ASSESSMENT OF THE VIRULENCE AND IMMUNOGENICITY OF *EDWARDSIELLA ICTALURI FHUCD* MUTANT

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ABSTRACT

The *fhuCD* system and its importance in *Edwardsiella ictaluri* virulence have not been studied yet. Previously, we constructed *E. ictaluri* Δ*fhuCD mutants* by in-frame deletion and allelic exchange. In the present study, the virulence of the *Ei*Δ*fhuCD* mutant was evaluated by immersion challenge using Specific Pathogen-Free channel catfish fingerlings and the ability of this mutant to protect the channel catfish against *E. ictaluri* was evaluated. Also, the ability of *EiΔfhuCD* mutant to grow in the presence of iron chelator 2,2' Dipyridyl was assayed. The results indicated that little or no attenuation in *Ei*Δ*fhu*CD (40.00% mortality) was observed as compared to the parent *E. ictaluri* (%46.42). The channel catfish treated with *Ei*Δ*fhuCD* mutant showed high relative percent survival rates (average 94.72%) after re-challenge with *E. ictaluri* wild type. It was also found that the deletion of the *E. ictaluri fhuCD* operon did not affect *E. ictaluri* growth under iron-replete and iron-restricted conditions. Taken together, such data suggest that *fhuCD* operon is partial essential in *E. ictaluri* full virulence in channel catfish.

Key word: *Edwardsiella ictaluri*; *fhuCD* mutant; virulence

1. INTRODUCTION

The main factor limiting the expansion and profitability of the commercial channel catfish (*Ictalurus punctatus*) industry is the disease control. Currently, the two most prevalent diseases are enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri* and columnaris disease caused by *Flavobacterium columnare* (USDA 2003a). ESC is estimated to cause losses at $50–80 million annually (Russo *et al.* 2009). The genus Edwardsiella is a member of the family Enterobacteriaceae. At the DNA level, the genus Edwardsiella is 20% related to *E. coli* and to other species of the family Enterobacteriaceae including *Salmonella, Shigella* and *Citrobacter* (Brenner 1978). ESC generally presents in two forms: acute septicemic form and chronic meningoencephalitis form, or "hole-in-the-head disease" (Miyazaki and Plumb 1985; Shotts *et al.* 1986).

Although Romet 30, Terramycin, and Aquaflor are approved antibiotics to treat infections in aquaculture, the antibiotic use is not considered the best treatment practice for several reasons, including development of anorexia in the sick catfish, development of antibiotic resistant strains, and antibiotic withdrawal times before consumption (Johnson 1991; Taylor and Johnson 1991; Hawke *et al.* 1998). Vaccination is an alternative disease prevention strategy for ESC, which reduces the need for antibiotic treatment. Previously, formalin killed *E. ictaluri* vaccine was reported not to be efficient in producing strong acquired immunity against *E. ictaluri* infection (Thune *et al.* 1994; Thune *et al.* 1997). Currently, a commercial live *E. ictaluri* vaccine (Klesius and Shoemaker 1999) is available from Intervet Inc. However, ESC is still the most prevalent disease in the industry. Different studies have developed live attenuated strains as potential vaccines against ESC (Lawrence *et al.* 1997; Thune *et al.* 1999; Lawrence and Banes 2005; Karsi *et al.* 2009; Santander *et al.* 2011).

