Transposon mutagenesis and identification of mutated genes in growth-delayed Edwardsiella ictaluri

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Abstract

Background: Edwardsiella ictaluri is a Gram-negative facultative intracellular anaerobe and the etiologic agent of enteric septicemia of channel catfish (ESC). To the catfish industry, ESC is a devastating disease due to production losses and treatment costs. Identification of virulence mechanisms of E. ictaluri is critical to developing novel therapeutic approaches for the disease. Here, we report construction of a transposon insertion library and identification of mutated genes in growth-delayed E. ictaluri colonies. We also provide safety and efficacy of transposon insertion mutants in catfish.

Results: An E. ictaluri transposon insertion library with 45,000 transposants and saturating 30.92% of the TA locations present in the E. ictaluri genome was constructed. Transposon end mapping of 250 growth-delayed E. ictaluri colonies and bioinformatic analysis of sequences revealed 56 unique E. ictaluri genes interrupted by the MAR2xT7 transposon, which are involved in metabolic and cellular processes and mostly localized in the cytoplasm or cytoplasmic membrane. Of the 56 genes, 30 were associated with bacterial virulence. Safety and vaccine efficacy testing of 19 mutants showed that mutants containing transposon insertions in hypothetical protein (Ei::004), and Fe-S cluster assembly protein (IscX, Ei::039), sulfurtransferase (TusA, Ei::158), and universal stress protein A (UspA, Ei::194) were safe and provided significant protection ($p < 0.05$) against wild-type E. ictaluri.

Conclusions: The results indicate that random transposon mutagenesis causing growth-delayed phenotype results in identification bacterial virulence genes, and attenuated strains with transposon interrupted virulence genes could be used as vaccine to activate fish immune system.

Keywords: pMAR2xT7, Enteric septicemia, Catfish, Virulence, Type III secretion system

Background

Enteric septicemia of catfish (ESC) is a devastating disease that causes significant production loss and treatment cost for the catfish aquaculture industry [1]. A few antimicrobials and a commercial live attenuated vaccine are available for treatment of ESC. However, treatment of sick catfish by medicated feed is not effective due to early onset of anorexia. The extensive use of antimicrobials can induce the appearance of resistant strains [2, 3]. The commercial ESC vaccine Aquavac-ESC has been available for the catfish industry for more than 15 years [4], but ESC is still one of the major diseases in the US catfish industry.

Edwardsiella ictaluri is well-adapted to channel catfish [5, 6] and some of the E. ictaluri virulence factors include lipopolysaccharide (LPS), flagella, outer membrane proteins (OMPs), and extracellular proteins [7–10]. There have been several reports on development of attenuated E. ictaluri strains by deleting genes involved in iron acquisition, tricarboxylic acid cycle, one-carbon metabolism, and amino acid biosynthesis [11–17]. However, virulence mechanisms of E. ictaluri are not understood well, and there is a need for identification of novel virulence-related genes to develop effective live attenuated vaccines.

Random transposon insertion is a high-throughput genetic manipulation tool that allows random mutation of genes at the genome level. Mariner family transposon Himar1 inserts itself randomly into “TA” nucleotide sequences [18, 19]. Mariner family transposons have

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been widely used to generate random mutagenesis in fish pathogen *Mycobacterium marinum*, and also human pathogens such as *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Leptospira interrogans*, and *Rickettsia prowazekii* [20–24].

In this research, *MAR2xT7* transposon, a Himar1 derivative [20], was used to identify genes required for *E. ictaluri* growth on a solid complex medium. We expect that colonies exhibiting attenuated growth on solid media will have transposon insertions in important bacterial genes, and these mutants may also show attenuated virulence in the catfish host and potentiate catfish immune responses [14]. Therefore, attenuation and vaccine efficacy of 19 transposon mutants were evaluated in channel catfish.

**Results**

**Transposon insertion library**

By using *MAR2xT7* transposon, an *E. ictaluri* transposon insertion library containing 45,000 transposants was constructed. Colonies with transposon insertion and delayed growth were observed on the BHI agar media after 48 h (Fig. 1). The initial overnight growth of these small colonies in BHI broth was also very slow compared to wild type, but this difference disappeared in later broth cultures (data not shown).

