Development and Characterization of a Novel Live Attenuated Vaccine Against Enteric Septicemia of Catfish

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INTRODUCTION

Channel catfish (Ictalurus punctatus) is the most significant aquaculture commodity in the United States, and Edwardsiella ictaluri causes enteric septicemia of channel catfish (ESC) (Hawke, 1979). The disease occurs as acute enteric septicemia or chronic encephalitis (Shotts et al., 1986). Use of antibiotic-added feed (Smith et al., 1994; Plumb et al., 1995) and feed restriction (Wise et al., 2004) are traditional means used to control ESC. Although these practices could reduce mortalities, feed restriction results in reduced production through lost feeding days. Medicated feed is expensive, useful only in fish that accept feed, and could yield antibiotic-resistant strains.

Vaccination is a vital prophylactic strategy for prevention of bacterial diseases in aquaculture (Shoemaker et al., 2009; Villumsen et al., 2014). Because E. ictaluri species has been shown to be very homogeneous (Plumb and Vinitnantharat, 1989; Bertolini et al., 1990), a vaccine strain could potentially provide wide-range of protection against different E. ictaluri strains in different fish species. The commercial ESC vaccine Aquavac-ESC (RE-33) was developed by serial passage in increasing concentrations of rifampicin (Klesius and Shoemaker, 1997). Aquavac-ESC is safe in catfish fry (Klesius and Shoemaker, 1999), but it has not been accepted widely due to marginal economic returns (Bebak and Wagner, 2012). Another live attenuated E. ictaluri vaccine was...
developed by serial passage on media containing increasing concentrations of rifamycin, which protected catfish fingerlings when added in feed and administered orally (Wise et al., 2015). Under current catfish production practices, catfish fry are transferred from hatchery to nursery ponds when they are 1–2-week-old. Therefore, there is an urgent need for an effective ESC vaccine that can be delivered to catfish fry before their release into nursery ponds.

Type six secretion system (T6SS) is a virulence factor for many pathogenic bacteria (Rao et al., 2004; Pukatzki et al., 2006). This system is highly conserved and widely distributed in Gram-negative bacteria as one or more copies (Bingle et al., 2008). T6SS delivers protein effectors into the periplasm of the target cells directly upon cell-to-cell contact. Therefore, contributing to different processes ranging from inter-bacterial killing to negative bacteria as one or more copies (Bingle et al., 2008). This system is highly conserved and widely distributed in Gram-negative bacteria as one or more copies (Bingle et al., 2008).

Our previous proteomics study showed that EvpB protein is differentially regulated during in vitro iron-restricted conditions (Dumpala et al., 2015). Thus, we hypothesized that EvpB protein could have a crucial role in the T6SS of E. ictaluri. In the current work, we report the construction of an evpB in-frame deletion mutant and its vaccine potential in catfish fingerlings and fry.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this work are listed in Table 1. Wild-type E. ictaluri strain 93–146 (EiWT) was cultured in brain heart infusion (BHI) agar or broth (Difco, Sparks, MD, United States) and incubated at 30°C throughout the study. E. coli strains CC118.pir and SM10.pir were cultured on Luria-Bertani (LB) agar or broth (Difco, Sparks, MD, United States) and incubated at 37°C throughout the study. When required, media were supplemented with the following antibiotics and reagents: ampicillin (amp: 100 mg/ml), colistin sulfate (col: 12.5 mg/ml), sucrose (5%), and mannitol (0.35%) (Sigma-Aldrich, St. Louis, MO, United States).

Sequence Analysis

The nucleotide sequences of the T6SS operon were obtained from the E. ictaluri strain 93–146 genome (GenBank accession: CP001600) (Williams et al., 2012). The Basic Local Alignment Search Tool (BLAST) was used to determine the sequence of the evpB open reading frame and adjacent sequences.