The iron uptake is essential for bacterial growth and colonization within the host, bacteria has developed different mechanisms to obtain this essential nutrient from the host that usually are siderophore mediated system or directly binding of iron from host (Ratledge and Dover 2000). The majors groups of siderophores include the catecholates or hydroxamates. The siderophores are excreted from the bacterial cell, bind iron with high-affinity, and are then taken up into the bacterial cell via specific transport system. In many pathogenic bacteria including *E. coli*, the Fhu region has been identified as the ferric hydroxamate siderophore uptake system (Mikael *et al.* 2002; Sebulsky *et al.* 2004; del Rio *et al.* 2006). It participates in the uptake of all four ferric hydroxamate compounds (ferrichrome, aerobactin, coprogen, and rhodotorulic acid) across the outer and the cytoplasmic membranes (Coulton *et al.* 1983; Mademidis and Koster 1998). The *E. coli* Fhu region composes of four consecutive genes named in the order of *fhuA, fhuC, fhuD,* and *fhuB* (Fecker and Braun 1983). The *fhuCD* operon participates in the uptake of ferric hydroxamate compounds from the periplasm into the cytoplasm across the cytoplasmic membrane. This has been shown for ferrichrome (Luckey *et al.* 1972; Braun *et al.* 1976; Kadner *et al.* 1980), ferric aerobactin (Braun *et al.* 1982), ferric coprogen, and rhodotorulic acid (Hantke 1983). FhuC is a hydrophilic protein localized at the inner side of the cytoplasmic membrane, which functions as the ATPase that energizes transport of ferrichrome and the other ferric hydroxamates across the cytoplasmic membrane (Burkhardt and Braun 1987; Coulton *et al.*

1987). FhuD is the periplasmic binding protein, which serves as a carrier for transport of ferric hydroxamates to the FhuB protein in the cytoplasmic membrane (Rohrbach *et al.* 1995; Mademidis *et al.* 1997; Braun and Killmann 1999).

The lack of siderophore synthesis has been shown to be correlated with loss of virulence, as seen in *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,* the deletion *of Fhu system* resulted in a strain, which was incapable of growth on iron hydroxamates as a sole source of iron, had a growth defect in iron-restricted laboratory media, and had a decreased virulence in a mouse kidney abscess model (Speziali *et al.* 2006). While, the iron uptake system of *E. ictaluri* is not characterized yet, it is reported that *E. ictaluri* grown under iron-limited conditions exhibited a low level siderophore activity (Thune *et al.* 1999).

We previously reported the construction of *E. ictaluri Ei*Δ*fhuCD* using in-frame deletion and allelic exchange (unpublished work yet). The objective of this study was to determine the role of *Ei*Δ*fhuCD* mutant in *E. ictaluri* virulence and also evaluated the potential utilization of this mutant to protect the channel catfish against *E. ictaluri*. Finally, the ability of the *Ei*Δ*fhuCD* to grow in the presence of the iron chelator 2, 2' Dipyridyl was assayed.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Fish.

A total number of two hundred forty Specific Pathogen Free channel catfish (SPF) fingerlings (13.88 ± 0.27 cm, 27.77 ± 1.04 g) were obtained from the SPF channel catfish laboratory at the College of Veterinary Medicine Mississippi State and maintained in aerated tanks supplied with a continuous flow of recirculation water at 26 °C. All the fish experiments were performed at the facilities of Department of Basic science at College of Veterinary Medicine Mississippi State University from period of September to December 2011.

2.1.2. Bacterial strains

E. ictaluri strain 93-146 is a clinical isolate that used at the Department of Basic science at the College of Veterinary Medicine Mississippi State University to investigate mechanisms of ESC. *E. ictaluri* WT isolated in 1993 from moribund channel catfish in a natural outbreak of ESC on a commercial farm Louisiana State University Aquatic Animal Diagnostic Laboratory (**Lawrence et al., 1997b).** *E. ictaluri* Δ*fhuCD* mutant was constructed by Hossam *et al.* 2012 (under publication).

2.1.3. Chemicals and media

1-Tricaine Methanesulphonate (MS-222 Sigma)
2-2,2′-dipyridyl (Sigma)
3. Difco™ Brain Heart Infusion Agar (Difco, Sparks, Maryland).
4. Bacto™ Brain Heart Infusion (Difco)

2.2. Methods

2.2.1. Fish rearing conditions.

Fish were allowed to acclimate for one week before the challenges. Fish were fed twice daily with commercial channel catfish feed. The fish were reared with a photoperiod of 12:12h (light/dark). The fish were euthanasia by Tricaine Methanesulphonate (MS-222 Sigma) at the end of the experiment by over-anesthetized.