The complete genome size of *E. ictaluri* strain 93–146 is 3,812,301 bp, which contains 3597 total genes. The number of TA locations in the entire *E. ictaluri* genome is 145,515. Thus, 45,000 transposants would saturate 30.92% of the potential *MAR2xT7* transposon insertion sites available. The number of TA locations in *E. ictaluri* open reading frames is 110,373, which represents 75.85% of all available *MAR2xT7* transposon insertion sites. Thus, random insertion events would saturate 23.45% of the potential *MAR2xT7* transposon insertion sites in the *E. ictaluri* open reading frames.

**Gene identification**

Transposon end amplification by single primer PCR yielded 151 samples with PCR products, of which 94 were sequenced successfully. After analysis, 56 unique genes containing transposon insertions were identified (Table 1). These unique genes contained a total number of 2235 *MAR2xT7* transposon insertion sites, and the exact number of *MAR2xT7* transposon insertion site in each gene was indicated in Table 1.

**Functional annotation**

Protein sequences of all 56 genes were annotated functionally and assigned to biological process (localization, cellular process, metabolic process, response to stimulus, biological regulation, signaling, multi-organism process, single-organism process, and biogenesis), cellular component (cell, macromolecular complex, and extracellular region), and molecular function (binding, transporter activity, catalytic activity, and nucleic acid binding transcription factor) (Fig. 2).

**Subcellular localization**

The locations of 15 proteins were unknown. Of the 41 proteins with known subcellular location, most were localized to the cytoplasm (20 proteins) and cytoplasmic membrane (16 proteins). Extracellular space, outer membrane, and periplasm contained very few proteins (3, 1, 1 proteins, respectively).

**Proteins involved in host-pathogen interactions**

Out of 56 identified unique proteins, 30 proteins had significant homology to Host-Pathogen Interaction Database (HPIDB) (Table 2). The proteins mostly matched to the *Enterobacteriaceae* (Yersinia pestis, *Escherichia coli K12*, *Shigella flexneri*), *Franciscellaceae* (*Francisella tularensis SCHU S4*), and *Bacillaceae* families (*Bacillus anthracis*).

**Proteins involved in bacterial virulence**

Out of 56 unique proteins, 30 matched significantly to known virulence-associated proteins from other Gram-negative and Gram-positive pathogenic bacteria in MVirDB (Table 3).

