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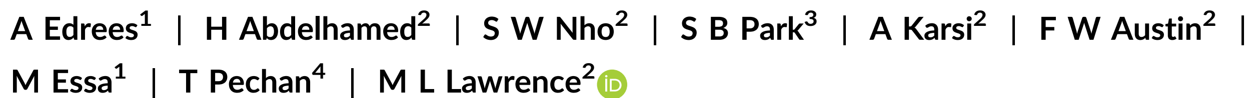
Construction and evaluation of type III secretion system mutants of the catfish pathogen *Edwardsiella piscicida*



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Abstract

Catfish is the largest aquaculture industry in the United States. Edwardsiellosis is considered one of the most significant problems affecting this industry. *Edwardsiella piscicida* is a newly described species within the genus *Edwardsiella*, and it was previously classified as *Edwardsiella tarda*. It causes gastrointestinal septicaemia, primarily in summer months, in farmed channel catfish in the south-eastern United States. In the current study, we adapted gene deletion methods used for *Edwardsiella* to *E. piscicida* strain C07-087, which was isolated from a disease outbreak in a catfish production pond. Four genes encoding structural proteins in the type III secretion system (T3SS) apparatus of *E. piscicida* were deleted by homologous recombination and allelic exchange to produce in-frame deletion mutants (*Ep*Δ*ssaV*, *Ep*Δ*esaM*, *Ep*Δ*yscR* and *Ep*Δ*escT*). The mutants were phenotypically characterized, and virulence and vaccine efficacy were evaluated. Three of the mutants, *Ep*Δ*ssaV*, *Ep*Δ*yscR* and *Ep*Δ*esaM*, were significantly attenuated compared to the parent strain ($p < .05$), but *Ep*Δ*escT* strain was not. Vaccination of catfish with the four mutant strains (*Ep*Δ*ssaV*, *Ep*Δ*esaM*, *Ep*Δ*yscR* and *Ep*Δ*escT*) provided significant protection when subsequently challenged with wild-type strain. In conclusion, we report methods for gene deletion in *E. piscicida* and development of vaccine candidates derived from a virulent catfish isolate.

KEYWORDS
aquaculture, catfish, *Edwardsiella piscicida*, type III secretion system, virulence

1 | INTRODUCTION

Edwardsiella piscicida is one of five species in the genus *Edwardsiella*. Prior to 2012, the genus consisted of three species: *Edwardsiella ictaluri*, *Edwardsiella hoshinae* and *Edwardsiella tarda*. However, based on collective evidence from genome sequencing and phenotypic comparisons, isolates from fish previously identified as *E. tarda* were reclassified into three different genetic groups: *E. tarda*, *E. piscicida* and *E. anguillarum*. All three species can grow in a temperature range from 25 to 37°C; however, the optimum temperature for growth of *E. piscicida* is 28–30°C (Abayneh, Colquhoun, & Sorum, 2013). Analysis of "*E. tarda*" isolates from channel catfish (*Ictalurus punctatus*) aquaculture in the southeast United States revealed that strains associated with gastrointestinal septicaemia, especially in summer months, are correctly classified as *E. piscicida* (Griffin et al., 2014). A quantitative PCR method specific for *E. piscicida* was developed and used to quantify the pathogen in various organs in both clinically and subclinically infected fish (Reichley, Ware, Greenway, Wise, & Griffin, 2015).

Edwardsiella piscicida was also isolated from farmed whitefish, *Coregonus lavaretus* (L.), in Finland during disease outbreaks in 2000, 2002 and 2013. The species was identified by biochemical tests, a species-specific PCR, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and multilocus sequence analysis (MLSA) (Shafiei

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et al., 2016). *Edwardsiella piscicida* was reported as the causative agent of Edwardsiellosis in cage-cultured sharpsnout sea bream (*Diplodus puntazzo*), indicating that *E. piscicida* is a possible serious threat for this Mediterranean aquaculture industry (Katharios, Kokkari, Dourala, & Smyrli, 2015).

Like many Gram-negative bacteria, *E. piscicida* utilizes a type III secretion system (T3SS) to modulate host cell functions (Blocker et al., 2008). Type III secretion system (T3SS) is a needle-like structure composed of several structural proteins encoded by genes typically located in a chromosome or plasmid locus. It is an important component in the pathogenesis of many Gram-negative bacteria, including several fish pathogens, and its function is to inject virulence proteins directly into host cells to disrupt normal functions (Tan, Zheng, Tung, Rosenshine, & Leung, 2005; Xie et al., 2014).

In aquaculture, bacterial infections are often treated with antimicrobial therapy. However, this strategy can result in the development of antimicrobial resistant *Edwardsiella* strains and subsequent treatment failures (Heuer et al., 2009; Lo, Lee, Wang, & Kuo, 2014; Wang et al., 2009). Furthermore, losses have already occurred when medicated feeds are applied; therefore, prevention is a more desirable strategy for farmers. Thus, there is increased interest in vaccination as a method to prevent fish diseases (Marsden, Vaughan, Foster, & Secombes, 1996; Temprano et al., 2005). The objective of the current study was to construct T3SS *E. piscicida* mutant strains by deleting genes that encode four structural proteins (AGH72974, AGH72975, AGH72976 and AGH72978) to determine the effects on phenotype and to evaluate their applicability as an attenuated vaccine against *E. piscicida* infection in catfish.

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	References
E. piscicida		
C07-087	Isolate from diseased catfish	Tekedar et al. (2013)
EpΔssaV	*E. piscicida; ΔssaV*	This study
EpΔesaM	*E. piscicida; ΔesaM*	This study
EpΔyscR	*E. piscicida; ΔyscR*	This study
EpΔescT	*E. piscicida; ΔescT*	This study
Escherichia coli		
CC118*λpir*	*Δ(ara-leu); araD; ΔlacX74; galE; galK; phoA20; thi-1; rpsE; rpoB; argE*(Am); *recAl; λpirR6K*	Herrero, de Lorenzo, and Timmis (1990)
SM10*λpir*	*thi; thr; leu; tonA; lacY; supE; recA;::RP4-2-Tc::Mu; Kmr; lpirR6K*	Miller and Mekalanos (1988)
BW19851	*RP4-2 (Km::Tn7, Tc::Mu-1), ΔuidA3::pir*$^+$*, recA1, endA1, thi-1, hsdR17, creC510*	Metcalf, Jiang, and Wanner (1994)
Plasmids		
pMEG-375	8,142 bp, sacRB mobRP4 oriR6K. Cm^R, Ap^R	Dozois, Daigle, and Curtiss (2003)
pEp*ΔssaV*	*ΔssaV*, pMEG-375	This study
pEp*ΔesaM*	*ΔesaM*, pMEG-375	This study
pEp*ΔyscR*	*ΔyscR*, pMEG-375	This study
pEp*ΔescT*	*ΔescT*, pMEG-375	This study
pAK*gfplux1*	pBBR1MCS4 with *gfpmut3* and *luxCDABE*	Karsi and Lawrence (2007)