2. 2.2. Assessment of virulence of the E. ictaluri ΔfhuCD mutant in catfish fingerlings

In vivo assessment of virulence and efficacy was conducted as described in our earlier work (Karsi *et al.* 2009) by following the institutional guidelines for animal care. Briefly, two hundred forty SPF channel catfish were stocked into 12 circular tanks at a rate of 20 fish/tank. There were three treatment groups (*EiΔfhuCD, E. ictaluri wild type* and BHI) and each treatment group was randomly assigned four replicate tanks. The first group was challenged with *EiΔfhuCD* mutant, the second group was challenged with *E.ictaluri* WT strain 93-146 as a positive control and the third group was challenged with BHI as a negative control. After lowering the tank water levels to ten liters and turning off the water flow, one hundred ml of overnight BHI broth cultures (3.32×10^7 CFU/ml) were added to each tank. The fish were exposed to the cultures for one hour and then the water flow was resumed. Mortalities were recorded daily for a total of 21 days post-treatment and the mortality percent rates were calculated for each group.

2.2.2. Evaluation of the efficacy E. ictaluri ΔfhuCD mutant in catfish fingerlings

To further determine the efficacy of the *EiΔfhuCD* mutant to protect the fish from *E. ictaluri*, the previous virulence experiment was extended and all the tanks in the three groups were re-challenged with *E. ictaluri* WT (3.83×10^7 CFU/ml) after 21 days post considering the second group as the negative control and the third group as positive control. Fish mortalities were recorded daily and relative percentage survival (RPS) of each group was determined according to the following formula (Amend 1981), which expresses the proportion of fish saved due to vaccination.

$$RPS = 100\% \times 1 \left(\frac{\% \text{ mortality in vaccinated fish}}{\% \text{ mortality in control fish}} \right)$$

2.2.3. Determination of the minimal inhibitory concentration of dipyridyl

Iron chelator 2,2′-dipyridyl was prepared as 10 mM stock solution by dissolving the compounds in distilled water followed by filter-sterilized prior the addition to autoclaved BHI broth. To determine minimum inhibitory concentration (MIC) of dipyridyl, *E. ictaluri* WT was inoculated into 5 ml BHI broth with 0, 20, 25, 50, 100, 125, 150, 200, and 400 μM dipyridyl. All experiments were carried out in triplicates for each concentration and the cultures were grown in a shaking incubator at 30°C for 18 hr and the optical density of each culture was measured at 600 nm. (On the basis of this work 120 μM DPD were chosen as suitable working concentrations).

2.2.4. Growth kinetic study of E. ictaluri ΔfhuCD mutant

Experiment was conducted to determine the ability of the *E. ictaluri* Δ*fhuCD* mutant to grow and proliferate on BHI. In triplicate independent assays, *E. ictaluri* wild-type and the *E. ictaluri ΔfhuCD* mutant were inoculated separately into the 15ml BHI and the cultures were grown in a shaking incubator at 30°C. The growth kinetics was studied over a period of 48 hrs and the OD_{600} measured every 6 hrs.

2.2.5. Growth kinetic study of E. ictaluri ΔfhuCD mutant under iron restricted condition (DPD)

Mutants' ability to grow and proliferate under iron restriction condition (DPD) was determined by inoculating *E. ictaluri* Δ*fhuCD* mutant and *E. ictaluri* WT into 15 ml BHI. Cultures were grown as described above.

3. RESULTS

3. 1. Virulence and efficacy of the E. ictaluri ΔfhuCD mutant

Immersion challenge of catfish fingerlings by the *E. ictaluri* Δ*fhuCD* mutant and wild type *E. ictaluri* revealed the level of virulence attenuation (Figure 1). After 21 days post challenge, *Ei*Δ*fhuCD* have revealed a very slight attenuation (40.00% mortality) as compared to that of *E. ictaluri* WT (46.91% mortality) and also there was no mortalities were observed in the catfish challenged with the BHI group (negative control). Mortality values among groups at day 21 were different significantly ($P < 0.05$). The catfish that died showed typical symptoms of ESC.