**Safety and vaccine efficacy of mutants in catfish**

Safety testing of transposon-derived mutants showed that all mutant strains were attenuated significantly compared to wild-type control ($p < 0.05$). *Eis::004*, *Eis::039*, *Eis::041*, *Eis::176*, and *Eis::194* caused less than 5% mortality, while...
| Mutant | Locus         | Product                                                                 | E-value | Frequency | TA Frequency |
|--------|---------------|--------------------------------------------------------------------------|---------|-----------|--------------|
| Eis001 | NT01EI_1281   | NAD-dependent malic enzyme (NAD-ME)                                       | 2.00E-72| 64        |              |
| Eis002 | NT01EI_1721   | PTS system, mannose/fructose/sorbose family, IIIB component              | 2.00E-106| 32        |              |
| Eis004 | NT01EI_0182   | Hypothetical protein                                                      | 7.00E-57| 26        |              |
| Eis006 | NT01EI_0085   | ATP-dependent DNA helicase Rep                                            | 2.00E-114| 46        |              |
| Eis009 | NT01EI_1236   | Coproporphyrinogen III oxidase, aerobic                                   | 6.00E-32| 24        |              |
| Eis011 | NT01EI_3690   | ABC transporter, periplasmic amino acid binding protein                    | 1.00E-74| 50        |              |
| Eis013 | NT01EI_2795   | Translocator protein, LysE family                                         | 2.00E-115| 36        |              |
| Eis018 | NT01EI_0377   | Aspartate ammonia-lyase                                                  | 7.00E-145| 59        |              |
| Eis024 | NT01EI_3505   | Dihydrouridine synthase Dus                                               | 1.00E-129| 59        |              |
| Eis027 | NT01EI_0408   | tRNA delta(2)-isopentenylpyrophosphate transferase                       | 5.00E-94| 22        |              |
| Eis028 | NT01EI_0277   | Transposase, IS4 family protein                                           | 1.00E-73| 17        |              |
| Eis029 | NT01EI_2683   | Membrane protein                                                          | 2.00E-88| 27        |              |
| Eis033 | pEl2_p2       | Putative Rep protein                                                      | 2.00E-123| 31        |              |
| Eis035 | pEl1_p4       | Putative RNA one modulator protein                                        | 9.00E-19| 12        |              |
| Eis037 | NT01EI_2355   | eseJ                                                                     | 3.00E-129| 88        |              |
| Eis038 | NT01EI_1334   | eseM                                                                     | 5.00E-67| 91        |              |
| Eis039 | NT01EI_3177   | FeS assembly protein tsX                                                  | 3.00E-38| 7         |              |
| Eis041 | NT01EI_0943   | eseC                                                                     | 8.00E-116| 53        |              |
| Eis048 | NT01EI_3148   | Hypothetical protein                                                      | 4.00E-80| 20        |              |
| Eis055 | NT01EI_2314   | Prophage lambda intasge                                                   | 8.00E-128| 56        |              |
| Eis059 | NT01EI_1941   | Hypothetical protein                                                      | 3.00E-70| 8         |              |
| Eis065 | NT01EI_2281   | Excinuclease ABC subunit C                                                | 3.00E-48| 64        |              |
| Eis068 | NT01EI_0448   | Polyprenyl synthetase                                                     | 1.00E-115| 37        |              |
| Eis080 | NT01EI_0981   | Hypothetical protein                                                      | 8.00E-129| 58        |              |
| Eis086 | NT01EI_1237   | N-acetylmuramoyl-L-alanine amidase AmiA                                    | 3.00E-67| 25        |              |
| Eis107 | NT01EI_0475   | DEAD box containing helicase                                              | 1.00E-112| 51        |              |
| Eis110 | NT01EI_1332   | eseL                                                                     | 4.00E-26| 73        |              |
| Eis131 | NT01EI_2157   | Hypothetical protein                                                      | 4.00E-66| 37        |              |
| Eis152 | NT01EI_0224   | Transporter, major facilitator family                                     | 1.00E-16| 47        |              |
| Eis154 | NT01EI_3522   | Selenate reductase, FAD-binding subunit                                   | 2.00E-73| 23        |              |
| Eis155 | NT01EI_0725   | Transcriptional regulator FruR                                           | 3.00E-134| 43        |              |
| Eis156 | NT01EI_2381   | Ribonuclease, RNaseE/RNaseG family                                       | 2.00E-04| 72        |              |
| Eis157 | NT01EI_0144   | Twin-arginine translocation protein subunit TatB                           | 8.00E-07| 13        |              |
| Eis158 | NT01EI_0022   | Sulfurtransferase, TusA                                                   | 8.00E-46| 10        |              |
| Eis171 | NT01EI_3723   | Magnesium-translocating P-type ATPase                                     | 0       | 71        |              |
| Eis172 | NT01EI_3786   | Hypothetical protein                                                      | 1.00E-26| 24        |              |
| Eis173 | NT01EI_5265   | Acyltransferase/AMP-dependent synthetase and ligase family                | 0       | 54        |              |
| Eis174 | NT01EI_3721   | Hypothetical protein                                                      | 3.00E-25| 7         |              |
| Eis175 | NT01EI_3774   | IS1 transposase                                                           | 7.00E-93| 19        |              |
| Eis176 | NT01EI_0962   | esaT                                                                     | 5.00E-129| 27        |              |
| Eis180 | NT01EI_3103   | UPF0126 domain protein                                                    | 7.00E-12| 16        |              |
| Eis183 | NT01EI_3105   | Chloride transporter, chloride channel (Cic) family                       | 6.00E-166| 53        |              |
| Eis184 | NT01EI_0419   | RNA methyltransferase, TrmH family, group 3                               | 9.00E-27| 21        |              |
| Eis185 | NT01EI_3386   | TRAP transporter, DctM subunit                                            | 7.00E-85| 47        |              |
The functional gene ontology analysis with Blast2GO predicted interaction with the channel catfish 

