CONSTRUCTION OF *EDWARDSIELLA ICTALURI FHUCD* IN-FRAME DELETION MUTANT

Abdelhamed H. *[1], Jingjun Liu[2], Neeti Dahali[2], Shaheen A. A.[1], Amany Abbass A.[1], Mark L. Lawrence[2] and Attila Karsi[2]
[1]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh 13736, Egypt
[2]Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762-6100, USA

ABSTRACT

A number of different strategies have been developed to achieve Site-directed mutagenesis. One of these methods is in-frame deletion technique which efficiently creates mutations at specific sites by deletion a number of nucleotides from a bacterial chromosome. The *fhuCD* operon consists of two genes (*fhuC* and *fhuD*) which are involved in the uptake of ferric hydroxamate siderophores across the bacterial membranes. In the present study, a construction of the *EiΔfhuCD* mutants was conducted. *EiΔfhuCD* mutant was achieved by performing the in-frame *fhuCD* fragment through overlapping extension PCR then digested the in-frame fragment by *Sac*I and *Xba*I restriction enzyme and ligated to the suicide plasmid pMEG-375. The recombination plasmid (p*EiΔfhuCD*) was transformed into *E. ictaluri* wild type strain by conjugation and integrated into the genomic DNA through two steps of homologous recombination. The colony PCR and DNA sequencing were used to genotypically confirm the deletion. Besides, it was successfully deleted a 1635bp/545aa from a 1707-bp of the *fhuCD* operon.

Key word: Edwardsiella ictaluri; fhuCD.

1. INTRODUCTION

The construction of site-directed mutagenesis on a bacterial chromosome by deletion mutations in DNA sequences is a powerful approach to analyzing the function and structure of genes and their products. The deletion mutation systems involve double selection in a two-step procedure with suicide plasmid integration of the target sequence by homologous recombination. Integration of the plasmids into the chromosome is selected by an antibiotic resistant marker. Excision of the integrated plasmid for allelic exchange is selected with counter-selectable markers (Reyrat *et al.* 1998). The most popular used counter selectable marker is the sucrose-sensitivity system that has been used to construct the mutant strains in many bacteria. The *Bacillus subtilis sacB* gene encodes levanesaccharase, which is lethal in most gram-negative bacteria in the presence of sucrose (Gay *et al.* 1983)

Horton and colleagues (Horton *et al.* 1990) described the technique of splicing overlap extension by the polymerase chain reaction (SOE by PCR). This technique is a well-accepted method for construction deleted mutants allowing for the discovery of virulence. The SOE allows the creation of large deletions without the use of restriction enzymes or ligase (Horton 1995). This system is based on the fact that PCR primers can be designed with extra sequences added to their 5' regions. These 'add on' sequences are complementary to the primers of a second PCR generated fragment. Thus one strand from the first PCR generated fragment can anneal with one strand from the second PCR generated fragment. The SOE by PCR procedure can be used for the creation of any kind of chimeric gene deletion mutant or site-directed mutant. This method represents a significant improvement over standard methods of site-directed mutagenesis because it is much faster, simpler and approaches 100% efficiency in the generation of mutant product. The general mechanism of overlap extension, as applied to site-directed mutagenesis, is illustrated in Figure (1).

The acquisition of iron is the main determinant as to whether the microorganism that finds themselves within an animal and is able to keep itself therein. Without such ability, the microorganism cannot grow and will effectively be eliminated through direct attack from the host defense mechanisms or will die of nutrient starvation. The *fhuC* and *fhuD* is the first two genes in an operon that also includes *fhuB* and *fhuA,* respectively in gene orders. In *E. coli,* the *fhu* gene region is known as the ferric hydroxamate uptake system. It participates in the uptake of all four ferric hydroxamate compounds (ferrichrome, aerobactin coprogen and rhodotorulic acid) across the outer membrane and the cytoplasmic membrane (Coulton *et al.* 1983; Mademidis and Koster 1998).

The importance of the Fhu system during invasive infections of the host is therefore questionable. Virtually nothing is known regarding the role of *fhuCD* during infection. More research is needed to understand and characterize the virulence factors of ferric hydroxamate system of *E. ictaluri* and their mechanisms in mediating fish diseases. The overall objective of this study was to construct *E. ictaluri* mutant through in-frame deletion of *fhuCD* operon from the *E. ictaluri* genome.

2. MATERIALS AND METHODS

3. 1.Materials

2.1. 1. Bacterial strains and plasmid.

