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An invasive and low virulent *Edwardsiella tarda esrB* mutant promising as live attenuated vaccine in aquaculture

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Abstract *Edwardsiella tarda* is a leading fish pathogen haunting worldwide aquaculture industry. In *E. tarda*, two-component system EsrA-EsrB positively regulates type III and VI secretion systems (T3SS and T6SS) and negatively regulates hemolysin EthA, which has been demonstrated to be essential for the invasion processes in fish. In order to develop a live attenuated vaccine (LAV) with high invasiveness to be practically and economically used as immersion-administered vaccine in aquaculture, here, we generated a random mutation library of *esrB* sequences by error-prone PCR and introduced them into the *E. tarda esrB* deletion mutant. The mutant YWZ47 with significantly increased hemolytic activity and low T3SS and T6SS secretion was screened. Phenotypes including extracellular protein profiles, invasion in macrophages, lethality toward fish, and infection kinetics were investigated in the wild-type strain EIB202 and the mutants Δ*esrB*, ΔT3SS, ΔT6SS, ΔT3SS/ΔT6SS, and YWZ47. Compared to the documented LAV strain Δ*esrB*, YWZ47 showed higher invasive capability and low in vivo virulence toward fish. Significantly higher relative percent survival (RPS) could be generated in turbot (*Scophthalmus maximus*) against the challenge of the wild-type EIB202 when inoculated through immersion route, and the RPS was comparable with that of Δ*esrB* through intraperitoneal (i.p.) injection inoculation. Two mutated points, K167M and H197L, were found by sequence analysis of $EsrB_{YWZ47}$ variant. These structural modifications underpin the variations in the regulatory functions of the mutant and wild-type EsrB. This study promoted understanding of virulence regulation by EsrB in *E. tarda* and presented a promising candidate of invasive attenuated vaccine used in aquaculture industries.

Keywords *Edwardsiella tarda* · T3SS · T6SS · *esrB* · Invasive phenotype · Vaccine

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Introduction

Edwardsiella tarda is a Gram-negative, facultative aerobic pathogen which inhabits a broad range of hosts including fish, amphibians, reptiles, birds, mammals, and even humans (Abbott and Janda 2006). As the leading pathogen claiming severe economic losses in aquaculture, *Edwardsiella tarda* causes systemic hemorrhagic septicemia, namely edwardsiellosis, in both freshwater and marine fish. During the process of infection in fish, *Edwardsiella tarda* colonizes in various organs, such as liver, spleen, kidney, and intestine in both extracellular and intercellular niches (Mohanty and Sahoo 2007).

Among the dozens of defined virulence determinants in *Edwardsiella tarda*, its pathogenicity mainly depends on functional type III secretion system (T3SS), type VI secretion system (T6SS), and hemolysin EthA that facilitates the host invasion processes (Leung et al. 2012; Park et al. 2012). T3SS is known to be essentially involved in *Edwardsiella tarda* survival and growth in phagocytes, and the related mutants are deficient in infecting the host (Srinivasa Rao et al. 2004; Tan et al. 2005). In *Edwardsiella tarda*, secreted T3SS translocon proteins EseB, EseC, and EseD might constitute a molecular syringe channeling specific effector proteins into the host cells in a way similar to their homologs (Srinivasa

Rao et al. 2004). T6SS also plays an important role in pathogenicity of *Edwardsiella tarda* (Zheng and Leung 2007; Wang et al. 2009a, b), which requires *evpA* and *evpB*, homologs of *vipA* and *vipB* in *Vibrio cholerae* (Bönemann et al. 2009; Basler et al. 2012), to facilitate the dynamic process of this specific secretion system. Moreover, it has been demonstrated that iron-regulated hemolysin EthA is essential for host cell adherence and internalization processes in *Edwardsiella tarda* (Hirono et al. 1997; Wang et al. 2010) and thus is regarded as the essential element for initial host invasion process.