| Mutant | Locus   | Product                                      | E-value | Frequency | TA Frequency |
|--------|---------|----------------------------------------------|---------|-----------|--------------|
| Eis192 | NT01EI_3147 | Hypothetical protein                         | 0       | 2         | 103          |
| Eis194 | NT01EI_1981 | Universal stress protein A uspA              | 9.00E-86| 3         | 20           |
| Eis195 | NT01EI_0376 | Anaerobic C4-dicarboxylate transporter DcuA   | 2.00E-159| 11        | 47           |
| Eis207 | NT01EI_1817 | Spermidine/putrescine transport system permease protein PotB | 4.00E-132| 22        |               |
| Eis210 | NT01EI_0800 | Prolipoprotein diacylglycerol transferase     | 5.00E-79 | 36        |               |
| Eis220 | NT01EI_2076 | Hypothetical protein                         | 6.00E-20 | 5         |               |
| Eis222 | NT01EI_0768 | Hypoxanthine phosphoribosyltransferase       | 5.00E-127| 24        |               |
| Eis223 | NT01EI_1086 | Extracellular solute-binding protein, family 5 | 2.00E-142| 48        |               |
| Eis230 | NT01EI_3769 | Phosphoglycerate transporter family protein   | 0       | 49        |               |
| Eis232 | NT01EI_0201 | Hypothetical protein                         | 2.00E-136| 3         | 58           |
| Eis233 | NT01EI_2530 | Putative permease, membrane region            | 4.00E-81 | 65        |               |
| Eis235 | NT01EI_3289 | Diaminopimelate decarboxylase                | 9.00E-36 | 38        |               |

aBlastx E-value
bmpAR2xT7 insertion frequency

to any stimulus sensing bacteria in the biological process. Although several proteins showed transporter activity, these proteins account for localization and are in the cytoplasm.

The subcellular locations predicted by PSORTb revealed that most of the identified proteins are found in the cytoplasm and cytoplasmic membrane. Although many well-known virulence proteins are located in the outer membrane or periplasm in Gram-negative bacteria, only three proteins of T3SS are located in extracellular space and outer membrane, and one of the ABC transporter proteins was found in the periplasmic space.

The host-pathogen interaction examined by HPIDB proved that many proteins have a high similarity to other virulence-associated proteins in different pathogenic bacteria including Y. pestis, S. flexneri, and B. anthracis. The pathogenic Gram-negative bacteria Y. pestis and S. flexneri share the same evolutionary lineage with Edwardsiella sp. in Enterobacteriaceae [28]. Thus, most of the virulence-associated proteins may have a similar role in E. ictaluri. Two T3SS effector proteins EseJ and EseM have a predicted interaction with the channel catfish ubiquitin-conjugating enzyme E2 (XP_017323313). These two T3SS-related effector proteins known as E3 ubiquitin ligase play an important role in manipulation of host ubiquitination pathways.

The interrupted genes eseJ (Eis::037), eseM (Eis::038), esaC (Eis::041), eseL (Eis::110), and esaT (Eis::176) are part of T3SS, which are involved in export of proteins inside the host immune cells [29]. EsaC and EsaT are the structural membrane associated proteins of T3SS. Eis::41, YscC ring-shaped structure protein in the outer membrane, is required for a stable oligomeric complex to shape a T3SS in the outer membrane [30]. YscT inner membrane-embedded component is located in the cytoplasm, which has extended
and helical regions that may form membrane-bound subunits. Insertions in the T3SS related genes, eseJ, eseM, and eseL, have been recently identified to be T3SS dependent effector proteins [31]. EseJ, EseM, and EseL proteins share high similarities with Salmonella T3SS effector proteins SspH2 and SlrP. They are involved in ubiquitination of proteins, an important process regulating inflammatory response in eukaryotes. As a part of novel E3 ligases (NELs) protein family, EseJ, E3 ubiquitin-protein ligase (SspH2), and EseL, a new class of E3 ubiquitin ligase, have a role in T3SS that provides a strategy to exploit host cell ubiquitin pathway [32]. EseM, T3SS leucine rich repeat protein (SlrP), is also required to form a complex ubiquitin ligase enzyme [33]. T3SS effector protein mutants eseJ, eseM, and eseL, and T3SS structural mutants esaC and esaT showed significantly decreased virulence. However, in comparison of protection level of those two main groups, T3SS structural proteins EsaC and EsaT have been caused less protection in catfish. Mutation in T3SS effector proteins provides better protection against pathogenic bacteria [34–36]. EseL has provided significant protection among other T3SS related effector proteins. T3SS effector proteins could contribute the bacterial survival inside host immune cells [37, 38].