Bacterial strains and plasmid used in this work are listed in Table (1). *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. coli* strain CC118*λpir* was used to maintain the delivery plasmids during mutation and for plasmid amplification. *E. coli* strain *SM10λpir* was used as the donor in conjugations for transfer of suicide plasmids into *E. ictaluri*. Suicide vector pMEG-375 (*sacRB mobRP4* R6K *ori* Cm^r Amp^r) was used to construct in-frame gene deletions in *E. ictaluri*.

2.1. 2. Chemical and media

1. Difco™ Brain Heart Infusion Agar (Difco, Sparks, Maryland).
2. Bacto™ Brain Heart Infusion (Difco)
3. Difco™ (Luria-Bertani) LB Agar, Miller (Difco)
4. Difco™ (Luria-Bertani) LB Broth, Miller (Difco)

5-Agarose gel (Promega Company)

6-Antibiotics (Sigma Sigma) were added to media and broth for the selection of resistant bacterial strains, at the following concentration: Colistin sulphate (12.5 mg/ml), Ampicillin (100mg/ml)

7- Sucrose (Sigma Company) was added to media at a concentration of 5% to select against plasmid carrying the Bacillus subtilis levansucrase gene

8-Mannitol (Sigma Company) was added to media at a concentration of 0.35% to select against plasmid carrying the Bacillus subtilis levansucrase gene

2.1. 3. Kits and reagents used in for construction of E. ictaluri mutant

1-Wizard® Genomic DNA kits (Promega Company) was used for isolation of the Genomic DNA from *E. ictaluri* wild type (WT).

2- QIAprep Spin Miniprep Kit (Promega Company) was used for the plasmid isolation from *E. coli* broth cultures.

3-QIAquick gel extraction kit (Qiagen Company) was used for purification of the DNA bands produced by PCR or restriction endonuclease digestion after separation on an agarose gel.

2.1. 4. Primers and reagents for polymerase chain reaction (PCR)

1. Oligonucleotide primers are shown in Table (2).
2. Taq® DNA polymerase (5u/µl) **(**Promega Company).

3. Deoxynucleoside triphosphates (10mM dNTPs mix) (Promega Company).
4. Taq® Reaction Buffer (Promega Company).
5. Nuclease Free water.

2.2. Methods

2.2.1. The strategy of in-frame deletion in E. ictaluri

The general mechanism of in-frame deletion mutant is illustrated in Figure (1). Briefly, the upstream and downstream homologous regions of the *fhuCD* were selected (~1kbp) from *E. ictaluri* genome. The upstream and downstream homologous region of the targeted sequence were amplified in two separate PCR reactions by using one flanking primer (A or D) and one internal primer (B or C). Then both fragments were mixed and used as a DNA template in the overlap-extension PCR reaction for production of in-frame deletion fragment (AD). The in-frame deletion fragment (AD) was excised and purified form agarose gel. The overlap fragments was digested with specific restriction enzymes and then ligated to pMEG-375 followed by electroporation into *E.coli* strain CC118λpir competent cells. Recombinant plasmid was isolated from the positive colonies and electroporated into conjugation strain *E.coli* strain SM10λpir. *E.coli* strain SM10λpir was subsequently used to transfer the mutated gene to *E. ictaluri* wild-type by conjugation and to allow homologous recombination and allelic exchange. The *E. ictaluri* mutant strain was selected by double crossover, confirmed genotypically by the colony PCR and then sequenced.

2.2.2. Bacterial culture

E. ictaluri strain 93-146 was cultured in BHI agar or broth and incubated at 30ºC throughout the study. *E. coli* strains were cultured on LB agar or broth and incubated at 37ºC throughout the study. When required, the following antibiotics and reagents were added to the culture medium.

2.2.3. Primer design for in-frame deletion of fhuCD operon

For in-frame deletion of each *E. ictaluri fhuCD* operon, *fhuCD* operon which includes two genes (*fhuC* and *fhuD*) was selected from the *E. ictaluri* genome Table (3). Four primers (two flanking primers (A and D) and two hybrid primers (B and C)) were designed using the primer 3 software (http://frodo.wi.mit.edu/).The primers are listed on Table (2). Primers were ordered from (Sigma Company). There were several important considerations in primer design. First, primers B and C would determine the deleted region of the gene (5' end and the 3' end of the *fhuCD* operon). Therefore primer B was placed just upstream of the start of the *fhuCD* operon and primer C needed to be downstream end of the *fhuCD* operon. Second, the primers A and D were 1kbp faraway from the beginning and the end of the gene, respectively. Both primers A and D included specific restriction sites to improve the efficiency of the cloning. The final consideration was the reverse complementary of the primer of B was added to end of the primer C (Figure 1 Bold line). This complementary region is necessary for the fusion of the fragments AB and CD in the second PCR step (Ho *et al.* 1989).