2 | MATERIALS AND METHODS

2.1 | Bacterial strains, plasmids and growth conditions

Bacterial strains and the plasmids used in this work are listed in Table 1. *Edwardsiella piscicida* C07-087 and mutant strains were cultured in brain–heart infusion (BHI) agar and broth (Becton Dickinson, Sparks, MD, USA) and incubated at 30°C. *Escherichia coli* strains were cultured in Luria–Bertani (LB) agar and broth (Becton Dickinson) and incubated at 37°C. *Escherichia coli* CC118*λpir* strain was used for cloning and maintenance of pMEG-375 plasmids, and *E. coli* BW19851 and SM10*λpir* strains were used for transferring plasmids into *E. piscicida* by conjugation. Ampicillin was used at 100 μg/ml to select for integration of pMEG-375 derivatives in the *E. piscicida* chromosome or for maintenance of pAK*gfplux*1. Colistin was used at 12.5 μg/ml for counterselection against *E. coli* following conjugation.

2.2 | Construction of in-frame deletion mutant strains

Four in-frame deletion mutant strains (*EpΔssaV*, *EpΔesaM*, *EpΔyscR* and *EpΔescT*) were constructed through allelic exchange as described for *E. ictaluri* (Abdelhamed et al., 2013; Dahal, Abdelhamed, Lu, Karsi, & Lawrence, 2013). All primers used in mutant construction are given in Table 2. Upstream and downstream fragments for each gene deletion were amplified using primer pairs A and B and primer pairs C and D, respectively, from each target gene. Fusion of the two fragments was done by overlap extension PCR using the mixed upstream and downstream products as a template DNA with primer pairs A and D. The resulting in-frame deleted fragments were purified using Gel/PCR DNA Fragment Extraction Kit (IBI Scientific, Peosta, IA, USA). Plasmid pMEG-375 was purified from *E. coli* CC118 using Hi-Speed Mini Plasmid Kit (IBI Scientific), and the plasmid and overlap extension PCR amplicons were digested with *Sac*1 and *Xba*1 (New England Biolabs, Ipswich, MA, USA) at 37°C for 8 hr. Amplicons were ligated into pMEG-375 by T4 ligase (New England Biolabs) at 16°C, generating *pEpΔssaV*, *pEpΔesaM*, *pEpΔyscR* and *pEpΔescT* (Table 1).

The resulting plasmids were transferred into *E. coli* BW19851 by electroporation and subsequently mobilized to *E. piscicida* C07-087 by filter conjugation (Karsi & Lawrence, 2007). Selection was on BHI agar plates with colistin and ampicillin. Colonies with ampicillin resistance were streaked on BHI plates with 5% sucrose and 0.35% mannitol (Sigma-Aldrich, St. Louis, MO, USA) and grown for 2–3 days to

TABLE 2 Primers used to generate and verify in-frame deletion of the *Edwardsiella piscicida* T3SS apparatus genes

Primer ID		Sequence (5′-3′)[b]	RE[a]
EpssaVF01	A	AAA**GAGCTC**ACGGTATGGGTCGAGCGTAT	*SacI*
EpssaVR87	B	CATGAAAACGGCCAGCAAC	
EpssaVF225	C	GTTGCTGGCCGTTTTCATGAGGCGTTGCTCTCACCCTAC	
EpssaVR01	D	AAA**TCTAGA**ATCCTGTCCCGCTCGGTAT	*XbaI*
EpssaVF01S		AGCAGCCTGACCCTGGAC	
EpesaMF01	A	AAA**GAGCTC**ACGAGAGGATCAGGACCAG	*SacI*
EpesaMR	B	GCCCATCAGTCGTAGCCAAAG	
EpesaMF	C	CTTTGGCTACGACTGATGGGCTAGCTGGCTACACAAACTCACC	
EpesaMR01	D	AAA**TCTAGA**CCGATGAAGATCAGGGTAGG	*XbaI*
EpesaMF01S		CGGTAACGAGAGGATCAGGA	
EpyscRF01	A	AAA**GAGCTC**CAGAGCCTTTGGTAATCACG	*SacI*
EpyscRR48	B	AGTATCTGCCGACAGTGTTCC	
EpyscRF48	C	GGAACACTGTCGGCAGATACTGCTATGGGAAAAGCACAGCTC	
EpyscRR01	D	AAA**TCTAGA**TACAGCAGGTGCCATAGGTG	*XbaI*
EpyscRF01S		CTGAAATCCACAGCGCATC	
EpecTF01	A	AAA**GAGCTC**GTGGAAGTGGGGAACACTG	*SacI*
EpecTR	B	CTTCCGGCATAGCTAAACAGC	
EpecTF33	C	GCTGTTTAGCTATGCCGGAAGTTGAGCGAGAAAACGGAACAG	
EpecTR01	D	AAA**TCTAGA**CTGCAGCGATGACTTGGTCT	*XbaI*
EpescTF01S		GCGATGACTCGCTGCTATTAC	

[a]RE: restriction endonuclease site added to the 5′ end of the primer sequence.
[b]Bold letters at the 5′ end of the primer sequence represent RE site. AAA nucleotides were added to the end of each primer containing a RE site to increase the efficiency of enzyme digestion. Underlined bases indicate reverse complemented primer sequence for overlap extension PCR.

allow curing pMEG-375 vector from the chromosome using *sacB* counterselection (Santander, Mitra, & Curtiss, 2011). After this, resulting colonies were inoculated in BHI broth and tested for ampicillin sensitivity to ensure loss of plasmid. Gene deletions were confirmed by PCR and sequencing using primers listed in Table 2. After confirmation, *Ep*Δ*ssaV*, *Ep*Δ*esaM*, *Ep*Δ*yscR* and *Ep*Δ*escT* mutants were labelled with bioluminescence using pAK*gfplux*1 plasmid (Karsi & Lawrence, 2007).

