WT except at 6 h (Fig. 2B and C). Addition of ferric iron sources to the iron-depleted media restored the growth of both mutant and WT to the level of bacterial growth observed in iron-replete media (Fig. 2C).

3.4. Virulence and efficacy of the E. ictaluri ΔfhuC mutant

Infection of catfish fingerlings by immersion using the *E. ictaluri* Δ*fhuC* mutant and WT *E. ictaluri* revealed the

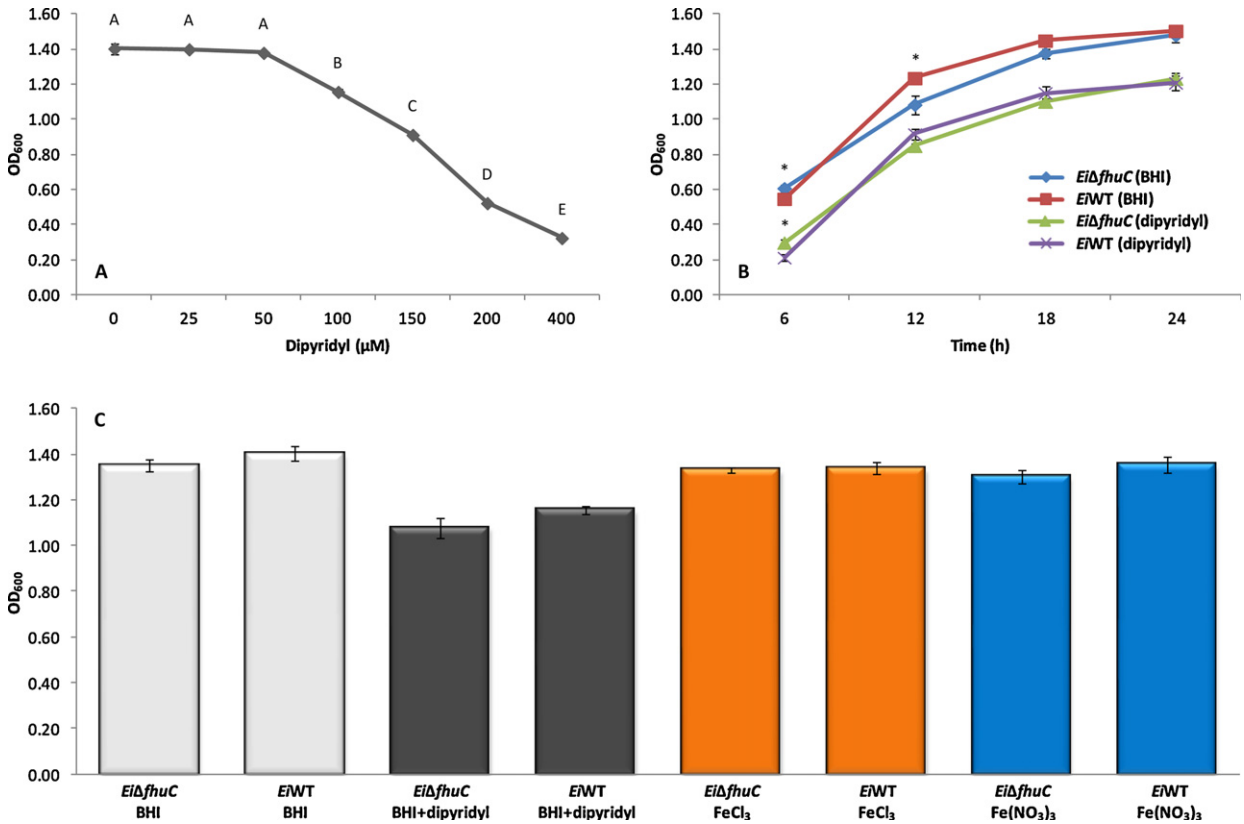

Fig. 2. Growth curve of the *E. ictaluri* WT in BHI with dipyridyl (A). Cultures were sampled after 18 h. Letters above the curve indicate statistical significance. Growth kinetics of the *E. ictaluri* Δ*fhuC* mutant and the *E. ictaluri* WT strain in BHI broth without and with dipyridyl (100 μM) (B). Star indicates significant difference between the *E. ictaluri* Δ*fhuC* mutant and WT. Growth of the *E. ictaluri* Δ*fhu* mutant and the *E. ictaluri* WT strain in BHI broth containing dipyridyl (100 μM) and different ferric iron sources (C).