Transport processes in bacterial cells through outer membrane and periplasmic space are linked to E. ictaluri metabolism to survive in the host environment as well as switching between various biochemical processes during different stages of ESC. Eis::157, tatB, is located in the periplasmic space and is involved in the translocation of proteins including the components of respiratory complexes using a proton gradient as an energy source [39]. tatB mutant exhibited slow growth under low-iron conditions and observed a 10-fold decrease in Legionella pneumophila growth [40]. Eis::086, amiA, is a Tat pathway dependent substrate encoding a cell wall amidase. Tat pathway mutant causes mislocalization of AmiA protein, preventing translocation in the periplasm [41, 42]. Eis::011, ABC transporter periplasmic amino acid binding protein, is an important antigenic factor involved in adhesion and aspartate/glutamate transport in the microaerobic environment in Campylobacter jejuni [43]. Eis::207, potB, encodes a protein associated with spermidine/putrescine transport system. Polyamines are mostly involved in stabilization of DNA for stress resistance, intracellular signaling processes, and swarming motility [44, 45]. Polyamines are also associated with the virulence in the intracellular pathogen Salmonella enterica [46]. Eis::002, PTS system IIB component, is a cytoplasmic component of the major carbohydrate transport system highly conserved through bacteria [47]. PTS system participates in a variety of virulence mechanisms including biofilm formation, modulating the virulence gene expression, and regulating carbohydrate metabolism in pathogenic bacteria [48–50]. Eis::171, magnesium-translocating P-type ATPase, is an inducible magnesium transport system when bacteria grow at the low concentration of magnesium. Although Mg$^{2+}$ is not essential for virulence, it participates in many cellular activities as a cofactor [51]. Magnesium is the part of the regulatory network that regulates the virulence-associated mechanisms in S. enterica [52]. Eis::195, dcuA, is encoded with aspartase in the same operon that is determined as an antiporter mechanism involved in the transport of aspartate under the anaerobic conditions [53]. DcuA function in the metabolic pathway under anaerobic
conditions contributes the pathogenicity for the colonization in the lower oxygen level [54].

Pathogenic bacteria adapted different carbohydrate metabolism, which is activated by oxygen presence in the host environment. *Eis*:018, aspartate ammonia-lyase, is involved in the production of fumarate activated specifically under anaerobic conditions while there is no available electron acceptor. Bacteria encodes aspartate ammonia-lyase to utilize alternative carbon sources in the host environment if there are no available carbon sources [55, 56].

Bacterial stress related proteins induce the protective mechanisms under a variety of stress conditions to protect the bacterial cell inside or outside of the host [57]. Universal stress protein A (*upsA*) in *Eis*:194, is one of the stress proteins found in intracellular pathogenic bacteria. *upsA* expression reaches a high level when bacteria are exposed to heat, starvation, antimicrobial, and oxidative agents [58, 59]. *UpsA* is a conserved protein that presents in Eubacteria, Archaea, plants, and fungi and the expression of *UpsA* is triggered by exposure to oxidative agents in growth arrested cells [60–62]. *UpsA* plays a significant role in the pathogenicity of bacteria, and *upsA* mutants are less virulent and sensitive to changes in the host environment. Mutation of *S. typhimurium C5 upsA* resulted in less virulence and more susceptibility to nutrient starvation oxidative agents [59]. In *Listeria monocytogenes*, *upsA* mutants were shown to have impaired activity in oxidative agent's exposure to low pH conditions [58]. Deletion of *upsA* gene in *Acinetobacter baumannii* revealed that it has a significant role in protecting the bacteria from H₂O₂ and low pH [63].