2.3 | Determination of growth kinetics

The growth kinetics of *E. piscicida* C07-087 and four mutant strains were determined in BHI broth (Liu, Dong, & Zhang, 2012). Briefly, overnight cultures in BHI were normalized based on optical density at 600 nm (OD_{600}), and pellets were washed twice in phosphate-buffered saline (PBS) before being subcultured at 1:1,000 dilution into 20 ml BHI. OD_{600} was measured at 30-min intervals until the onset of stationary phase. All growth kinetics experiments were performed four times from two independent starter cultures.

2.4 | Biochemical characterization

Edwardsiella piscicida C07-087 and mutant strains were suspended in PBS for Gram stain, catalase, oxidase and motility tests. Other biochemical tests were performed using the API 20E kit (BioMerieux Inc., Durham, NC, USA) according to the manufacturer's instructions. Pure cultures were transferred aseptically to lysine iron agar slant (Remel, Lenexa, KS, USA) and incubated for 24 hr at 35°C to determine H_2S production.

2.5 | Biofilm assay

Biofilm formation was determined as described (Wakimoto et al., 2004) with minor modifications. Briefly, overnight cultures of *E. piscicida* C07-087 and mutant strains were diluted 1:100 in BHI supplemented with 1% glucose (Sigma-Aldrich), and 100 μl of the bacterial suspension was transferred into the wells of a 96-well microtitre plate (Coster, USA). Plates were incubated at 30°C for 72 hr, wells were gently washed two times with PBS, and they were stained with 0.1% crystal violet (Sigma-Aldrich) for 10 min at room temperature. Plates were rinsed with 100 μl PBS and dried at room temperature, and 100 μl of 70% ethanol was used for destaining. Finally, biofilm formation was quantified by measuring absorbance at 538 nm with a SpectraMax M5 ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Biofilm formation was determined five times for each strain.

2.6 | Haemolysin activity

Haemolysin activity was determined in triplicate as described (Xu et al., 2010) with minor modifications. Sheep erythrocytes

(Innovative Research, Novi, MI, USA) were washed three times with haemolysis buffer [0.85% physiological saline, 0.01 M $CaCl_2$ (Sigma-Aldrich)] and resuspended in the same buffer to make a final concentration of 3% sRBCs (Watson & White, 1979). *Edwardsiella piscicida* C07-087 and mutant strains were cultured in BHI for 24 hr at 30°C with shaking, diluted 1:100 in BHI and incubated at 30°C for 2, 4, 6, 8, 10 and 12 hr. Bacteria were harvested by centrifugation and washed two times in haemolysis buffer. Assay tubes containing 0.2 ml sRBCs, 250 μl bacteria and 1.55 ml haemolysis buffer were incubated at 30°C for one hour and then pelleted by centrifugation. Absorbance of the supernatant at 560 nm was measured to determine haemolytic activity. Assay tubes containing 1.8 ml haemolysis buffer and 0.2 ml sRBCs were included as negative control, and positive control consisted of 1.8 ml sterile water and 0.2 ml sRBCs. Percent haemolysis was calculated as follows:

[(Absorbance of sample − Absorbance of negative control)/Absorbance of positive control] × 100.

2.7 | Extracellular proteins

Extracellular proteins (ECPs) from *E. piscicida* C07-087 and mutant strains were extracted as described (Wang, Wang et al., 2010) with some modifications. One hundred microlitres of cultured bacteria was spread on BHI agar medium and incubated at 30°C for 48 hr. Bacteria were collected, suspended in PBS and pelleted by centrifugation. Supernatant was filtered through a 0.22-μm syringe filter (VWR, Radnor, PA, USA). ECPs were precipitated with trichloroacetic acid (TCA, Sigma-Aldrich) and dissolved in PBS. After centrifugation, supernatants were kept, and protein concentrations were determined using Bradford assay (Bio-Rad). One half volume of tricine sample buffer (Bio-Rad, Hercules, CA, USA) was added, and mixture was sonicated by Fisher Scientific Sonic Dismembrator (Waltham, MA, USA). A total of 100 μg of ECPs was loaded on precast gels (Bio-Rad) for SDS–PAGE, and gels were stained with Coomassie Brilliant Blue (Bio-Rad).

In-gel digestion was performed as described (Nho et al., 2011) with minor modifications. Briefly, sliced protein bands were completely destained in a mixture of equal volumes of 75 mM ammonium bicarbonate (ABC) and 40% ethanol with vortexing. Gel strips were reduced with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate (ABC) for 1 hr and alkylated by incubating with 55 mM iodoacetamide (IAA) in 25 mM ABC for 45 min in the dark. Supernatants were discarded, and 100 mM ABC was added to the gel bands, followed by dehydration with 100% acetonitrile (ACN). This process was repeated once, and then gels were dried using a vacuum dryer. Trypsin solution (0.02 μg/ml; Promega, Madison, WI, USA) was added to the dried gel particles. After incubating on ice for 40 min, 25 mM ABC was added, and samples were incubated at 37°C for 14 hr. Fresh 25 mM ABC was added, samples were vortexed for 30 min, and supernatants were transferred to fresh tubes. Gels were re-extracted by adding extraction solution (0.1% [v/v] formic acid in 25% [v/v] ACN) and vortexing for 30 min. Supernatants were transferred to the tubes containing tryptic peptide mixture from the first extraction, and samples were stored at −20°C until LC-MS/MS analysis.

2.8 | Liquid chromatography–tandem mass spectrometry

Mass spectra were collected and analysed (Takac et al., 2016) using an UltiMate 3000 nano-HPLC system linked to a LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). Protein tryptic digest was separated on an Acclaim PepMap C18 column (75 μm × 150 mm, ThermoFisher Scientific) in 60 min linear gradient of acetonitrile (2%–55% for 35 min, 95% for 10 min, 2% for 15 min), with a flow rate of 300 nl/min. Intact and fragmented peptide ions were measured utilizing the linear trap mass detector in data-dependent acquisition mode with dynamic exclusion being applied.

The raw mass spectral data were matched to *Edwardsiella piscicida* protein database using the SEQUEST algorithm of Proteome Discoverer 1.1 (Thermo Fisher Scientific). The target database (7,667 referenced sequences as of March 2016) was obtained from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The reversed copy (created automatically by Proteome Discoverer) served as a decoy database to calculate false discovery rate (FDR). To obtain high confidence protein identification, results were filtered with FDR value of $<1.0\%$.