3. 2. Immersion immunization trial

We evaluated the immune protection of the catfish 21 days post immersion challenges with *E.ictaluri* Δ*fhuCD* mutant. It was found that 94.72% RPS for the catfish treated with Δ*fhuCD* mutant. The non-treated control catfish presented 41.70% RPS, while the catfish treated by *E.ictaluri* WT was calculated to have 92.54% RPS (Figure 2) ($P < 0.05$).

3. 3. Minimal inhibitory concentration of dipyridyl

In order to obtain iron depleted conditions, various concentrations of the iron chelator dipyridyl were added to the BHI broth. It was found that the MIC that inhibited *E. ictaluri* growth was 120μM (Figure 3) and this concentration was subsequently used for further experiments.

3. 4. Growth kinetics and iron source utilization of the E. ictaluri Δfhu mutants

The *E. ictaluri* Δ*fhuCD* did not showed any significant growth defects in BHI broth as compared to *E. ictaluri* WT (Figure 4). Also, the growth rate of the *E. ictaluri ΔfhuCD* was the same as that of *E. ictaluri* wild-type in the presence of the iron chelator 120μM dipyridyl (Figure 5).

4. DISCUSSION

Little is known about the FhuCD operon and its role in the pathogenesis of *E. ictaluri* in channel catfish host. To the best of our knowledge, this is the first study that explores the role of *fhu* genes on the *E. ictaluri* virulence. In the present study, our virulence assessment demonstrated that *Ei*Δ*fhuCD* showed a very slight attenuation (40.00% mortality) as compared to that of *E. ictaluri* WT (46.91% mortality). Evaluation of mutant's efficacy indicated high RPS (94.72%) values among mutants, which were similar to that of the WT strain. These results are in agreement with a previous study, in which the mice challenged with a *S. aureus fhuCBG* mutant strain did not exhibit significantly differences in kidney abscess score, percent weight loss and bacterial load in the kidneys as compared to parent strain (Speziali *et al.* 2006). Further, an *fhuA* deletion in *A. pleuropneumoniae* serotype 7 did not appear to be profitable for the construction of a live vaccine strain because no significant differences were observed between the disease caused by the wild type strain and the mutant strain (Baltes *et al.* 2003).

An effective live attenuated vaccine must be safe but *Ei*Δ*fhuCD* showed high accumulated mortalities in catfish. This result suggested that Δ*fhuCD* mutant strain is not good candidates as a live attenuated vaccine against ESC. It is not obvious from this data whether *fhuCD* is unnecessary in iron transport because sufficient iron sources are available in the host or because alternative methods of iron acquisition are being utilized. Our results suggested that *E. ictaluri fhuCD* operon do not play a significant role in *E. ictaluri* virulence.

Acquisition of iron is essential for virulence in bacterial pathogens; thus, inactivation of iron acquisition systems correlates with reduced virulence in animal models (Henderson and Payne 1994; Bearden *et al.* 1997; Takase *et al.* 2000; Cabrera *et al.* 2001; Cendrowski *et al.* 2004; Dale *et al.* 2004; Visser *et al.* 2004). Little information is known about the growth of *E. ictaluri* under iron restricted conditions. In this study, we found 120μM dipyridyl was sufficient to promote the growth of *E. ictaluri* WT. The MIC of iron chelator dipyridyl for *E. ictaluri* WT was equal to other bacteria such as *Renibacterium salmoninarum* (Grayson *et al.* 1995), *Acinetobacter baumannii,* and *Vibrio anguillarum* (Dorsey *et al.* 2004). Also, we found that the growth rates of the *Ei*Δ*fhuCD* mutant strains was the same as that of the *E. ictaluri* WT in BHI broth suggesting that loose of *fhuCD* operon did not cause any effect on *E. ictaluri* growth in media (in vitro).