2.2.4. PCR amplification and Splicing by Overlap extension PCR

Genomic DNA was isolated from *E. ictaluri* using a Wizard Genomic DNA Kit. To delete the functional *fhuCD* operon of *E. ictaluri*, gene splicing by overlap extension method were used according to (Horton *et al.* 1989). Briefly, the upstream (AB fragment) and downstream (CD fragment) regions were amplified using 50-100 ng *E. ictaluri* genomic DNA as template in 25µl PCR reactions, containing 1.25 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM primers, and 0.2 mM dNTP mix. The thermocycler conditions were: 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.

The upstream and downstream fragments were mixed and 4µl were used as a template in the subsequent 50µl overlap extension PCR reactions, which used the outside flanking primers (A and D) to generate the overlapped product (AD fragment). The conditions of the overlap extension PCR were: 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 20 min.

2.2.5. Cloning of the overlapped fragments (AD) into pMEG-375 plasmid

After confirming the size of the fusion products on agarose gel, the remaining PCR reactions were separated on agarose gel and purified by using a QIAquick Gel Extraction Kit. Purified fusion fragments were digested with *Sac*I *and Xba*I restriction enzymes and cleaned up using a Wizard SV Gel and PCR Clean-Up Kit. The suicide plasmid pMEG-375 was purified from an overnight culture by a QIAprep Spin Miniprep Kit and compatible ends were produced by with *Sac*I *and Xba*I restriction enzyme cut. The digested plasmids were run on an agarose gel and purified using a QIAquick Gel Extraction Kit. Then, fragment containing the in-frame deleted *fhuCD* operon was ligated into the linearized pMEG-375 by T4 DNA Ligase.

2µl ligation reaction was electroporated into *E. coli* C118 λ*pir* competent cells using a Gene Pulser II system (Bio-Rad, Hercules, California) set to 1.8 kV, 25 µF, and 400 Ω. Then, cells were recovered in SOC medium for 1 h at 37ºC and spread on selective LB agar plates with ampicillin to select plasmid bearing clones. After overnight growth on agar plates, Amp^r colonies were picked and inoculated into LB broth with ampicillin. The recombinant plasmid was minipreped from the selected positive colonies and run on an agarose gel alongside the empty pMEG-375 plasmid for insert verification. Plasmids that are larger than the empty pMEG-375 were chosen for further confirmation of successful cloning by restriction enzyme digestion (*Sac*I *and Xba*I) and were visualized on a 1% agarose gel. The recombinant plasmid p*EiΔfhuCD* was electroporated into donor *E. coli* SM10λ*pir* as previously described.

2.2.6. Transformation of recombinant plasmid into E. ictaluri and selection of EiΔfhuCD mutant

The recombinant plasmids were introduced into *E. ictaluri* WT by conjugation to allow homologous recombination and allelic exchange to occur between the cloned fragment with in-frame deleted *fhu* gene and flanking homologous regions to the *E. ictaluri* chromosome. The selection of the *E. ictaluri* Δ*fhuCD* mutant was conducted in two steps. In the first step, the colonies with entire plasmid insertion by a single crossover were selected on BHI agar plate with ampicillin and colistin. In the second step, the single crossover mutants were propagated on LB with 5% sucrose and 0.35% mannitol to allow the loss of the suicide vector. At each step, a colony PCR was conducted to check for the correct single and double crossover mutants using the (A and D primers) specific for each gene. The correct mutant with in-frame deleted gene was tested for ampicillin sensitivity to ensure the loss of the plasmid.

2.2.7. Sequence analysis of EiΔfhuCD mutant

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, California) and a gene specific sequencing primer (Table 3) in an Applied Biosystems 310 genetic Analyzer (Applied Biosystems).