Construction of EiΔevpB In-frame Deletion Mutant

In-frame deletion of the E. ictaluri evpB gene (NT01E1_RS11895) was accomplished by following the procedures described previously (Abdelhamed et al., 2013). Briefly, 1,210 bp upstream and 1,155 bp downstream regions of evpB were amplified from E. ictaluri strain 93–146 genomic DNA with A/B and C/D primer pairs (Table 2), respectively. These PCR products were mixed, diluted, and used as template in a splicing overlap extension PCR (Horton et al., 1990) with the A/D primers to generate ΔevpB deletion fragment (2,365 bp). The resulting ΔevpB deletion fragment was cloned into pMEG-375 suicide plasmid. The resulting plasmid, pEIΔevpB, was transformed into E. ictaluri by conjugation, and transformants were selected on BHI agar containing Amp and Col at 30°C for 2 days. To allow the second homologous recombination, a single Amp-resistant merodiploid colony was plated on BHI agar with sucrose and mannitol and incubated at 30°C for 3 days. Amp-sensitive colonies were

### Table 1 | Bacterial strains and plasmids.

| Bacterial Strain | Strains | References |
|------------------|---------|------------|
| Edwardsiella ictaluri 93–146 | Wild type; pEI1+; pEI2+; Col<sup>+</sup> | Lawrence et al., 1997 |
| EiΔevpB | 93–146 derivative; pEI1+; pEI2+; Col<sup>+</sup>; ΔevpB | This study |
| EiΔevpB+pEIevpB | ΔevpB; pEIevpB | This study |

### Table 2 | Primers used to generate and verify in-frame deletion of the E. ictaluri evpB gene.

| Primers | Sequence<sup>a</sup> | RE<sup>b</sup> |
|---------|----------------------|--------------|
| EvpB-F01 | AATCTAGAGGAGCGACTCACTT | XbaI |
| EvpB-R189 | TAAGTCAAGCGAATGTCAC | |
| EvpB-F1375 | GTCAGAAGCTTGGTCACTAGTGAATC | |
| EvpB-R01 | AATCTAGAGTTCATGCTACTGGAATGTC | XbaI |
| EvpB-Seq | GCTCCCAAGCTGAAAGAC | SmaI |
| EvpB-F01-Comp | AAACCCGGATGAGCGAAACAGAATGTGC | |
| EvpB-R01-Comp | AATCTAGGTGGCAGCACCAAGTGAAG | XbaI |

<sup>a</sup> Bold letters at the 5’ end of the primer sequence represent restriction enzyme (RE) site added. AA nucleotides were added to the end of primers containing a RE site to increase the efficiency of enzyme cut. Underlined bases in primer C indicate reverse complemented primer B sequence. <sup>b</sup> RE stands for restriction enzyme.
screened by colony PCR using the A/D primers, and further confirmation was done by sequencing of A/D fragment.

**Complementation of the evpB Gene**

The 1,464 bp open reading frame of the evpB gene was amplified using primers listed in Table 2. The amplicon was cloned into a pBBR1-MCS4 plasmid (Kovach et al., 1995) at the Smal and XbaI restriction sites. The resulting plasmid, pEievpB, was transferred to EiΔevpB by conjugation. Successful transformation was verified by observing plasmid profile of EiΔevpB. The resulting strain was designated as EiΔevpB+pEievpB.

**Determination of Safety and Efficacy of EiΔevpB in Catfish Fingerlings**

All fish experiments were approved by the Institutional Animal Care and Use Committee at Mississippi State University (protocol numbers: 12-042, 15-043, and 17-288). Virulence and vaccine efficacy of the EiΔevpB strain were assessed, as described (Abdelhamed et al., 2013). Briefly, 240 specific-pathogen-free (SPF) channel catfish fingerlings (13.88 ± 0.27 cm, 27.77 ± 1.04 g) were stocked in 40 1 flow-through tanks (20 fish/tank) with constant aeration and allowed to acclimate for 1 week. Water temperature was maintained at 26 ± 2°C during the experiment. The tanks were randomly assigned into three groups, and each group contained four replicates. The three groups were EiΔevpB, EiWT (positive control), and BHI (sham control). The fish were vaccinated by immersion for 1 h in water containing approximately $3.32 \times 10^7$ CFU/ml, and then flow-through conditions were resumed. Mortalities were recorded daily, and the presence of E. ictaluri was confirmed by streaking anterior kidney onto BHI plates. At 21-days post-immunization, the vaccinated fish were challenged with EiWT ($3.83 \times 10^7$ CFU/ml in water) by immersion for 1 h as described above. Mortalities were recorded daily.