Two-component systems (TCSs) are essential for bacterial pathogens to sense and response to the stress signals from environment and host. *Edwardsiella tarda* genome encodes 33 TCSs (Lv et al. 2012; Wang et al. 2009a, b), and EsrA-EsrB, homologs to SsrA-SsrB in *Salmonella* (Zheng et al. 2005), represents as primary virulence regulator controlling the expression of major virulence determinants, i.e., T3SS, T6SS, and hemolysin EthA in vivo and in vitro (Wang et al. 2009a, b, 2010; Zheng et al. 2005). While displaying upregulated EthA expression, *esrB* deletion mutant is defective in T3SS and T6SS and is significantly attenuated in vivo, demonstrating as an ideal live attenuated vaccine (LAV) candidate against *Edwardsiella tarda* infection in turbot (Mo et al. 2007). However, it still remains unclear how EsrB orchestrates these virulent determinants to optimize its in vitro and in vivo infection processes. At the same time, *esrB* mutant has to be intraperitoneally (i.p.) administered to mount high protective efficacy in turbot (Mo et al. 2007), and significant low protection was observed when inoculated via immersion route (our unpublished data) that is widely accepted as a labor-saving and cost-effective vaccination method for flatfish.

Aiming at improving understanding of EsrB regulation on pathogenesis and screening invasive LAV candidates that can be used as immersion vaccine in aquaculture industry, we here generated a library of *esrB* variants by error-prone PCR and investigated the roles of these gene variants in regulating major virulence determinants in the context of Δ*esrB* mutant strain. Two strains were obtained with dramatically shifted expression patterns of T3SS, T6SS, and hemolytic activity, and one of the strains intriguingly displayed high protection rate when administered by immersion route. This study will facilitate dissecting the regulation mechanism of EsrB on the virulence determinants in *Edwardsiella tarda* and developing a promising invasive and low virulent vaccine.

Materials and methods

Bacterial strains, plasmids, and media

The bacterial strains used in this study are listed in Table 1. *Edwardsiella tarda* strains were routinely grown in tryptic soy broth (TSB), tryptic soy agar (TSA), or Dulbecco's modified essential medium (DMEM) at 30 °C, while *Escherichia coli* strains were cultured in Luria broth (LB) at 37 °C. *Escherichia coli* Top10F′ and *Escherichia coli* cc118 *λpir* were used for plasmid harvest. TSB containing 20 % (*v*/*v*) glycerol was used as a medium for microbe preservation. When necessary, colistin (Col), ampicillin (Amp), chloramphenicol (Cm), and kanamycin (Km) were supplemented at final concentrations of 10, 100, 25, and 50 μg/ml, respectively.

Construction of site-directed mutants

In order to construct an *evpAB* site-directed deletion mutant, PCR amplification was performed to generate the upstream and downstream fragments of *evpAB* with primer pairs evpABup-for/evpABup-rev and evpABdown-for/evpABdown-rev, respectively (Table 2). The PCR products were then obtained with next run of overlap PCR with primer pair evpABup-for/evpABdown-rev, generating a PCR product containing in-frame deletion fragment from 7 to 516 bp in *evpA* and from 1 to 583 bp in *evpB*. The overlap product was sequenced and cloned into the *Bgl*II/*Sph*I sites of the suicide vector pDMK (Table 1), which carried R6K *ori*, *sacB* sucrose-sensitivity gene, Cm-resistance gene, and Km-resistance gene. The resulting plasmid pDMKdevpAB (Table 1) was transformed into *Escherichia coli* cc118 *λpir* and *Escherichia coli* SM10 *λpir*. Then, the plasmid was mated from *Escherichia coli* SM10 *λpir* into *Edwardsiella tarda* EIB202 by conjugation. The unmarked in-frame deletion mutants were selected in two sequential homologous recombination processes on TSA medium containing Col, Cm, and Km and then on TSA with 10 % (*v*/*v*) sucrose. The targeted in-frame deletion mutants were confirmed by sequencing of the deleted region, and it was designated as ΔT6SS. The ΔT3SS/ΔT6SS double mutation strain was obtained by another two runs of counter selection using ΔT6SS mutant and *Escherichia coli* SM10 *λpir* harboring plasmid pDMK-TED (Xiao et al. 2013) (Table 1).