3. RESULTS

3.1. Production of the upstream (AB), downstream fragment (CD) and in-frame overlap fragment (AD).

In the present study, the in-frame deletion was introduced in vitro to *fhuCD* operon successfully and cloned them into pMEG-375 suicide plasmid (Figure 2). The 1073 bp upstream and the1136 bp downstream homologous regions of the *E. ictaluri fhuCD* genes were amplified successfully (Figure 2A). A 2209 Overlap extension product containing the in-frame deletion region was produced by using flanking primer pairs (A-D) along with the PCR products of the first two primary amplifications (AB) and (CD) fragment regions as a template DNA. The agarose gel pictures (Figure 2B) confirmed the predicted size of the amplified overlapped fragment (AD).

3.2. Cloning of transformed E.coli CC118 λ pir plus insert

The in-frame deletion fragments (AD) was successfully ligated into linearized pMEG-375 yielding the following plasmids p*EiΔfhuCD*. The recombinant plasmid successfully introduced onto *E. coli* strain CC118λpir by electroporation for maintenance and plasmid amplification. Transformants were initially selected by LB plus ampicline plates. The growth of the colonies indicated the correct electroporation due to the plasmid pMEG-375 contains the ampicline resistance gene. The *E. coli* cell that had taken up the recombinant plasmid grown on LB plus ampicline plates while, non-transformed competent cells did not grow on this medium.

Further the recombinant plasmid was examined by miniprep and restriction digests. Figure (2C) showed that the recombinant plasmid p*EiΔfhuCD* was higher than the empty plasmids. Also as expected, cutting of the recombinant plasmid p*EiΔfhuCD* revealed the presence of two distinct bands that identified as the cut vector close to (~8 Kb) and the insert (~2 Kb) as shown on Figure (2D). This result confirmed the successfully ligation of the insert into plasmid and successfully transformed of the appropriate plasmid into *E. coli* CC118λpir.

3.3. Construction and selection of EiΔfhuCD mutant

The *E. ictaluri* mutant designated as *EiΔfhuCD* were obtained successfully by allelic exchange. The agarose gel picture of the colony PCR after the single crossover (Figure 2 E) confirmed that the presence of two bands the wild type band and the mutant band. The correct *EiΔfhuCD* mutant strain was genotypically confirmed by the colony PCR based on the size of the amplified PCR products. The agarose gel picture of the colony PCR after the double crossover mutant confirmed that the size of the product in the *EiΔfhuCD* mutant was smaller than the *E. ictaluri* wild-type by the amount that was deleted (Figure 2 E). The expected size fragment for the mutation with primers A-D was 2209 bp while the wild type band was 3391bp.

3. 4. Sequence analysis

The final confirmation of the genetic construct mutant as *EiΔfhuCD* was obtained by sequencing the PCR products. Results from sequencing of the *EiΔfhuCD* mutant strain PCR products revealed a precise deletion between primers B and C exactly as expected (Figure 3). We were able to delete 1635bp/545aa from a 1707-bp of the *fhuCD* operon. The result of sequence alignment between *EiΔfhuCD* and *E. ictaluri* wild type genomic DNA confirmed the deletion of each targeted gene (Figure 4).

4. DISCUSSION

The overall objective of this study was to create *EiΔfhuCD* mutant through in-frame deletion of *fhuCD* operon based on in-frame deletion method (Horton *et al.* 1990). The general mechanism of inframe-deletion mutants is illustrated in Figure (1). To achieve this aim, *fhuCD* operon was selected from *E. ictaluri* genome. To best to our knowledge mutant construction by in-frame deletion derived from *E. ictaluri* strain 93-146 is for the first time reported in the present study.

In this work, a pair of primer was designed to amplify approximately 1kb the upstream and the downstream regions of 5 and 3 end of *fhuCD* operon. It was found that a length of 1 kbp of the upstream and the downstream regions were high enough for homologous recombination event while 500 bp were not as efficient as 1 kbp for homologous recombination to occur (unpublished data). The optimum length of homology required for homologous recombination has not been determined but recombination can be achieved with fragments of 700 bp (Parish *et al.* 1999).

In the present study, the overlap extension PCR was used for fusion of the upstream and downstream homologous fragments. When fragment AB and fragment CD were mixed together at similar concentrations, the 3 end of fragment AB and the 5 end of fragment CD lower strand were hybridized due to the complementary extremities of the primers B and C (21bp reverse complement) without relying on restriction sites and produce 2209bp fragments. This result confirmed that the efficiency for successful SOE by PCR was high. It is agreement with the claim of 100% efficiency by (Horton et al. 1989).