**Determination of Safety and Efficacy of EiΔevpB in Catfish Fry**

Nine hundred 14-day-old SPF channel catfish fry were stocked in 18 tanks (50 fry/tank). Tanks were randomly assigned to six treatment groups with three replicates per group. Treatment groups consisted of high ($3.32 \times 10^7$ CFU/ml in water) and low ($3.32 \times 10^6$ CFU/ml in water) doses of EiΔevpB, EiWT, and BHI. Immersion vaccination was conducted same as fingerling challenge described above. At 21 days post-vaccination, fry were challenged with EiWT by immersion exposure at approximately $3.10 \times 10^7$ CFU/ml in water. Mortalities were recorded daily.

**Evaluation of Various Challenge Doses of EiΔevpB in Catfish Fry**

Vaccine efficacies of three separate doses of EiΔevpB were evaluated in 7-day-old fry to determine the optimal dose. Briefly, 750 fry were stocked into 15 tanks (50 fry/tank). The tanks were divided into five groups with three replicates per group. Vaccinated groups consisted of three doses of EiΔevpB ($3.72 \times 10^6$, $3.72 \times 10^7$, and $3.72 \times 10^8$ CFU/ml in water), EiWT (positive control), and BHI (sham control). Fish were monitored daily, and mortalities were recorded from each tank. After 30 days post-vaccination, fry were challenged with the EiWT by immersion in water ($3.80 \times 10^7$ CFU/ml) for 1 h. Mortalities were recorded daily.

**Comparison of EiΔevpB and Aquavac-ESC in Catfish Fingerlings**

Vaccine efficacy of EiΔevpB strain was compared with Aquavac-ESC in catfish fingerlings. Briefly, 320 channel catfish fingerlings (7.75 ± 0.08 cm, 4.50 ± 0.014 g) were stocked into 16 tanks (20 fish/tank). Each group included four replicate tanks. Vaccination groups consisted of Aquavac-ESC, EiΔevpB, EiWT (positive control), and BHI (sham control). Fingerlings were vaccinated by immersion in water containing approximately $4.5 \times 10^7$ CFU/ml for 1 h. Fish were monitored, and dead fish were removed daily. After 21 days, immunized fish were challenged with EiWT by immersion in water with $3.80 \times 10^7$ CFU/ml for 1 h. Mortalities were recorded daily.

**Comparison of EiΔevpB and Aquavac-ESC in Catfish Fry**

Vaccine efficacy of EiΔevpB was compared with Aquavac-ESC in 14-days post-hatch fry. Briefly, 800 channel catfish fry were stocked into 16 tanks (50 fry/tank). Each group included four replicate tanks. Vaccination groups consisted of EiΔevpB strain, Aquavac-ESC, EiWT (positive control), and BHI (sham control). Fry were vaccinated by immersion ($3.72 \times 10^7$ CFU/ml in water) for 1 h. Fish were monitored, and dead fish were removed daily. After 21 days, immunized fish were challenged with EiWT by immersion ($3.80 \times 10^7$ CFU/ml in water) for 1 h. Mortalities were recorded daily.

**Comparison of EiΔevpB and Complemented Strain**

Virulence of EiΔevpB+pEievpB strain was compared to EiΔevpB in 12-days post-hatch fry. Briefly, 360 channel catfish fry were stocked into 12 tanks (30 fish/tank). Experimental groups consisted of EiΔevpB, EiΔevpB+pEievpB, EiWT (positive control), and BHI (negative) each group included three replicate tanks. Fry were challenged by immersion ($4.60 \times 10^7$ CFU/ml in water) for 1 h. Mortalities were recorded daily.

**Statistical Analysis**

The percent mortality and survival values were arcsine transformed, and pairwise comparison of the means was performed with Tukey procedure. Analysis of variance (ANOVA) was done using PROC GLM in SAS for Windows v9.4 (SAS Institute, Inc., Cary, NC, United States) to assess significance. An alpha level of 0.05 was used in all analyses.

**RESULTS**

**T6SS in E. ictaluri Genome**

Analysis of the E. ictaluri genome revealed the presence of a 20,724 bp operon containing 16 genes (evpP, plus genes from
Construction of the EiΔevpB Mutant

We successfully introduced an in-frame deletion to the evpB gene in the E. ictaluri chromosome. The resulting EiΔevpB strain contained a deletion of 1,167 bp out of 1,488 bp open reading frame (78.42%), resulting in loss of 389 amino acids from the E. ictaluri evpB gene. This in-frame deletion was verified by PCR and sequencing of the amplified fragment from the EiΔevpB strain.