In addition, the growth rates of the *E.ictaluri* Δ*fhuCD* mutant were the same like that of *E. ictaluri* WT under the iron restricted conditions. The lack of difference between the two is correlated with the fact that *E. ictaluri* does not synthesize detectable siderophores (Thune *et al.* 1999; Santander *et al.* 2012) . Santander et al (Santander *et al.* 2012) found that *E. ictaluri* does not secrete either catechol or hydroxylamine related siderophores, or heme binding molecules, although the *E. ictaluri* chromosome contains the siderophore receptors, a ferric enterobactin transport protein and a TonB-dependent ferrichrome receptor protein, within respective operon. In agreement with our work, Fhu operon in *A. pleuropneumoniae* did not show any significant change in expression in response to iron restricted conditions, indicating that the Fhu operon is not regulated by iron levels in the environment and these three genes appears to be iron independent (Mikael *et al.* 2002; Mikael *et al.* 2003; Deslandes *et al.* 2007). Also, Del Rio and colleagues (del Rio *et al.* 2006) found that FhuA protein expression in *Haemophilus parasuis* is not affected under iron-restricted conditions.

Previously, Speziali and others (Speziali *et al.* 2006) indicated that *S. aureus* strains with *fhuG: fhuD1: fhuD2* mutations did not have an obvious growth defect in iron-deficient media. However, the growth of *fhuC mutant strain alone* was significantly retarded compared to the growth of the wild-type strain in iron-deficient media.

In conclusion, in vitro growth and in vivo virulence of the *E. ictaluri* Δ*fhuCD* mutant seems correlated, which suggests that the FhuCD system of *E. ictaluri* is partially important in growth and virulence in catfish. The FhuCD system in *E. ictaluri* may not be the essential iron uptake system but rather could be a complementary system to other iron uptake pathways. Based on our findings in this research, *fhuCD* deletion does not appear to be advantageous for the construction of a live vaccine strain and the safety of these mutants can be increased further by introducing more than one attenuating phenotype. Further research on this aspect is in progress

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Figure legends

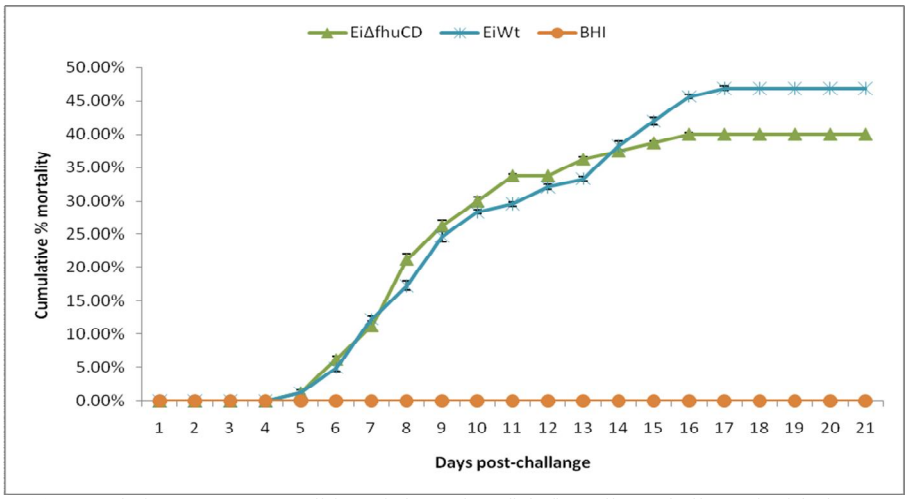


Figure (1) Cumulative percent mortalities of channel catfish fingerlings challanged with the *E. ictaluri* Δ*fhuCD* mutant and wild type.