Several studies have demonstrated the use of suicide plasmids for allelic exchange in construction of in-frame deletion mutants. In this work, it was found that pMEG-375 is suitable as suicide vectors to construct the *E. ictaluri* mutant. The pMEG-375 vector has certain advantages which made it easy to use for *E. ictaluri* mutant construction. The pMEG-375 has ampicline resistant genes inducing antibiotic resistance. Basically, *E. coli* and *E. ictaluri* are sensitive to ampicline since bacterial cells that uptake the plasmid will be able to grow on selective plates plus ampicline. Also, the suicide plasmids pMG-375 able to replicate on *E. coli* strains but incapable of replication in the *E. ictaluri*. These is because the replication of the pMG-375 vector requires the protein from an integrated pir gene as encoded in the *E. coli* strain *CC118λpir* and *SM10 λpir* but *E. ictaluri* do not encode the pir gene (Santander *et al.* 2007; Santander *et al.* 2010). Finally, the pMEG-375 contains *Bacillus subtilis sacB* gene encoding for levansucrase. The expression of the *sacB* gene encodes levanesaccharase is toxic for gram-negative bacteria when grown in the presence of sucrose providing a direct selection for loss of the plasmid (Gay *et al.* 1983; Donnenberg and Kaper 1991).

In the present study the sucrose selection by *sacB* gene is useful for construction mutant on *E. ictaluri* strain 93-146. Similarly to our results, *sacB* gene counter-selection method has been found to be used to construct mutants on *Mycobacterium tuberculosis, Helicobacter pylori, Bordetella pertussis* and many other bacteria (Steinmetz *et al.* 1983; Pelicic *et al.* 1996).

This and other studies conducted in our laboratory, we reported the successful transformation of the recombinant plasmids onto of *E. ictaluri* using conjugation while the attempts to transform the recombinant plasmid by electroporation little or no success and the subsequent homologous recombination event failed (data not shown). These results are correlated with (Lawrence *et al.* 2001; Maurer *et al.* 2001). This is might because the transformation efficiency of *E. ictaluri* is very low and also the big size of the recombinant plasmids (11kbp). Maurer *et al.* (2001) optimized a conjugation procedure for transfer of foreign DNA into *E. ictaluri* using a kanamycin resistant plasmid as a suicide vector. Lawrence *et al.* (2001) successfully used this conjugation procedure to transform wild-type *E. ictaluri* strain 93–146 by mixing donor *E.coli* SM10γpir with the recipient in the presence of MgSO4 to construct a lipopolysaccharide mutant strain of *E. ictaluri*. In the other hands Russo et at (Russo *et al.* 2009) reported the successful transformation of seven strains of *E. ictaluri* using electroporation and two different chemical procedures.

Homologous recombination events can be occurred into one-step or two-step methods. Using a one-step method, gene replacement is directly selected after introduction of the recombinant plasmids. In the two-step method, first single cross-over mutant strain allowed the whole recombinant plasmids integrated into the chromosome DNA and the second recombination event is then allowed to isolation of the double crossovers carried out (Muttucumaru and Tanya 2004). In this study, double selection in two steps has been successfully applied for construction of *EiΔfhuCD* mutant by using the two counter selectable markers (ampicline resistant gene and *Bacillus subtilis sacB* gene). Firstly, the entire plasmid is integrated into the *E. ictaluri* chromosome by a single-crossover between the homologous *fhuCD* regions producing a chromosomal duplication. Secondly, the chromosomal duplication is segregated by homologous recombination between the flanking direct repeats, ultimately leaving one copy of the gene on the chromosome either the wild-type copy or the mutant copy. The results of this work suggest that homologous recombination in *E. ictaluri* is as efficient enough for construction of in-frame deletion *EiΔfhuCD* mutant.

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Table (1) Bacterial strains and plasmid used in the present study

Strain	Relevant Characteristics	References
Edwardsiella ictaluri		
93146	Wild type; pEI1^{+}; pEI2^{+}; Col^{r}	(Lawrence *et al.* 1997)
EiΔfhuCD	93146 derivative; pEI1^{+}; pEI2^{+}; Col^{r}; Δ*fhuCD*	This study
Escherichia coli		
CC118λ*pir*	Δ(*ara-leu*); *araD*; Δ*lacX74*; *galE*; *galK*; *phoA20*; *thi-1*; *rpsE*; *rpoB*; *argE*(Am); *recA1*; 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	(Simon *et al.* 1982)
Plasmid		
pMEG-375	8142 bp, Amp^{r}, Cm^{r}, *lacZ*, R6K *ori*, *mob incP*, *sacR sacB*	(Dozois *et al.* 2003)
P*EiΔfhuCD*	10351 bp, Δ*fhuCD*, pMEG-375	This study

Table (2) Primers used to generate and verify in-frame deletions and sequence of the deleted regions.