Virulence and Vaccine Efficacy of EiΔevpB in Catfish Fingerlings

The virulence of EiΔevpB (0% mortality) was significantly lower than those fish challenged with EiWT (46.91% mortality) (p < 0.5) (Figure 2A). At 21 days post-vaccination, the EiΔevpB vaccinated group had significantly higher survival compared to the sham-vaccinated group (100% vs. 40.69% survival, respectively) (p < 0.05) (Figure 2B).

Virulence and Efficacy of EiΔevpB in Fry

No mortality was observed in the fry vaccinated with EiΔevpB at 10⁶ and 10⁷ CFU/ml in water. In contrast, 98.67 and 100% mortalities were observed in the fry exposed to EiWT at 10⁶ and 10⁷ CFU/ml in water doses, respectively (Figure 3A). The fry vaccinated with EiΔevpB showed 34.24% survival at 10⁶ CFU/ml in water, and 80.34% survival at 10⁷ CFU/ml in water at 21 days post-vaccination. On the contrary, the sham-vaccinated group had 1.79% survival (Figure 3B).

Optimal Vaccine Dose of EiΔevpB in Catfish Fry

Mortality in 7-day old fry vaccinated with EiΔevpB (10⁶, 10⁷, and 10⁸ CFU/ml in water) and sham group ranged between 2.53 and 5.13%, which was not statistically different. Dead fish collected from these treatments did not show any pathology, and E. ictaluri was not present in the fish. On the other hand, very high mortality (95.71%) was observed in EiWT exposed (10⁷ CFU/ml in water) fry (Figure 4A). At 30-day post-vaccination with three different doses (10⁶, 10⁷, and 10⁸ CFU/ml in water), the percent survival in fry challenged with EiWT were significantly higher (57.74, 62.74, and 71.06%, respectively) compared to the sham-vaccinated fry (12.16% survival) (p < 0.05) (Figure 4B).

Comparison of EiΔevpB to Aquavac-ESC in Catfish Fry

EiΔevpB, Aquavac-ESC, and sham control showed low mortalities (5.14, 1.45, and 1.67%, respectively) in catfish fingerlings. On the other hand, EiWT challenge caused 81.83% mortality (Figure 5A). Catfish fingerlings vaccinated with EiΔevpB elicited significantly higher protection (96.20% survival) compared to that of Aquavac-ESC (45.51% survival) and sham-vaccinated (6.67% survival) groups (p < 0.05) following EiWT challenge (Figure 5B).

Comparison of EiΔevpB to Aquavac-ESC in Catfish Fingerling

EiΔevpB, Aquavac-ESC, and sham control showed low mortalities (5.14, 1.45, and 1.67%, respectively) in catfish fingerlings. On the other hand, EiWT challenge caused 81.83% mortality (Figure 5A). Catfish fingerlings vaccinated with EiΔevpB elicited significantly higher protection (96.20% survival) compared to that of Aquavac-ESC (45.51% survival) and sham-vaccinated (6.67% survival) groups (p < 0.05) following EiWT challenge (Figure 5B).

DISCUSSION

The primary objective of this study was to develop a live attenuated E. ictaluri vaccine strain based on mutation of the evpB gene, which is the second gene in the T6SS operon. The E. ictaluri EvpB protein was previously annotated as Eip55 (Moore et al., 2002), but it was not known that Eip55 was part of the T6SS. Eip55 is expressed during E. ictaluri infection and is antigenic to channel catfish. The percent sequence identity at the amino acid level between E. ictaluri and E. tarda EvpB (Rao et al., 2004) is high (96.5%). The first step of our work was construction of the EiΔevpB strain by in-frame deletion of the evpB gene leaving 189 bp at the 5′ end and 132 bp at the 3′ end. Mutant construction did not introduce any selective antibiotics to the EiΔevpB strain. Introduction of extraneous antibiotic resistance in vaccine strains is not desirable to avoid spread of antibiotic resistance genes.