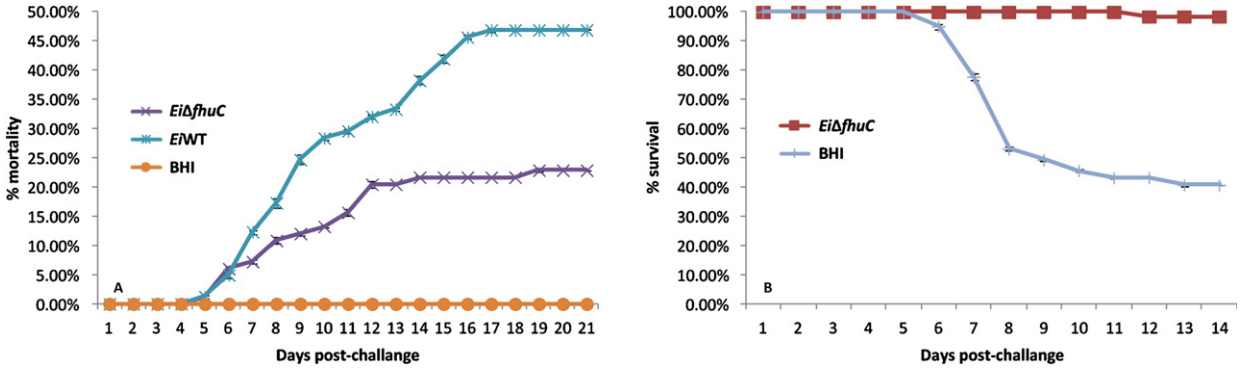

Fig. 3. Cumulative percent mortalities of channel catfish fingerlings challenged with the *E. ictaluri* Δ*fhuC* mutant and WT (A). Percent survival of the channel catfish fingerlings vaccinated with the *E. ictaluri* Δ*fhuC* mutant and challenged with the *E. ictaluri* WT strain (B).

level of virulence attenuation (Fig. 3A). After 21 days post challenge, catfish mortality was 22.89% in the *Ei*Δ*fhuC* challenged fish, whereas *Ei*WT caused 46.91% mortality. Evaluation of the *E. ictaluri* Δ*fhuC* mutant's efficacy after vaccination indicated that survival rate in the *Ei*Δ*fhuC* vaccinated group was 2.42-fold higher than that of the non-vaccinated group when re-challenged with the *E. ictaluri* WT (98.44% vs 40.74%) (Fig. 3B). RPS of the *Ei*Δ*fhuC* vaccinated group was 97.50%, which expresses the proportion of fish that were protected due to vaccination.

4. Discussion

Acquisition of iron is essential for virulence in bacterial pathogenesis. Thus, inactivation of iron acquisition systems correlates with reduced virulence in animal models (Cendrowski et al., 2004; Dale et al., 2004; Henderson and Payne, 1994; Visser et al., 2004). In this research, our purpose was to determine whether the *fhuC* gene, involving in the uptake of hydroxamate type siderophores, was important in the virulence of *E. ictaluri* in catfish.

We identified that the *E. ictaluri* genome contains four *fhu* genes organized in an operon structure in the following order: *fhuC* → *fhuD* → *fhuB* → *fhuA*. The Fhu system of *E. ictaluri* is similar to that of other *Enterobacteriaceae*, such as *E. tarda* and *Salmonella*. It is similar also in *Haemophilus parasuis* (del Rio et al., 2006) and *Actinobacillus pleuropneumoniae* (Mikael et al., 2002). In contrast, *fhuA* gene in *E. coli* is located first in the *fhu* operon (*fhuA* → *fhuC* → *fhuD* → *fhuB*) (Fecker and Braun, 1983). *Sequence alignment of E. ictaluri, E. tarda FL6-60, and E. coli K-12 MG165 fhu genes confirmed the close evolutionary relationship between E. ictaluri and E. tarda.*

In-frame deletion of functional genes allows studying gene function in vivo without affecting other downstream genes. In this study, we were able to delete a large portion of the *fhuC* gene by using gene splicing by overlap extension method. As electroporation of short linear DNA fragments and suicide plasmids into *E. ictaluri* is not yet efficient, we used the conjugation method to introduce p*Ei*Δ*fhuC* into *E. ictaluri* WT. It appeared that the allelic exchange procedure worked well when sequences flanking the in-frame deletion were about 1 kb. We have some difficulty in obtaining mutants with shorter flanking regions. It is possible that other mutation techniques, such as lambda Red recombinase system, could be used in *E. ictaluri*.