Primers		Sequence 5'-3'[b]	RE[a]
*EifhuCD*F01	A	AA**GAGCTC**ACTTGGACATGCCCTGTAGAC	*Sac*I
*EifhuCD*F1626	B	TGTTTTCGCTAACCAACCTAGAGATGGCGGAAAGCCTGATG	
*EifhuCD*F894	C	<u>CTGTTCTCCCTGTTTTCACCCAT</u>GGCGGAAAGCCTGATG	
*EIfhuCD*R01	D	AA**TCTAGA**CTCTGGCTGGTAGGTCAGGTA	*Xba*I
*EifhuCD*F01S		GCCAAGTGGAAAAGGTGAATA	

[a]Restriction enzymes (RE) added to the 5' end of the primer sequence.
[b]RE are represented by bold letters in primer sequences. AA nucleotides were also added to the end of each primer containing a RE site to increase the efficiency of enzyme cuts. Underlined bases indicate reverse complemented B primers were added to primer C to provide an overlapped region.

Table (3) Properties of *E. ictaluri fhuC* and *fhuC* genes and their similarities to *E. coli* and *E. tarda.*

Genes	Locus tag	ORF (bp)	Location in *E. ictaluri* genome (bp)	% Similarity *E. coli*	% Similarity to *E. tarda*
fhuC	NT01EI_0713	774	705427..706200	44.7%	95.6%
fhuD	NT01EI_0714	933	706191..707123	27.9%	94.4%

Figure legends

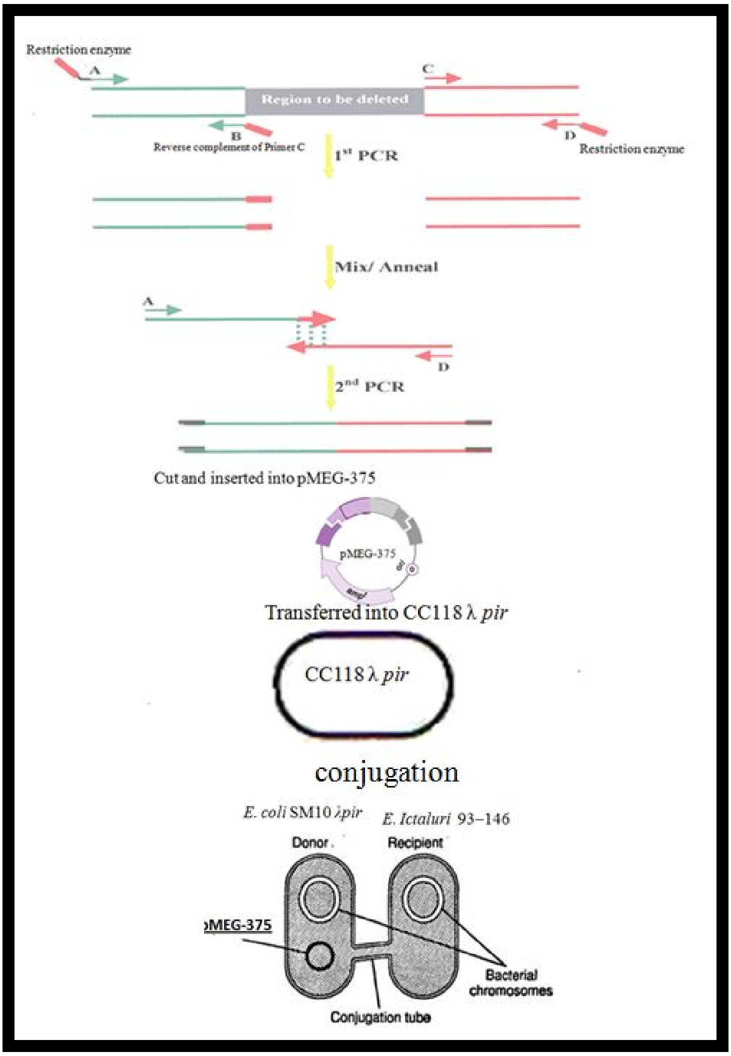

Figure (1) Schematic diagram explains the construction of *fhuCD* in-frame deletion mutant of *E. ictaluri*. The fragments AB (upstream of the *fhuCD* operon) and the fragment CD (downstream of the *fhuCD* operon) were amplified in two separate PCR step. Then, the two fragments were mixed and were used as a template in overlap extension PCR reactions to generate the overlapped product (AD fragment) by the outside flanking primers (A and D). Further, the overlap product (AD fragment) cloned into pMEG-375 plasmid and transferred into *E. ictaluri* by conjugation to allow homologous recombination event.