### Table 2 Genes involved in host-pathogen interactions

| Mutant | Accession Nu. | Protein | Organism | E-value |
|--------|---------------|---------|----------|---------|
| Eis152 | YP_019321.1   | Oxalateformate antiporter, putative | *Bacillus anthracis* | 2.00E-20 |
| Eis155 | YP_017710.1   | Sugar-binding transcriptional regulator, LacI family | *Bacillus anthracis* | 2.00E-21 |
| Eis207 | YP_002347936.1 | Inner membrane permease T of sulfate/thiosulfate ABC transporter | *Yersinia pestis* | 1.00E-12 |
| Eis011 | YP_017492.1   | Amino acid ABC transporter, amino acid-binding protein | *Bacillus anthracis* | 1.00E-15 |
| Eis013 | NP_670988.1   | Putative threonine efflux protein | *Yersinia pestis* | 5.00E-11 |
| Eis171 | YP_002345523.1 | Putative cation transport protein | *Yersinia pestis* | 4.00E-88 |
| Eis223 | YP_00234598.1 | HTH-type transcriptional regulator SgrR | *Yersinia pestis* | 2.00E-46 |
| Eis176 | NP_857736.1   | Yop proteins translocation protein T | *Yersinia pestis* | 9.00E-31 |
| Eis107 | YP_022388.1   | ATP-dependent RNA helicase, DEAD/DEAH box family | *Bacillus anthracis* | 1.00E-84 |
| Eis024 | NP_842644.2   | tRNA-dihydouridine synthase | *Bacillus anthracis* | 6.00E-62 |
| Eis110 | NP_858359.2   | E3 ubiquitin-protein ligase ipaH9.8 | *Shigella flexneri* | 6.00E-90 |
| Eis086 | NP_667964.1   | N-acetylmuramoyl-L-alanine amidase II | *Yersinia pestis* | 1.00E-39 |
| Eis184 | YP_016695.1   | RNA methyltransferase, TrmH family, group 3 | *Bacillus anthracis* | 6.00E-43 |
| Eis180 | YP_002345138.1 | Putative membrane protein | *Yersinia pestis* | 4.00E-20 |
| Eis006 | YP_170066.1   | ATP-dependent DNA helicase | *Francisella tularensis* | 1.00E-171 |
| Eis009 | YP_170044.1   | Coproporphyrinogen-III oxidase, aerobic | *Francisella tularensis* | 9.00E-52 |
| Eis027 | YP_169650.1   | tRNA dimethylallyltransferase | *Francisella tularensis* | 8.00E-76 |
| Eis041 | YP_002345337.1 | Possible type III secretion protein | *Yersinia pestis* | 4.00E-154 |
| Eis173 | NP_994169.1   | Bifunctional protein aas | *Yersinia pestis* | 0 |
| Eis235 | YP_00234585.1 | Diaminopimelate decarboxylase | *Yersinia pestis* | 7.00E-177 |
| Eis156 | NP_669066.1   | RNase E | *Yersinia pestis* | 0 |
| Eis183 | NP_668136.1   | H(+)/Cl(−) exchange transporter ClcA | *Yersinia pestis* | 0 |
| Eis065 | NP_669748.1   | UvrABC system protein C | *Yersinia pestis* | 0 |
| Eis002 | YP_002346757.1 | PTS enzyme IIAB, mannose-specific | *Yersinia pestis* | 3.00E-149 |
| Eis222 | YP_646612.1   | Hypoxanthine phosphoribosyltransferase | *Yersinia pestis* | 6.00E-84 |
| Eis068 | YP_491372.1   | Octaprenyl-diphosphate synthase | *Escherichia coli* | 2.00E-157 |
| Eis001 | YP_002346527.1 | NAD-dependent malic enzyme | *Yersinia pestis* | 0 |
| Eis230 | YP_001608410.1 | Putative regulatory protein | *Yersinia pestis* | 0 |
| Eis018 | NP_667943.1   | Aspartate ammonia-lyase (Aspartase) | *Yersinia pestis* | 0 |
| Eis233 | YP_002346351.1 | Putative transport protein YPO1326/y2857/YP_1266 | *Yersinia pestis* | 0 |
IscX in Eis::S039, acts as a regulator for the Fe–S (iron-sulfur) cluster, which encodes proteins essential for cell activities [64]. FeS assembly protein IscX (YfhJ) is a part of the iron-sulfur cluster (ISC) mediated FeS cluster, which is a small acidic protein that binds IscC and Fe, and acts as a Fe donor in FeS cluster [65, 66]. ISC mediated FeS biogenesis is involved in survival of bacteria that face with iron starvation and oxidative stress. In S. flexneri, ISC mutants were less invasive and cannot form plaques on Henle cells monolayers [67]. ISC transcriptional regulator iscR mutant in Pseudomonas aeruginosa caused more susceptibility to oxidative agents and a significant decrease in virulence [68]. The importance of ISC system in bacterial virulence has been emphasized in different studies. However, limited information is known about the role of IscX in bacterial virulence.