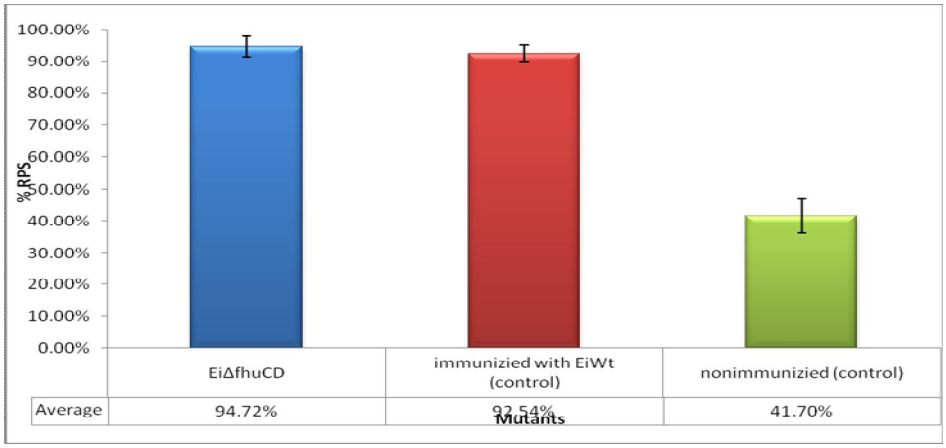


Figure (2) Reletive perent survival of channel catfish fingerlings vaccinated with the *E. ictaluri* Δ*fhuCD* mutant and challanged with the *E. ictaluri* WT strain.

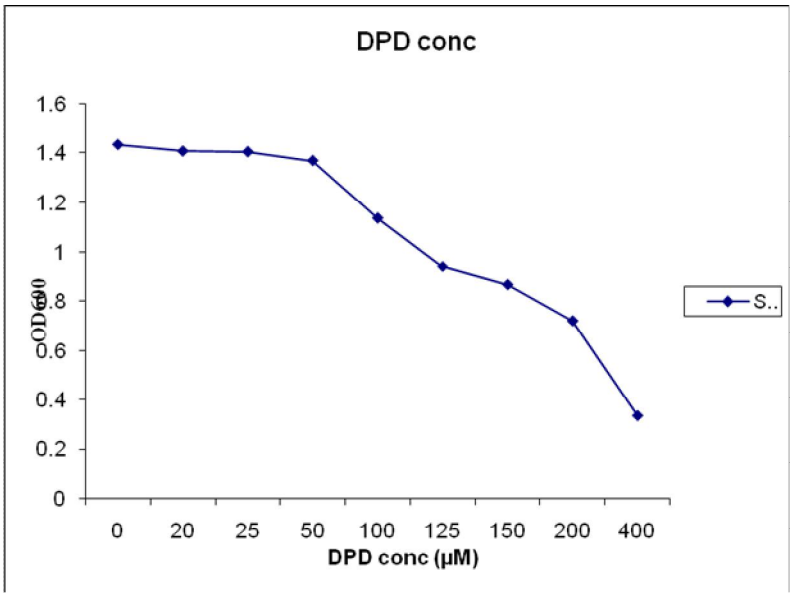
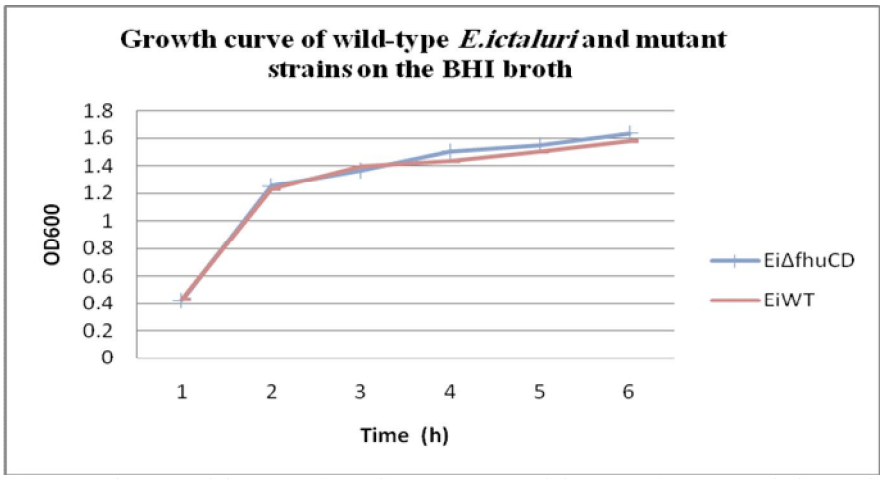