Little information is known about the growth of *E. ictaluri* under iron-restricted conditions. In this study, we found up to 50 μM dipyridyl bacterial growth was not affected significantly. Therefore, we used 100 μM dipyridyl to achieve iron-restricted conditions, which is also the preferred concentration to prepare iron-restricted growth media in many bacterial species, including *E. ictaluri* (Santander et al., 2012). This suggests that the Fhu system may not be essential for *E. ictaluri* growth in vitro. In support of this, addition of ferric iron sources into iron-depleted medium completely restored the growth of both *E. ictaluri* Δ*fhuC* mutant and the *E. ictaluri* WT. A recent study reported that no siderophore production was detected in *E. ictaluri* and no siderophore genes were found in the *E. ictaluri* genome (Santander et al., 2012). The presence of the Fhu operon supports that *E. ictaluri* may capture iron, when needed, from siderophores secreted by other bacteria. In contrast to our findings, significantly retarded growth was observed in *S. aureus* Δ*fhuC* mutant in iron-deficient media. However, the growth defect was not a result of the inability to transport ferric hydroxamates, but was a result of an inability to transport iron complexed to staphylobactin (Speziali et al., 2006).

No information is available on the Fhu system and its role in *E. ictaluri* pathogenesis in the catfish host. Furthermore, studies linking the Fhu system with bacterial virulence are limited. Our virulence assessment demonstrated that *Ei*Δ*fhuC* showed significant attenuation (22.89% mortality) compared with that of *Ei*WT (46.91% mortality). In *S. aureus*, deletion of the Fhu system resulted in a strain with decreased virulence in a mouse kidney abscess model (Speziali et al., 2006). In contrast, *fhuA* deletion in *A. pleuropneumoniae* resulted in no significant differences between the disease caused by the WT strain and by the mutant strain (Baltes et al., 2003).

A previous study in *V. cholerae* has shown that the virulence for mutant strains defective in heme transport, vibriobactin biosynthesis, and vibriobactin transport is weakly affected (Henderson and Payne, 1994; Tashima et al., 1996). In a similar study, two iron-enterobactin transport system deficient mutants of *V. cholerae* were able

to multiply and produce disease in mice, and the virulence was indistinguishable from the parent strains (Sigel et al., 1985). In *Shigella*, mutations in individual iron transport systems were tested for the ability to invade and form plaques in cultured cells. Neither heme transport (Mills and Payne, 1997) nor aerobactin-mediated siderophore transport (Lawlor et al., 1987) was found to be essential for invasion or plaque formation.

Our study shows that the loss of the *fhuC* gene causes approximately 50% reduction in virulence of *E. ictaluri* in catfish. As completely attenuated live vaccines are desirable in commercial scale catfish vaccination, *Ei*Δ*fhuC* strain may be further attenuated by targeting other iron acquisition systems. Analysis of the *E. ictaluri* genome indicated presence of other iron uptake systems, including ferric iron uptake system (*afuABC*) and TonB energy transducing system (TonB-ExbB-ExbD). Therefore, it is possible that inactivation of these iron acquisition systems may cause greater attenuation of the *E. ictaluri* Δ*fhuC* mutant. In *Salmonella enterica*, virulence of the single and double enterobactin deficient mutant strains were similar to the virulence of parent strain, but a triple mutant was significantly attenuated (Rabsch et al., 2003).

In conclusion, our results indicate that although deletion of the *fhuC* gene caused significant growth defect in *E. ictaluri* only at early stages of in vitro growth, in vivo virulence of the *E. ictaluri* Δ*fhuC* mutant diminished significantly, which suggests that the Fhu system of *E. ictaluri* may be used to capture hydroxamate siderophores secreted by other bacteria.

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