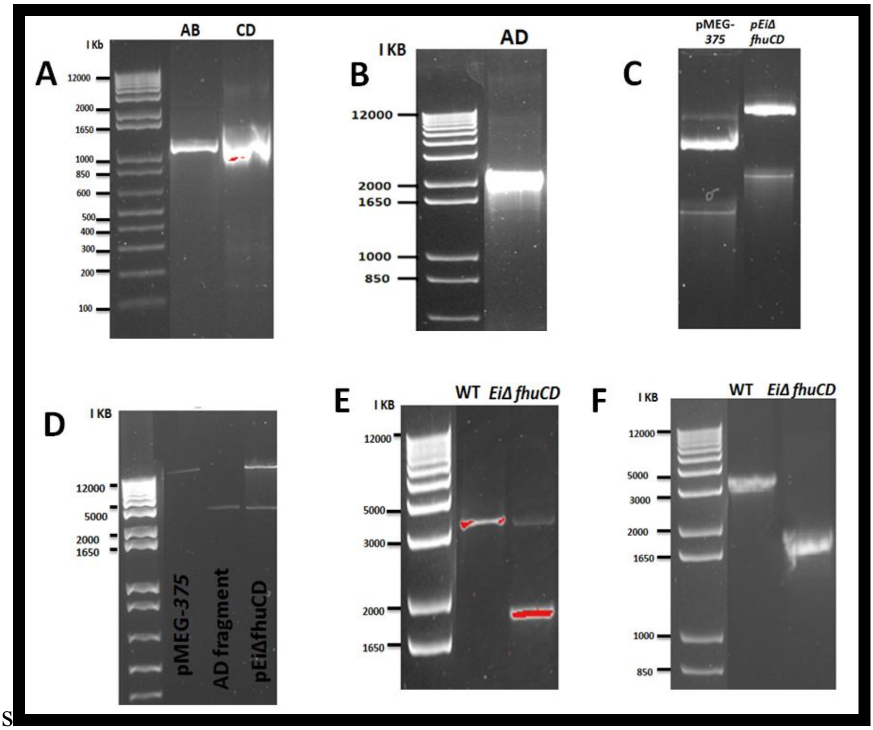

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Figure (2) Construction of in vitro and in vivo in-frame gene deletions. (A) the upstream (AB) and do)wnstream (CD) fragments of *fhuCD* operon amplified from the *E. ictaluri* WT genomic DNA. (B) the in-frame deleted fusion fragment (AD). (C) Screening of the suicide plasmid with inserts. Lane one, empty pMEG-375; lane two is the recombinant positive plasmid pEiΔfhuCD. (D) Insert verification by RE digestion. Lane one, 1 Kb ladder; lane two, linear pMEG-375; lane three, purified insert; lane four, the positive recombinant plasmid. (E) The colony PCR products amplified from the single cross-over mutants and the wild-type parent strain. (F) The colony PCR products amplified from the *E. ictaluri* Δ*fhu* mutants.

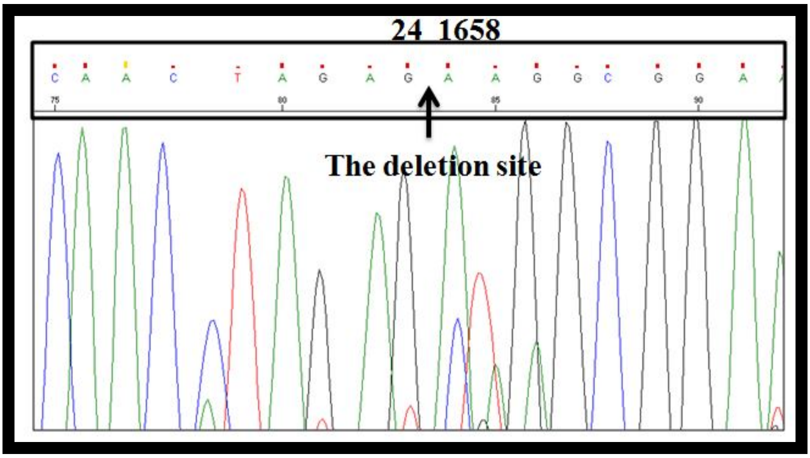

Figure (3) Chromatogram obtained from the *E. ictaluri* Δ*fhuCD* mutant, indicating the in-frame deletions site.