The EiΔevpB strain was completely avirulent in catfish fingerlings. Moreover, vaccination of fingerlings with EiΔevpB provided full protection against subsequent challenge with EiWT at 21 days post-vaccination. However, U.S. catfish production practices limit immersion vaccination to 7–14 days post-hatch. At this age, 1000s of fry are housed in hatchery tanks and can be vaccinated cost-effectively. To conform to industry practices, the EiΔevpB strain was assessed in 14-day-old fry by immersion at two different doses (10⁶ and 10⁷ CFU/ml in water). The result demonstrated that the EiΔevpB strain was completely attenuated in channel catfish fry at both doses. Following vaccination, we found 80.34% survival at the 10⁷ CFU/ml in water dose and 34.24% survival at the 10⁶ CFU/ml in water dose. These results demonstrate that 10⁷ CFU/ml in water dose of EiΔevpB could provide excellent protection levels in catfish fry against ESC.

Immunization of 7-day-old catfish fry with increasing doses of EiΔevpB (10⁶, 10⁷, and 10⁸ CFU/ml in water) demonstrated that the protection levels of EiΔevpB, although was not statistically
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FIGURE 1 | Type VI secretion system organization in the E. ictaluri genome. Arrows indicate the direction of transcription, and numbers at the beginning and the end indicate genomic coordinates.

FIGURE 2 | Results of virulence (A) and vaccine efficacy (B) trials of $Ei\Delta\text{evpB}$ in catfish fingerlings. Lowercase letters indicate significant differences ($p < 0.05$).

FIGURE 3 | Results of virulence (A) and vaccine efficacy (B) trials in catfish fry vaccinated with two different doses of $Ei\Delta\text{evpB}$ ($10^6$ and $10^7$ CFU/ml in water). Lowercase letters indicate significant differences ($p < 0.05$).

FIGURE 4 | Results of virulence (A) and vaccine efficacy (B) trials of three different vaccination doses ($10^6$, $10^7$, and $10^8$ CFU/ml in water) of $Ei\Delta\text{evpB}$ in catfish fry. Lowercase letters indicate significant differences ($p < 0.05$).
We also compared vaccine efficacy of $Ei\Delta evpB$ to the commercial vaccine Aquavac-ESC in channel catfish fingerlings and fry by immersion. In both trials, $Ei\Delta evpB$ provided better protection than Aquavac-ESC. Besides the superior performance of $Ei\Delta evpB$ compared to Aquavac-ESC, $Ei\Delta evpB$ does not have an added antibiotic resistance gene while Aquavac-ESC is rifampicin resistant. Also, $Ei\Delta evpB$ has a known genotype, while the genetic basis for Aquavac-ESC attenuation is not described.

The results from fry and fingerling experiments showed that evpB is vital in $E. ictaluri$ virulence, which is consistent with findings in $E. tarda$ PPD130/91, where deletion of evpB led to reduced virulence in blue gourami and impaired replication in gourami phagocytes (Rao et al., 2004). Several T6SS proteins are important for bacterial pathogenesis. However, the function of most T6SS proteins remains unknown (Filloux et al., 2008; Silverman et al., 2012). This is the first report to our knowledge that evpB is required for $E. ictaluri$ virulence. This is also the first study that linked T6SS and virulence in $E. ictaluri$.

The results shown here suggest that deletion of evpB gene provides an excellent live attenuated vaccine candidate. Live attenuated bacterial vaccines activate immune responses by mimicking the route of natural infection, possess intrinsic adjuvant properties, and can be administrated as mucosal vaccines. In the present study, only the immersion route of exposure was tested, which is the preferred vaccination method.
in commercial settings, because large numbers of small fish can be vaccinated quickly and cheaply (Stevenson, 1997; Chettri et al., 2013).