Figure (3) The minimum inhibition concentration (MIC) of the dipyridyl for *E.ictaluri* growth



FIigure (4) Growth curve of the *E. ictaluri* Δ*fhuCD* mutant and the *E. ictaluri* WT strain in BHI broth.

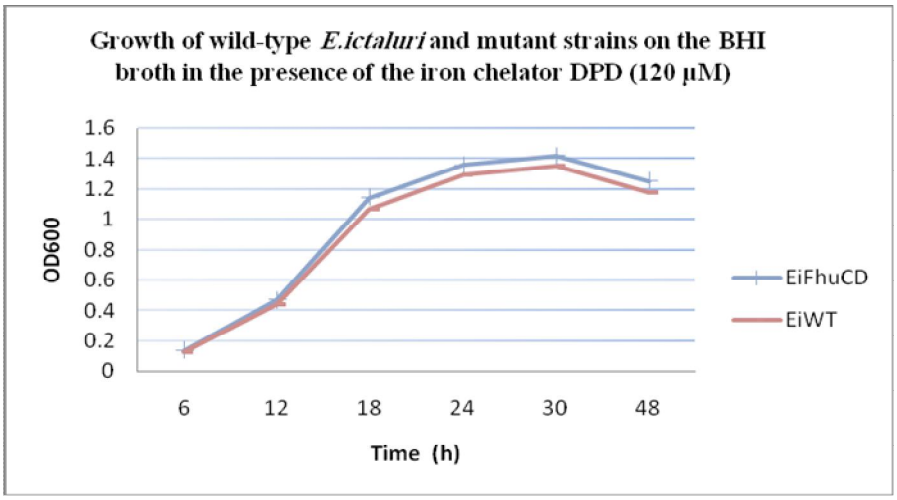


Figure (5) Growth curve of the *E. ictaluri* Δ*fhuCD* mutant and the *E. ictaluri* WT strain in BHI broth containing 120 µM dipyridyl.

تقيم الضروة والمناعة فى طفرة fhuCD الادوارد سيللا اكتالورى

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اهمية نظام fhuCD فى ضراوة ميكروب الادواردسيلا اكتالورى لم يتم دراستها بعد وفى دراسة سابقة تم تصميم طفرة fhuCD بواسطة حزف وتبادل الجينات فى الكروموسومات المتشابهة . وقد تم بهذه الدراسة تقيم الضراوة لهذه الطفره

عن طريق العدوى بالغمر باستخدام اصباعيات قرموط القنوات الخالى من مسببات العدوى وقدره هذه الطفرة على حمايه قرموط القنوات من ميكروب الادواردسيلا اكتالورى . تم اختبار قدره الطفرة على النمو فى وجود مزيلات الحديد (Dipyridyl 2.2) واثبتت النتائج اضعاف قليل فى ضراوة الطفرة (40% نفوق) مقارنة بالبكتيريا المعزولة من السمك البرى (46.42%) بعد معاملتها بالعترة المعزولة من السمك البرى وقد اثبتت الدراسة ان حذف نظام fhuCD

لم يؤثر على نمو الطفرة فى وجود او عدم وجود مزيلات الحديد . ومن النتائج المجمعة يتضح ان النظام fhuCD له دور جزى فى الضراوة الكاملة لميكروب الادوارسيلا اكتالورى