Construction of *esrB* deletion and complement strains

Error-prone PCR was performed to generate an *esrB* mutant library with primer pair esrB-for1/esrB-rev targeting the open reading frame (ORF) region of *esrB* gene (Table 2). Error-prone PCR is similar to routine PCR in procedure but with different reaction mixture. In brief, in a routine PCR reaction mixture of 50 μl, the following regents were added: 2 μl Taq polymerase (5000 U/ml) (New England Biolabs, Beijing), 2 μl $MnCl_2$ (10 mM), 2.4 μl $MgCl_2$ (10 mM), 0.4 μl dCTP (100 mM) (NEB, Beijing), and 0.4 μl dTTP (100 mM) (NEB, Beijing). The pool of error-prone PCR product was ligated with the ~600-bp promoter region of *esrB* gene with overlapping PCR with primer pairs esrB-for/esrB-rev1 and esrB-for/esrB-rev and *pfu* polymerase. The PCR amplification products

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Reference
Edwardsiella tarda		
EIB202	Wild-type strain, CCTCC M208068, Col^r, Cm^r	Xiao et al. 2008
Δ*esrB*	EIB202, in-frame deletion of *esrB*, Col^r, Cm^r	Lv et al. 2012
esrB$^+$	Δ*esrB* containing pUTatesrB, Col^r, Cm^r, Amp^r	This study
ΔT3SS	EIB202, deletion of *eseB-eseD*, Col^r, Cm^r	This study
ΔT6SS	EIB202, deletion of *evpA-evpB*, Col^r, Cm^r	This study
ΔT3SS/ΔT6SS	EIB202, deletion of *eseB-eseD* and *evpA-evpB*, Col^r, Cm^r	This study
YWZ15	Δ*esrB*, with pUTat containing *esrB* variant obtained by error-prone PCR, CCTCC M453679, Col^r, Cm^r, Amp^r	This study
YWZ47	Δ*esrB*, with pUTat containing *esrB* variant obtained by error-prone PCR, CCTCC M453680, Col^r, Cm^r, Amp^r	This study
Escherichia coli		
Top10F'	F'[lacIq Tn10(TetR) *mcr* Φ180 *lacZ* Δm15Δ*lac* X74 *deoR recA*]	Invitrogen
cc118 *λpir*	*λpir* lysogen Δ(*ara-leu*) *araD* Δ(*lacX74*) *phoA20 thi-1 rpoB argE* (*am*) *recA1*	Dennis and Zylstra 1998
SM10 *λpir*	*thi thr leu tonA lacy supE recA::RP4-2-Tc::Mu λpir* Kan^r	Liang et al. 2003
Plasmids		
pMD19-T	PCR cloning vector, Amp^r	TaKaRa
pUTat	Medium copy number cloning vector, pAT153 replicon, Amp^r	Xiao et al. 2011
pDMK-TED	pDMK derivative containing 2208-bp deletion in the *eseB-eseD* locus, Cm^r, Kan^r	Xiao et al. 2013
pDM4	Suicide plasmid, *pir* dependent, R6K, SacBR, Cm^r	Wang et al. 2002
pmCherry	mCherry gene carrying vector	Clontech
pDMK	pDM4 derivative with Km resistance, Km^r, Cm^r	Xiao et al. 2009
pUTatesrB	pUTat derivative containing *esrB*, Amp^r	This study
pDMKdevpAB	pDMK derivative containing 1089-bp deletion in the *evpAB* locus, Cm^r, Km^r	This study
pUTatRFP	pUTat derivative containing fragment of P_{BAD}-*mCherry*, Amp^r	This study
pUTat15RFP	pUTat derivative encoding $EsrB_{YWZ15}$ and P_{BAD}-*mCherry*, Amp^r	This study
pUTat47RFP	pUTat derivative encoding $EsrB_{YWZ47}$ and P_{BAD}-*mCherry*, Amp^r	This study

were digested with *Bam*HI and *Pst*I and then cloned into the medium copy vector pUTat (Xiao et al. 2011) treated with the same enzymes. The derivative plasmid was then electroporated into Δ*esrB* mutant (Table 1). The transformants were selected on LB agar containing Col and Amp and were confirmed by routine PCR with primer pair M14-for/M14-rev (Table 2). The *esrB* complement strain was obtained by a similar way with routine PCR and *pfu* polymerase (Thermo Fisher Scientific, China) to amplify the authentic *esrB* gene. The complement strain was designated as *esrB*$^+$.