Live attenuated vaccines must achieve a precise balance between lack of pathogenicity and sufficient immunogenicity to provide protective immunity. An important consideration for the success of vaccination in catfish fry is that the immune system of fry may not be fully developed (Ellis, 1988). Therefore, fry may not be able to respond to vaccination effectively. The protective immunity in channel catfish is determined by cellular immune mechanisms, which generally precedes the development of humoral immunity (Shoemaker et al., 1997; Thune et al., 1997; Petrie-Hanson and Ainsworth, 2001). In previous studies, it has been reported that catfish fry failed to produce a significant antibody response before 3 weeks of age due to the poor organization of secondary lymphoid tissue (Patrie-Hanson and Jerald Ainsworth, 1999; Petrie-Hanson and Ainsworth, 2001). This could elucidate why attempts of early vaccination of fry are less likely to succeed. However, our results showed that vaccination of 2-week-old catfish fry with EiΔevpB could provide very high protection. It is clear that use of EiΔevpB at three or 4-week-old fry may provide full protection against EiWT, but current catfish practices do not permit housing fry in hatcheries beyond 10–14 days.

CONCLUSION

In conclusion, EiΔevpB described in this work is entirely safe and provides full protection for catfish fingerlings. Also, EiΔevpB is safe and highly protective in catfish fry. Further, EiΔevpB is well characterized genetically and does not carry any additional antibiotic resistance. Field trials in earthen ponds will allow us to assess the commercial potential of EiΔevpB under production conditions. Understanding the responses of the catfish immune system to EiΔevpB vaccination will help us develop a better vaccination strategy.

AUTHOR CONTRIBUTIONS

AK and ML supervised the study. HA, ML, and AK designed the experiments and analyzed and interpreted the data. HA and AK performed the experiments. All authors wrote and approved the manuscript.

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REFERENCES

Abdelhamed, H., Lu, J., Shaheen, A., Abbass, A., Lawrence, M. L., and Karsi, A. (2013). Construction and evaluation of an Edwardsiella ictaluri fhuC mutant. Vet. Microbiol. 162, 858–865. doi: 10.1016/j.vetmic.2012.11.006

Bebak, J., and Wagner, B. (2012). Use of vaccine vaccination against enteric septicemia of catfish and columnaris disease by the U.S. catfish industry. J. Aquat. Anim. Health 24, 30–36. doi: 10.1089/08979659.2012.667048

Bertolini, J. M., Cipriano, R. C., Pyle, S. W., and McLaughlin, J. J. A. (1990). Serological investigation of the fish pathogen Edwardsiella ictaluri, cause of enteric septicemia of catfish. J. Wildl. Dis. 26, 246–252. doi: 10.7589/0090-3558-26.2.246

Bingle, E. L., Bailey, C. M., and Pallen, M. J. (2008). Type VI secretion: a beginner’s guide. Curr. Opin. Microbiol. 11, 3–8. doi: 10.1016/j.mib.2008.01.006

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., and Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? BMC Genomics 10:104. doi: 10.1186/1471-2164-10-14

Cascales, E. (2008). The type VI secretion toolkit. EMBO Rep. 9, 735–741. doi: 10.1038/embor.2008.131

Chettri, J. K., Deshmukh, S., Holten-Andersen, L., Jafaar, R. M., Dalsgaard, I., and Buchmann, K. (2013). Comparative evaluation of administration methods for a vaccine protecting rainbow trout against Yersinia ruckeri O1 biotype 2 infections. Vet. Immunol. Immunopathol. 154, 42–47. doi: 10.1016/j.vetimm.2013.04.001

Curtiss, R. III (2003). Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic Escherichia coli strain. Proc. Natl. Acad. Sci. U.S.A. 100, 247–252. doi: 10.1073/pnas.232686799

Dumplap, P. R., Peterson, B. C., Lawrence, M. L., and Karsi, A. (2015). Identification of differentially abundant proteins of Edwardsiella ictaluri during iron restriction. PLoS One 10:e0132504. doi: 10.1371/journal.pone.0132504

Ellis, A. E. (1988). “Ontogeny of the Immune System in Teleost Fish,” in Fish Vaccination, ed. A. E. Ellis (London: Academic Press), 20–31.

Filloux, A., Hachani, A., and Bleves, S. (2008). The bacterial type VI secretion machine: yet another player for protein transport across membranes. Microbiology 154, 1570–1583. doi: 10.1099/mic.0.016840-0

Hawke, J. P. (1979). A Bacterium associated with disease of pond cultured channel catfish, Ictalurus punctatus. J. Fishery Res. Board Can. 36, 1508–1512. doi: 10.1139/f79-219

Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. J. Bacteriol. 172, 6557–6567. doi: 10.1128/JB.172.11.6557-6567.1990

Ho, B. T., Dong, T. G., and Mekalanos, J. J. (2014). A view to a kill: the bacterial type VI secretion system. Cell Host Microbe. 15, 9–21. doi: 10.1016/j.chom.2013.11.008

Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1990). Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques, 8, 528–535.