2.9 | Bioluminescence imaging

Bioluminescence imaging (BLI) was conducted using an IVIS Imaging System (IVIS Lumina XRMS In Vivo Imaging System, PerkinElmer, Waltham, MA, USA; Karsi, Menanteau-Ledouble, & Lawrence, 2006). Five fish per bacterial strain were anesthetized and injected IP with 3.7×10^6 CFU/fish. For imaging, fish were anesthetized and transferred to the imaging chamber; total photon emissions from the whole fish body were collected using an exposure time of one minute. Following BLI, fish were returned to well-aerated water for recovery. BLI was conducted at 0, 2.5, 6, 12, 24 and 36 hr post-infection.

2.10 | Virulence/vaccine efficacy of *E. piscicida* T3SS mutants

All fish experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University. Specific pathogen-free (SPF) channel catfish fingerlings (19 ± 2.04 g, 11 ± 0.67 cm) were obtained from the SPF Channel Catfish Laboratory at Mississippi State University's College of Veterinary Medicine. Fish were maintained in aerated tanks supplied with a continuous flow of recirculating water (flow rate: 1-L/min) at water temperature 28 ± 1°C, and they were fed twice daily with commercial channel catfish feed. Anaesthesia was provided using tricaine methane sulphonate (MS-222) when needed.

We first determined the 50 percent lethal dose (LD_{50}) of *E. piscicida* C07-087. Eight treatment groups were included, each with

three tanks with ten fish in each. Overnight cultures of *E. piscicida* C07-087 strain in BHI broth were harvested by centrifugation, washed twice with sterile PBS and then serially diluted. Fish were injected intraperitoneally (IP) with 0.1 ml of 1.9×10^3, 9.3×10^3, 4.7×10^4, 2.3×10^5, 1.2×10^6, 5.8×10^6, 2.9×10^7 and 1.5×10^8 CFU/fish of *E. piscicida* C07-087 strain. One more group was used as a negative control group that was handled similarly but injected IP with sterile PBS. Fish were observed for 7 days, and any fish that died were removed for routine bacteriological examination. The LD_{50} was calculated (Reed & Muench, 1938).

Based on the LD_{50} determination, a virulence/vaccine trial was conducted. For the virulence determination, about 180 six-month-old SPF channel catfish fingerlings were stocked into 18 tanks at a rate of 10 fish/tank. Each treatment had three replicate tanks. Treatments consisted of *E. piscicida* C07-087, *EpΔssaV*, *EpΔesaM*, *EpΔyscR* and *EpΔescT* and negative control (sterile PBS). Catfish were experimentally infected by IP injection (0.1 ml per fish). Injection doses were approximately 9×10^5, 7.5×10^5, 1.14×10^6, 9×10^5 and 1.6×10^6, and CFU/fish for *E. piscicida* C07-087, *EpΔssaV*, *EpΔesaM*, *EpΔyscR* and *EpΔescT*, respectively. Mortalities were recorded for 21 days. Following the virulence trial (21 days post-exposure from the first challenge), all animals from the mutant-exposed and negative control treatment groups were experimentally infected by IP injection with *E. piscicida* C07-087 (1.2×10^6 CFU/fish). Mortalities were recorded for 7 days, and any fish that died were removed for routine bacteriological examination.

2.11 | Statistical analysis

Linear regression analysis in PROC MIXED of SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC, USA) was used for growth kinetics, biofilm, haemolysis and bioluminescence assays where the mutant strains were compared to *E. piscicida* strain C07-087. Separate models were developed for each analytical test using manual forward selection, and each included mutant type and hour as fixed effects. Rep was included as a random effect. Adjustment for multiple comparisons was made using the SIMULATE option. Results are reported as least square means ± standard error.

Exact logistic regression was used in PROC LOGISTIC of SAS for Windows 9.4 (SAS Institute, Inc.) to evaluate percent mortalities during the pathogenicity and vaccine trials. The outcome was the number of deaths over the total number of trials. For the challenge analysis, comparisons were made to the negative control, and separate models were developed for low and high doses. Odds ratios and 95% confidence limits are reported. For the pathogenicity analysis, comparisons were made to strain C07-087.

3 | RESULTS

3.1 | Construction of *E. piscicida* mutant strains

Genes *ssaV*, *esaM*, *yscR* and *escT* encode structural proteins that contribute to formation of the T3SS apparatus of *E. piscicida* C07-087 (Table 3). These four genes were deleted to obtain four mutant strains: *EpΔssaV*, *EpΔesaM*, *EpΔyscR* and *EpΔescT* (Table 1). All mutant strains were ampicillin sensitive, and each was confirmed by PCR (Figure 1) and sequencing.

3.2 | Growth kinetics

Growth kinetics of *E. piscicida* C07-087 and mutant strains were determined in BHI broth in quadruplicate. No significant difference in growth kinetics was detected between the parent wild-type strain and any of the mutants (data not shown).

3.3 | Biochemical characterization

Edwardsiella piscicida C07-087 and mutant strains were all cytochrome oxidase negative and catalase positive, and all had the same pattern in API20E (positive reaction on ortho-nitrophenyl-ßD-galactopyranosidase, ornithine decarboxylase, indole production, citrate utilization and glucose utilization; negative reaction on gelatin liquefaction, mannose, sucrose, arabinose, sorbitol, inositol, rhamnose and melibiose utilization). For H_2S production, all strains showed positive reaction except *EpΔesaM* strain, which had a weak positive reaction. This finding was confirmed by streaking an isolated colony from each mutant and the parent strain C07-087 on lysine iron agar slant with incubation at 35°C for 24 hr, where *EpΔesaM* showed the lowest H_2S production.

3.4 | Biofilm formation

None of the wild-type or mutant strains had significant biofilm formation at 24 or 48 hr. All four mutants and parent strain C07-087 had significant biofilm formation at 72 hr ($p < .05$), but there were no significant differences in biofilm formation between strains (Figure 2).

3.5 | Haemolysin activity

Haemolysin activity was low for all strains at 2-hr incubation and was near 100% for all strains at 6-hr incubation. No significant differences between strains were detected at these time points.