Hypothetical protein in Eis::004 is located in the cytoplasm. There is no available information about the function of this hypothetical protein in any virulence related mechanisms. However, decreased virulence and significant protection against ESC revealed that Eis::004 mutant could be considered as a vaccine candidate for live attenuated vaccine development.

**Conclusions**

In summary, these results showed that random transposon mutagenesis in the E. ictaluri genome resulted in colonies with delayed growth on complex solid media, and many of the disrupted genes have important functions.
and potentially contribute to *E. ictaluri* virulence. Fish experiments showed that *Eis*:004, *Eis*:039, *Eis*:110, *Eis*:158, *Eis*:176, *Eis*:194, and *Eis*:195 mutants were significantly attenuated, and *Eis*:004 and *Eis*:194 provided good immunization in catfish.

**Methods**

**Bacterial strains, plasmids, and growth conditions**

Bacterial strains and plasmids used in this work are listed in Table 4. *Edwardsiella ictaluri* 93–146 carrying pAKgfplux1 [69] was grown at 30 °C using brain heart infusion (BHI) broth and agar plates (Difco, Sparks, MD). *Escherichia coli* SM10λpir donor strain carrying pMAR2xT7 [20] was grown at 37 °C using Luria-Bertani (LB) broth and agar plates (Difco). Antibiotics were added to the culture medium at the following concentrations: ampicillin (100 μg/ml), colistin (12.5 μg/ml), and gentamicin (12.5 μg/ml).

**Construction of transposon insertion library**

Transposon insertion library was constructed by conjugation using the donor *E. coli* SM10λpir carrying pMAR2xT7 and the recipient *E. ictaluri* 93–146 wild type (WT) containing pAKgfplux1 [69]. Transposon insertion mutants were selected on selective BHI agar plates containing 100 μg/ml of

**Table 4** Bacterial strains and plasmids

| Strain or plasmid | Description | Source |
|-------------------|-------------|--------|
| *Escherichia coli* | SM10λpir: km<sup>+</sup>; thi; thr; leu; tonA; lacY; supE; recA; :RP4–2-Tc::Mu; λpir R6K | [75] |
| *Edwardsiella ictaluri* | 93–146: wild-type; pEI1; pEI2; Col<sup>R</sup> | [11] |
| Plasmids | pMAR2xT7: R6K replicon; Himar I; T7 promoters; Amp<sup>R</sup>; Gen<sup>R</sup> | [20] |
ampicillin, 12.5 µg/ml of gentamicin, and 25 µg/ml colistin. Various sizes of gentamicin resistant transposon insertion colonies were observed on the selective BHI plates, and 250 smallest colonies compared to normal colony size were cultured in the BHI broth with colistin and gentamicin at 30 °C for 2 days. Finally, bacterial stocks were prepared in 20% glycerol and stored at −80 °C freezer.

Transposon end mapping
Genomic DNA was isolated from the frozen E. ictaluri transposon insertion mutants using the heat denaturation method. Briefly, 100 µl frozen culture were added in 1 ml ddH₂O and mixed well. Bacteria were collected by centrifugation and water was removed completely. After dissolving the bacterial pellet in 100 µl ddH₂O, each sample was transferred to 200 µl PCR tubes and tubes were incubated at 100 °C for 10 min by using an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Grand Island, NY). Samples were mixed well by vortexing, and bacterial cell debris was pelleted by centrifuging at 14,000 rpm for 5 min. The supernatant containing the genomic DNA was used as template in subsequent PCR reactions. Single primer PCR was performed by using a transposon-specific R1 primer (5′-CCGTATGCCCAAAT TTGATATAG-3′) to amplify the transposon end and flanking bacterial DNA [70]. Before sequencing, the PCR products were cleaned by using ExoSAP-IT for PCR Product Cleanup (Affymetrix, Santa Clara, CA). Sequencing was conducted at Eurofins MWG Operon LLC (Huntsville, AL) using a transposon-specific nested R3 primer (5′- TCTC GGCTTTGAACGAATTGTT-3′).