Klesius, P. H., and Shoemaker, C. A. (1997). Heterologous isolates challenge of channel catfish, Ictalurus punctatus, immune to Edwardsiella ictaluri. Aquaculture 157, 147–155. doi: 10.1016/S0044-8486(97)00768-8

Klesius, P. H., and Shoemaker, C. A. (1999). Development and use of modified live Edwardsiella ictaluri vaccine against enteric septicemia of catfish. Adv. Vet. Med. 41, 523–537. doi: 10.1603/0065-3519(99)00039-1

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., et al. (1995). Four new derivatives of the broad-host-range cloning vector pBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176. doi: 10.1016/0378-1119(95)00584-1
Lawrence, M. L., Cooper, R. K., and Thune, R. L. (1997). Attenuation, persistence, and vaccine potential of an Edwardsiella ictaluri purA mutant. Infect. Immun. 65, 4642–4651.

Lin, J. S., Ma, L. S., and Lai, E. M. (2013). Systematic dissection of the agrobacterium type VI secretion system reveals machinery and secreted components for subcomplex formation. PLoS One 8:e67647. doi: 10.1371/journal.pone.0067647

Miller, V. L., and Mekalanos, J. J. (1988). A novel suicide vector and characterization of Edwardsiella ictaluri proteins expressed and recognized by the channel catfish Ichthiopterus punctatus immune response during infection. Dis. Aquat. Organ. 52, 93–107. doi: 10.3354/dao052093

Moore, M. M., Fernandez, D. L., and Thune, R. L. (2002). Cloning and characterization of Edwardsiella ictaluri type VI secretion to target bacterial competitors. J. Bacteriol. 184, 5605–5609. doi: 10.1128/JB.05671-11

Patrie-Hanson, L., and Jerald Ainsworth, A. (1999). Humoral immune responses of channel catfish (Ictalurus punctatus) fry and fingerlings exposed to Edwardsiella ictaluri. Fish Shellfish Immunol. 9, 579–589. doi: 10.1006/fsim.1999.0215

Petrie-Hanson, L., and Ainsworth, A. J. (2001). Ontogeny of channel catfish lymphoid organs. Vet. Immunol. Immunopathol. 81, 113–127. doi: 10.1016/S0165-2427(01)00331-2

Plumb, J. A., Shoreinger, C. C., Shryock, T. R., and Goldsby, T. (1995). Susceptibility of six bacterial pathogens of channel catfish to six antibiotics. J. Aquat. Anim. Health 7, 211–217. doi: 10.1577/1548-8667(1995)007<0211:SOSBPO>2.3.CO;2

Plumb, J. A., and Vinitnatharat, S. (1989). Biochemical, biophysical, and serological homogeneity of Edwardsiella ictaluri. J. Aquat. Anim. Health 1, 51–56. doi: 10.1577/1548-8667(1989)001<0051:BBASPO>2.3.CO;2

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., et al. (2006). Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system. Proc. Natl. Acad. Sci. U.S.A. 103, 1528–1533. doi: 10.1073/pnas.0510322103

Rao, P. S., Yamada, Y., Tan, Y. P., and Leung, K. Y. (2004). Use of proteomics to identify novel virulence determinants that are required for Edwardsiella tarda pathogenesis. Mol. Microbiol. 53, 573–586. doi: 10.1111/j.1365-2958.2004.04123.x

Shoemaker, C. A., Klesius, P. H., Evans, J. I., and Arias, C. R. (2009). Use of modified live vaccines in aquaculture. J. World Aquac. Soc. 40, 573–585. doi: 10.1111/j.1749-7345.2009.00279.x

Shoemaker, C. A., Klesius, P. H., and Plumb, J. A. (1997). Killing of Edwardsiella ictaluri by macrophages from channel catfish immune and susceptible to enteric septicemia of catfish. Vet. Immunol. Immunopathol. 58, 181–190. doi: 10.1016/S0165-2427(97)00026-3

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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