TABLE 3 Properties of the selected *Edwardsiella piscicida* T3SS apparatus proteins

Protein	Locus tag	GI	ORF (bp/aa)
Secretion system apparatus protein SsaV	ETAC_04215	469762556	2,058/685
EsaM	ETAC_04220	469762557	336/111
Type III secretion system protein YscR	ETAC_04225	469762558	648/215
Type III secretion apparatus protein EscT	ETAC_04235	469762560	783/260

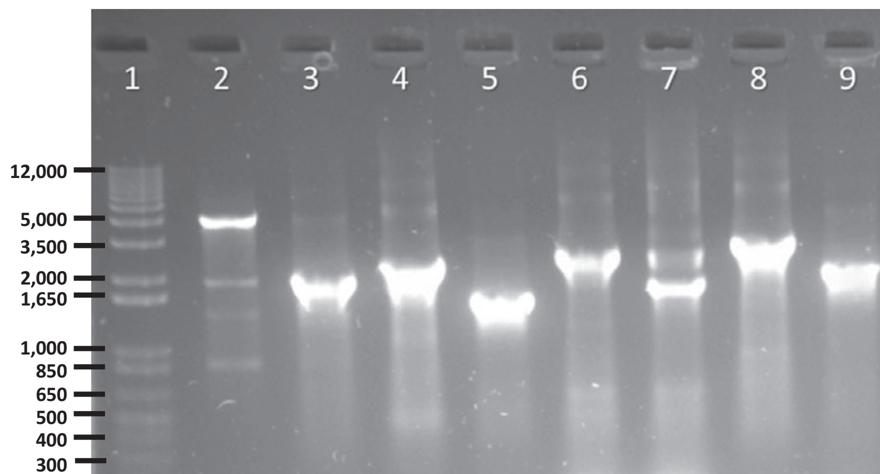

FIGURE 1 Genotypic confirmation of *Edwardsiella piscicida* mutant strains by PCR using A and D primer pair. Lane 1: 1KB plus DNA ladder; Lane 2: 4,081 bp *E. piscicida* wild-type strain *ssaV* gene; Lane 3: 1,922 bp *EPΔssaV* mutant; Lane 4: 2,198 bp *E. piscicida* wild-type strain *esaM* gene; Lane 5: 1,865-bp *EpΔesaM* mutant; Lane 6: 2,426 bp *E. piscicida* wild-type strain *yscR* gene; Lane 7: 1,748 bp *EpΔyscR* mutant; Lane 8: 2,685 bp *E. piscicida* wild-type strain *escT* gene; Lane 9: 1,872 bp *EpΔescT* mutant

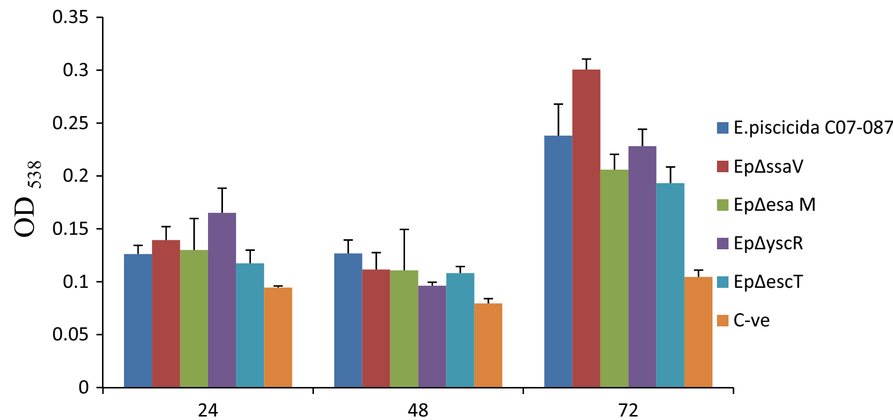

FIGURE 2 Mean biofilm formation of *Edwardsiella piscicida* C07-087 and mutant strains as measured by absorbance at 538 nm to detect crystal violet staining of biofilm. The data represent means of five replicates plus or minus standard error. *Edwardsiella piscicida* C07-087 = parent strain, and C-ve = negative control (wells with no bacteria). All strains had significant biofilm formation at 72 hr compared to the negative control [Colour figure can be viewed at wileyonlinelibrary.com]

However, at 4-hr incubation, mutant strains *EpΔesaM* and *EpΔescT* had significantly higher level of haemolytic activity than wild-type strain C07-087 (p = .0002) (Figure 3).

3.6 | Extracellular proteins

SDS–PAGE of ECPs from *E. piscicida* C07-087 and mutant strains indicated a band with molecular weight of approximately 150 kDa was present in *E. piscicida* C07-087, *EpΔssaV* and *EpΔyscR*, but it was missing in *EpΔescT* and *EpΔesaM* (Figure 4). For comparison, the band from C07-087 and the same gel region from strain *EpΔescT* were excised and analysed by the same method. Mass spectrometry results are shown in Table 4. Parent strain C07-087 exhibited a dominant presence of flagellin protein as indicated by a high number of spectra (>200) matched to peptides belonging to four individual protein entries in the database. On the contrary, only two flagellin peptides were detected in mutant strain *EpΔescT*. RNA polymerase subunit was also detected in both strains.

3.7 | Bioluminescence imaging

Bioluminescence (quantified as photon counts) from the catfish infected with each of the T3SS mutants (*EpΔssaV*, *EpΔesaM*, *EpΔyscR* and *EpΔescT*) was significantly lower than that of strain C07-087 for at least one time point (Figure 5). Strain *EpΔssaV* was the most attenuated and had significantly lower bioluminescence at every time point after 2.5 hr post-infection. Strain *EpΔesaM* had significantly lower bioluminescence at early time points (6 and 12 hr post-infection), and strain *EpΔescT* was similar (significantly lower at 12 hr post-infection). Strain *EpΔyscR* had a different pattern with significantly lower bioluminescence at later time points (24 and 36 hr post-infection).