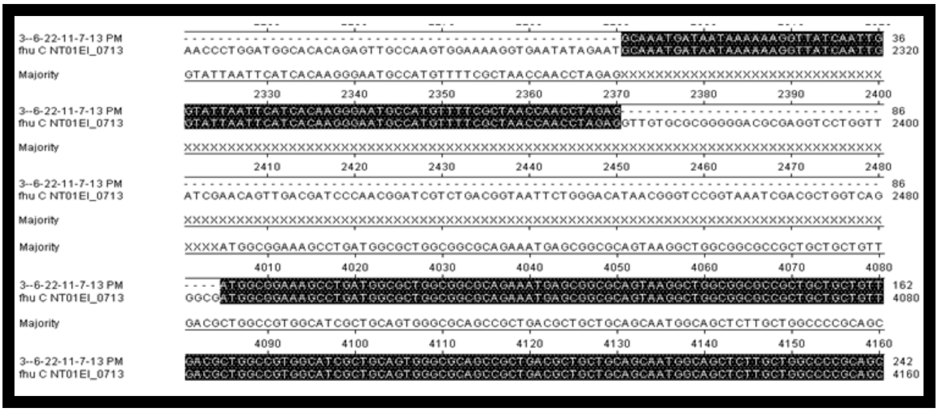

Figure (4) Nucleotide sequence alignment of mutant constructed of *E. ictaluri* Δ*fhuCD* mutant and *E. ictaluri* wild type. The top line is a nucleotide region of on the chromosome *E. ictaluri* wild type. The bottom line is nucleotide region of Δ*fhuCD* mutants. Nucleotides that are similar between two lines are indicated by shaded box. While the (xxxx) indicates missing nucleotides.

انشاء طفرة الادوارد سيللا اكتالورى بحذف *fhuCD*

حسام عبد الحميد[1] ، عادل شاهين[1] ، امانى عباس[1]
Jingjun Liu[2] , Mark L. Lawrence[2] , Attila Kars[2] , Neeti Dahali[2]
[1] قسم امراض ورعاية الاسماك - كلية الطب البيطرى - جامعة بنها - مشتهر - طوخ - ج م ع
[2] قسم العلوم الاساسية - كلية الطب البيطرى - جامعة ولاية الميسيسبى - ولاية الميسيسبى - الولايات المتحدة الامريكية

هناك عدد من الاستراتيجيات المختلفة لتحقيق الطفرات الموجهة الموقع. إحدى هذه الطرق هي تقنية الحذف في الإطار التي تخلق الطفرات عالية الكفاءة في مواقع محددة بواسطة حذف عدد من النيوكليوتيدات من كروموسوم البكتيريا. يتكون نظام (fhuCD) من اثنين من الجينات (fhuC وfhuD) التي تشارك في امتصاص الحديد من (hydroxamate siderophores) عبر الأغشية البكتيرية. في هذه الدراسة تم انشاء طفرة (EiΔfhuCD). وقد تحقق انشاء هذة الطفرة بواسطة حذف جزء في الإطار من نظام (fhuCD) من خلال (Overlapping extension PCR) وقد تم قطع جزء في الإطار بواسطة انزيم الهضم (SACI وXbaI) و قد تم ارتبطها إلى البلازميد المتحلل(pMEG-375). وقد تم نقل البلازميد المعاد تكوينة (pEiΔfhuCD) الي عترة الادوارد سيللا اكتالورى المعزولة من الاسماك البرية عن طريق الدمج وقد تم تداخلها في الحمض النووي الجيني من خلال خطوتين من إعادة التركيب المتماثل. تم استخدام تفاعل البلمرة الجزية وتسلسل الحمض النووي (DNA) لتأكيد الحذف الجيني المطابق. الى جانب ذلك تم حذف (1635من النيوكليوتيدات- 545احماض بروتنية) من (1707من النيوكليوتيدات) من نظام (fhuCD)