Bioinformatics analyses
Transposon sequence removal and sequence trimming based on sequence quality scores were done by using the Sequencher DNA sequence analysis software v4.10.1 (Gene Codes Corp., Ann Arbor, MI). Trimmed sequences were searched against the available E. ictaluri 93–146 genome [29] by using basic local alignment search tool (Blast) at the National Center for Biotechnology Information (NCBI) for gene identification. Using the GI numbers, a FASTA file containing all protein sequences were downloaded from the Batch Entrez database of NCBI and used for downstream analysis. Gene Ontology (GO) annotation, visualization, and metabolic and cellular processes were determined by using Blast2GO [71] at the cut-off level 2. Subcellular localization of proteins was predicted by using PSORTb version 3.0.2 [72]. E. ictaluri proteins involving in host-pathogen interactions were identified by using the Host-Pathogen Interaction Database (HPIDB) at the cut-off level 0.0001. Bacterial proteins interacting with channel catfish proteins were determined at the cut-off level 0.00001, at identity filter 50% in bacterial proteins, and 70% in channel catfish proteins. [73]. The potential E. ictaluri virulence proteins were identified using the Microbial Virulence Database (MVirDB) at the cut-off level 0.5 [74]. TA sequence frequencies in the entire E. ictaluri genome, open reading frames, and genes with transposon insertion were calculated using CLC genomics workbench 11.0.1 (Qiagen, Redwood City, CA).

Safety and vaccine efficacy testing of mutants in catfish
Specific pathogen free (SPF) channel catfish was obtained from the fish hatchery of the College of Veterinary Medicine at Mississippi State University. All fish experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee at Mississippi State University (protocol number 12–042). In vivo experiments were conducted using catfish infection model to test 19 mutants. Briefly, four-month-old pathogen free channel catfish (11.58 ± 0.23 cm, 15.29 ± 0.95 g) were stocked at a rate of 20 fish/tank into 40 L tanks and maintained at 26 ± 2 °C throughout the experiment. Each transposon mutant, positive (E. ictaluri wild-type), and negative (BHI) controls were assigned to three or four tanks randomly. Catfish were challenged/vaccinated by immersion exposure using transposon mutants or wild type (3.09 × 10⁷ CFU/ml of water) using published procedures [15]. Catfish mortalities were recorded for 21 days. After 21 days of the first vaccination, both vaccinated, and sham-vaccinated catfish were infected with E. ictaluri wild type by immersion exposure (3.27 × 10⁷ CFU/ml of water). Catfish mortalities were recorded for two weeks.

Statistical analysis
We used SPSS V25 (IBM Corp., Armonk, NY) to conduct statistical analysis. For each strain, mean percent mortalities were calculated and arcsine-transformed. The one-way analysis of variance at significance level 0.05 was conducted using the “Univariate” function, in which strains were independent and arcsine-transformed mortalities were dependent variables. Because our data included different sample sizes, and variances were not equal, Games-Howell post hoc test was selected to identify significant differences between mutants and wild type or mutants and sham vaccinated group in virulence and efficacy experiments, respectively.

Abbreviations
ESC: Enteric septicemia of catfish; HPIDB: Host-Pathogen Interaction Database; IACUC: Institutional Animal Care and Use Committee; MVirDB: Microbial Virulence Database; PSORTb: Subcellular Localization Prediction Tool; T3SS: Type III Secretion System; UspA: Universal Stress Protein A

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
Conception and design of the study: MLL, AK. Performed experiments: SK, JL, HA, HCT. Analyzed data: SK. Wrote the manuscript: SK, MLL, AK. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All fish experiments were conducted under a protocol approved by the Institutional Animal-Care and Use Committee at Mississippi State University (protocol number 12–042).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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