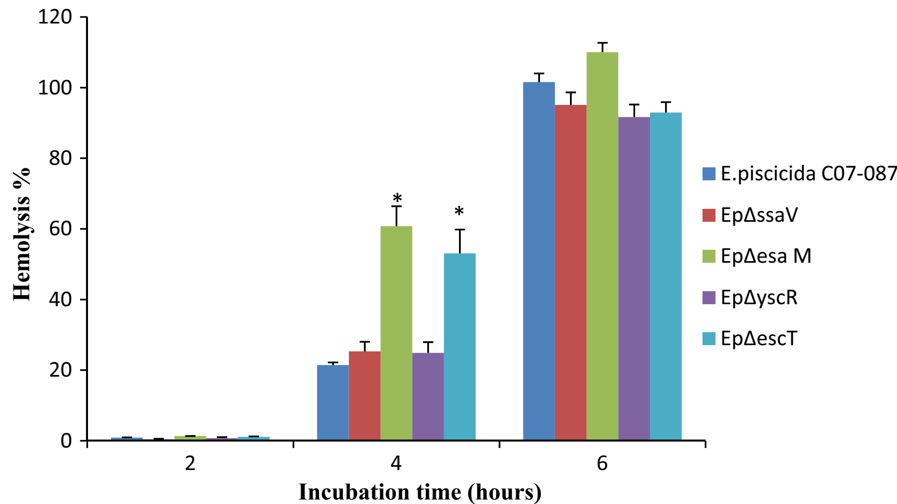

FIGURE 3 Percent haemolysis of *Edwardsiella piscicida* C07-087 and mutant strains. Error bars indicate standard deviation for three triplicate samples. * indicates statistically significant difference compared to parent strain C07-087 ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

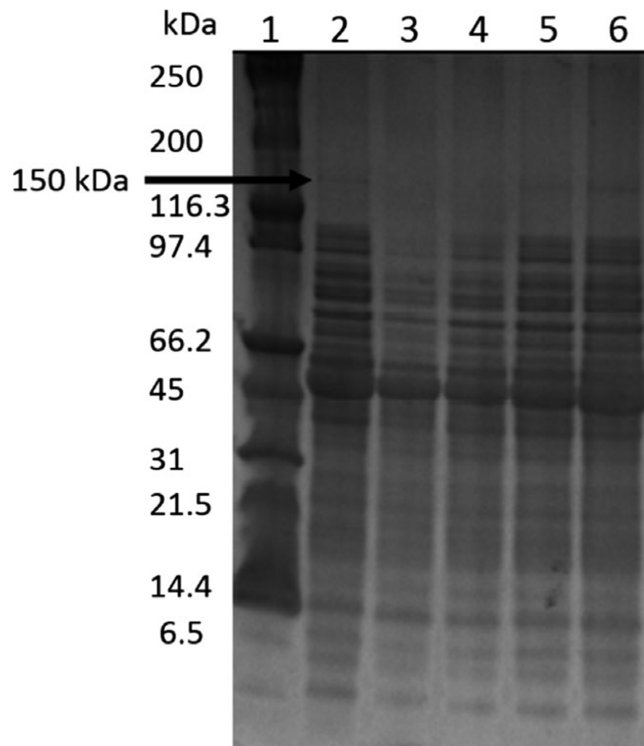

FIGURE 4 SDS–PAGE analysis of ECP of *Edwardsiella piscicida* strain C07-087 and T3SS mutants. Lane 1: Molecular weight marker; Lane 2: *E. piscicida* C07-087; Lane 3: *Ep*Δ*escT*; Lane 4: *Ep*Δ*esaM*; Lane 5: *Ep*Δ*yscR*; Lane 6: *Ep*Δ*ssaV*. The arrow refers to the approximately 150 kDa band missing in *Ep*Δ*escT* and *Ep*Δ*esaM*

3.8 | Virulence/vaccine efficacy of *E. piscicida* T3SS mutants

The LD_{50} of *E. piscicida* strain C07-087 was 3.3×10^5 CFU/fish, and infected fish died within 6 days with typical signs of haemorrhagic septicaemia. In the virulence comparison, significantly ($p < .05$) higher mortalities occurred in fish infected with parent strain C07-087 (26%) compared with mortalities in fish infected with strains *Ep*Δ*ssaV* (0%), *Ep*Δ*yscR* (0%) and *Ep*Δ*esaM* (6%) (Figure 6a). By contrast, there was no significant difference ($p > .05$) in mortalities between fish injected with *Ep*Δ*escT* (16%) and parent strain C07-087. To determine potential vaccine efficacy of *E. piscicida* T3SS mutants, fish were challenged with *E. piscicida* C07-087 by IP injection three weeks post-immunization. Fish were protected significantly ($p < .001$) after vaccination with all four mutants. After the second challenge, no mortalities occurred in fish vaccinated with *Ep*Δ*esaM*, *Ep*Δ*yscR* and *Ep*Δ*escT*, whereas *Ep*Δ*ssaV*-vaccinated group had some mortalities (6%) compared with 100% mortalities in sham-vaccinated group (Figure 6b).

4 | DISCUSSION

The newly described *Edwardsiella* species *E. piscicida* has been isolated from diseased fish of multiple species (Griffin et al., 2014; Katharios et al., 2015; Shafiei et al., 2016). T3SS is a nanosyringe that forms a channel across the bacterial membrane and the host cell membrane to transport proteins into the host cell. There are four types of T3SS proteins: apparatus, effectors, chaperons and regulators. The apparatus is complex and typically comprised of almost 25 proteins (Hueck, 1998). SsaV, YscR, EsaM and EscT are four T3SS apparatus proteins in the inner membrane that are collectively necessary for organization of the inner membrane rings. The individual functions of these proteins are still unclear (Galán, Lara-Tejero, Marlovits, & Wagner, 2014; Notti & Stebbins, 2016).

In *E. piscicida* C07-087, the *esaM* gene was annotated as encoding a hypothetical protein, but it showed 99.9% identity with a *E. tarda* EIB202 T3SS apparatus gene. The four genes encoding these proteins are located in adjacent divergently transcribed operons with *ssaV* and *esaM* located in one operon and *yscR* and *escT*

TABLE 4 Extracellular proteins of *Edwardsiella piscicida* identified in 150 kDa region of SDS–PAGE. Two strains (C07-087 and *Ep*Δ*escT*) are compared

Protein characterization					C07-087		*Ep*Δ*escT*	
Accession number (gi)	Proteins name	MW [kDa]	pI	aa	Peptides	Coverage	Peptides	Coverage
505274692	Flagellar biosynthesis protein	43.5	5.21	417	55	56.59	2	8.39
505274691	Flagellar biosynthesis protein	43.6	5.30	419	55	56.32	2	2.24
779983474	Flagellin	43.5	5.22	416	48	43.03	–	–
502611981	Multispecies: flagellin	43.7	5.22	416	43	43.51	–	–
779991656	DNA-directed RNA polymerase subunit beta	150.6	5.26	1,342	2	1.86	–	–
Total peptides					203		4	

(–) indicated absences of protein in *Ep*Δ*escT* mutant strain. (aa) is total amino acids in protein. (pI) is the isoelectric point of the protein.

located in another. These genes were selected for deletion to determine the effects of inner membrane rings on T3SS function and virulence. To our knowledge, these proteins have not been characterized in any *Edwardsiella* species, and in general they are not well studied. The current study also demonstrates that gene deletion methods developed for *E. ictaluri* are effective in *E. piscicida*.

Similar to previous studies on the *E. tarda* T3SS, disruption of the *E. piscicida* T3SS did not interfere with growth kinetics in BHI at 30°C (Lan et al., 2007; Wang, Mo et al., 2010). Disruption of genes encoding T3SS structural proteins also does not affect growth of plant pathogens on plant cells (Sun, Liu, & Bent, 2011). Using API20E strips, the four T3SS mutants were also biochemically homogenous and similar to parent strain C07-087.

The one exception to the biochemical homogeneity is that strain *Ep*Δ*esaM* showed lower production of H_2S, which was confirmed in lysine iron agar. The H_2S production test measures the ability of bacteria to reduce sulphur-containing compounds to sulphides during metabolism. For example, certain genera of Enterobacteriaceae are able to reduce tetrathionate during respiration as a terminal electron acceptor (as an alternative to oxygen). This is controlled by *ttr* genes, which in *Salmonella* are located within pathogenicity island 2 (SPI-2). SPI-2 also encodes a T3SS that facilitates pathogenesis of *Salmonella* (Figueira & Holden, 2012). Transcription of the *ttr* operon is controlled by a TtrS sensor and TtrR response regulator (Hensel, Hinsley, Nikolaus, Sawers, & Berks, 1999). It is possible that the difference in H_2S production in *Ep*Δ*esaM* is not due to a difference in effects on T3SS inner membrane ring function; it may be due to a secondary function of EsaM. Interestingly, although EsaM has no similarity with proteins encoded by any known *ttrRSBCA* genes, it has 33.7% identity with putative transcriptional regulator MerR in *Mycobacterium* sp. JLS. If EsaM has a secondary function as a transcriptional regulator, it may have a regulatory effect on the *ttr* operon.

Biofilm formation is important for bacterial survival in adverse environmental conditions, including escape from the host immune system (Wakimoto et al., 2004). Therefore, biofilm formation can contribute to disease pathogenesis and persistence in the host (Sundell & Wiklund, 2011). Biofilm formation requires multiple steps (Watnick & Kolter, 2000). In *P. aeruginosa*, T3SS is required for cell-associated aggregation on the surface of polarized epithelial cells, but it is not required for aggregation on abiotic surfaces (Tran et al., 2014). Therefore, it is possible that we did not detect significant differences in biofilm formation in our T3SS mutants because the assays were performed on plates.

Several virulence factors contribute to *E. tarda* pathogenicity including secretion systems, flagella, fimbriae, haemagglutinins, chondroitinases, haemolysins and iron scavenging system (Park, Aoki, & Jung, 2012; Wang et al., 2009; Wang, Wang et al., 2010). In the current study, we investigated effects of the selected T3SS apparatus genes on haemolytic activity of *E. piscicida*. Our results revealed that haemolysis requires 6 hr of incubation at 30°C in *E. piscicida*, which is similar to previously reported results from *E. tarda* (Watson & White, 1979). Interestingly, *Ep*Δ*esaM* and *Ep*Δ*escT* had increased haemolytic activity compared to parent strain C07-087. In *E. tarda* EIB202, a *ΔesrB* mutant strain had higher haemolytic activity than its parent strain (Wang, Wang et al., 2010) due to upregulation of EthA, a haemolysis-associated protein. Although *E. tarda* EsrB does not have any similarity to *E. piscicida* EsaM or EscT, it is possible that increased haemolysin activity in *Ep*Δ*esaM* and *Ep*Δ*escT* is due to a similar effect in *E. piscicida*.

Virulent and avirulent strains of *E. tarda* have differences in extracellular protein expression (Tan et al., 2002). We expected that mutation of T3SS genes in *E. piscicida* could affect ECP expression. On SDS–PAGE of ECPs, *Ep*Δ*escT* and *Ep*Δ*esaM* mutants showed a missing protein band with a molecular mass of approximately 150 kDa. Analysis of the 150 kDa region by band excision, in-del

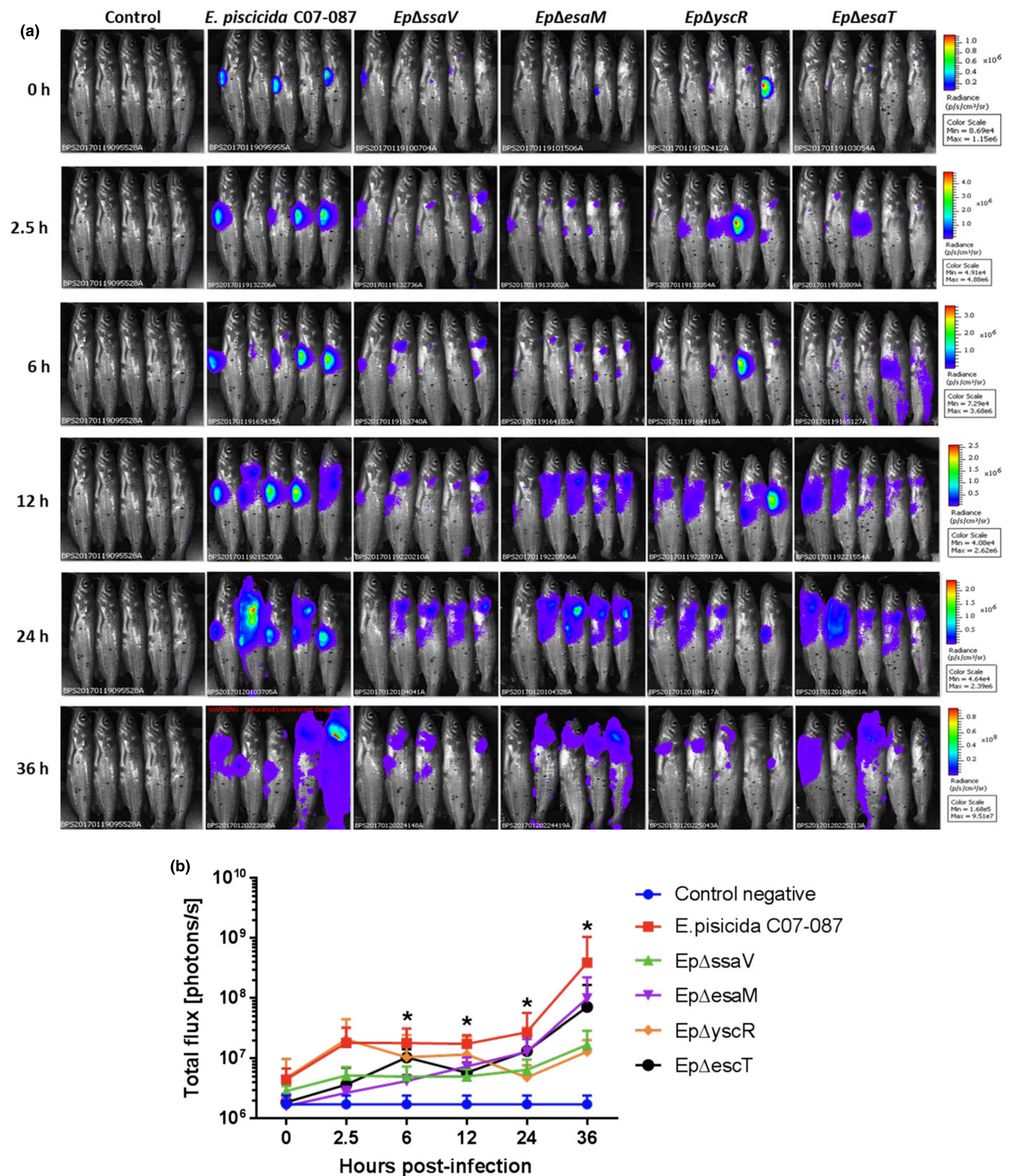

FIGURE 5 Bioluminescent imaging of live catfish after IP injection. (a) BLI of catfish. (b) mean total photon emissions at each time point (S = second; Sr = steradean). *indicates significant difference between *Edwardsiella piscicida* C07-087 and mutant strains [Colour figure can be viewed at wileyonlinelibrary.com]

digestion and LC-MS/MS showed high abundance of flagellin proteins in *E. piscicida* C07-087, while only traces of flagellin were detected in *EpΔescT*. Theoretical Mr of the flagellins is given as 43.5–43.7 kDa, which is in discrepancy with experimentally observed Mr (~150 kDa). However, unless steps are taken in the protein isolation to disaggregate flagellins into monomers, it is common that

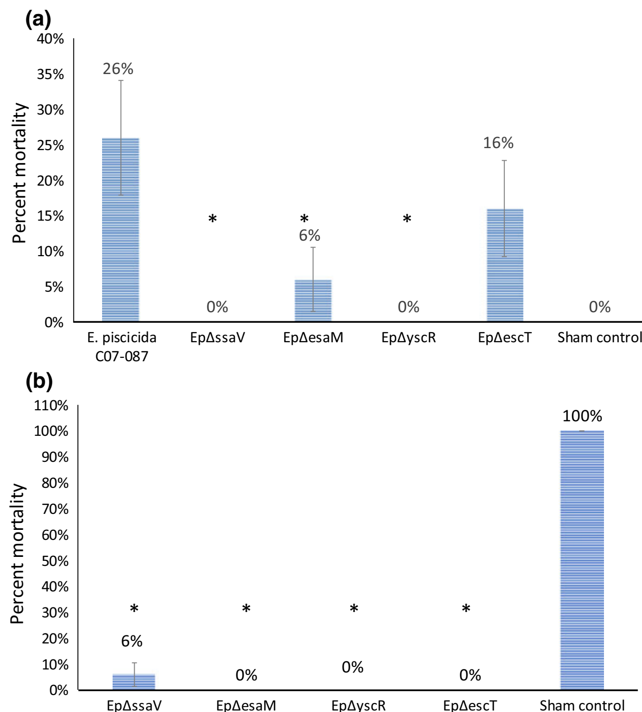

FIGURE 6 (a) Percent mortalities of catfish fingerlings challenged with *Edwardsiella piscicida* C07-087 and T3SS mutant strains.
(b) Percent mortalities of channel catfish fingerlings vaccinated with the T3SS mutants and challenged with wild-type strain C07-087 at 21 days post-vaccination. Data are presented as means $\pm$ *SE*.
*indicates significant difference compared to parent strain C07-087 [Colour figure can be viewed at wileyonlinelibrary.com]

flagellin proteins can exist in multimeric form (Simon et al., 2014), so it is plausible that flagellin trimer(s) would be detectable in the 150 kDa range. In *Yersinia*, T3SS apparatus protein YscT is linked with flagella formation (Ghosh, 2004). EscT is homologous to YscT, so it is not surprising that EscT would participate in *E. piscicida* flagellar formation.

Although *E. piscicida* C07-087 and T3SS mutants display close phenotypic characteristics, virulence comparison in channel catfish showed significant attenuation of *Ep*Δ*ssaV* and *Ep*Δ*yscR* compared to the parent strain. Mortalities for the fish challenged with strain C07-087 were lower than expected (26%) despite using a dose higher than our calculated LD_{50}, but both *Ep*Δ*ssaV* and *Ep*Δ*yscR* caused no mortalities. Moreover, bioluminescence imaging illustrated that *Ep*Δ*ssaV* and *Ep*Δ*yscR* were able to invade, establish infection and then be cleared without causing mortalities. Low mortalities were observed in catfish injected with strains *Ep*Δ*esaM* (6% mortality) and *Ep*Δ*escT* (16% mortality) compared to parent strain C07-087 (26% mortality). However, bioluminescence imaging showed that these two mutations have significant impact on *E. piscicida* during earlier stages of infection, but by 24 and 36 hr post-infection, these mutants had similar tissue quantities as the parent strain. Finally, our second challenge with parent strain C07-087 indicated that the four mutant strains provided significant protection against wild-type infection. Altogether, *Ep*Δ*ssaV*, *Ep*Δ*esaM* and *Ep*Δ*yscR* are safe and provided protection, suggesting they may be viable live attenuated vaccine candidates.

In conclusion, four T3SS mutants (*Ep*Δ*ssaV*, *Ep*Δ*esaM*, *Ep*Δ*yscR* and *Ep*Δ*escT*) were successfully constructed by homologous recombination and allelic exchange, illustrating that the methods used for deletion mutation of *E. ictaluri* are effective in *E. piscicida*. Phenotypic comparisons showed some minor differences in the T3SS mutants in H_2S production, haemolysin activity and flagellin expression. Importantly, all four mutants provided significant protection against infection with the virulent strain C07-087. Strains *Ep*Δ*ssaV* and *Ep*Δ*yscR* were the safest and caused no vaccination mortalities, suggesting they may have potential as live attenuated vaccines.

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