Construction of RFP-labeled strains

For construction of red fluorescence protein (RFP)-labeled strains, a plasmid pUTat RFP expressing mCherry was constructed. Plasmids pBAD33 and pmCherry (Table 1) were used for PCR amplification of P_{BAD} and *mCherry* gene fragments with primer pairs P_{BAD}-for/P_{BAD}-rev and RFP-for/RFP-rev (Table 2), respectively. The P_{BAD} fused to *mCherry* gene fragment was then obtained in next run of overlap PCR with primer pair P_{BAD}-for/RFP-rev, which was then cloned into the *Eco*RI/*Hin*dIII sites of the vector pUTat (Table 1). The recombinant plasmid was electroporated into competent cells of EIB202, Δ*esrB*, ΔT3SS, ΔT6SS, and ΔT3SS/ΔT6SS. For the RFP labeling of strains YWZ15 and YWZ47, the P_{BAD}-*mCherry* gene fragment was further fused to the corresponding *esrB* mutant alleles by a second run of overlap PCR with primer pair of RFPfuse-for/RFP-rev and then cloned into the plasmid pUTat. The plasmids were electroporated into the Δ*esrB* competent cells.

Hemolytic activity assay

β-Hemolytic activity of each strain was tested on 1.5 % agar base supplemented with 5 % (*v*/*v*) sheep blood. Ten-microliter cell suspension of each strain was inoculated in the hole punched on the agar and incubated at 30 °C for 24 h. The diameter of a clear colorless zone surrounding the hole indicated β-hemolytic activity of the strains (Chen et al. 1996).

Quantitative hemolytic activity assay was also performed. The sheep blood was added into 10 ml of fresh M9 medium at a final concentration of 5 % (*v*/*v*) to make a hemolytic activity

Table 2 Primers used in this study

Name	Sequence (5′ to 3′)
esrB-for	CGGGATCCTCCCAATCGCATGACACAAGGCACC
esrB-rev1	AATAGAAATAGTCATATTTAAAGGGTACTCCGA
esrB-for1	TCGGAGTACCCTTTAAATATGACTATTTC
esrB-rev	AACTGCAGTTAAAACTCCAGAACCCCCAGGCGG
M14-for	CGCCCAATACGCAAACCGCCTCT
M14-rev	TTCTCTCATCCGCCAAAACAGCC
evpABup-for	GAAGATCTTAGTTCATTGGCAAGGTC
evpABup-rev	GCCGAACATTCCTGCGCTGGTGCCATGAAGTCATCTCCGT
evpABdown-for	ACGGAGATGACTTCATGGCACCAGCGCAGGAATGTTCGGC
evpABdown-rev	GGCATGCTTGGCGGAGTCACTGTCAT
P_{BAD}-for	CGGAATTCTTATGACAACTTGACGGCTACATCA
P_{BAD}-rev	TATCCTCGCCCACCATGGTTAATTCCTCCTGTTAGCCCAAAA
RFP-for	ATGGTGGGCGAGGATAGCGTGCTGA
RFP-rev	CCCAAGCTTGCTGTGACCCAGCTTGCTCGGCAGG
RFPfuse-for	AACTGCAGTTAAAACTCCAGAACCCCCAGGCGG

assay medium. Overnight bacterial culture was adjusted to optical density at 600 nm (OD_{600}) of 0.1, and 100 μl was taken to inoculate into the hemolytic activity assay medium and the blank M9 medium, respectively, and then to incubate at 30 °C. One hemolytic unit (HU) was defined as the amount of hemolysin that would produce 1 % hemolysis. Triton X-100 was added into the hemolytic activity assay medium at a final concentration of 1 % (*v*/*v*), and the supernatant of culture was determined spectrophotometrically at OD_{540}. Each sample was tested at 3-h intervals. Five hundred microliters of the supernatant in hemolytic activity assay medium was collected by centrifugation at 5000×*g* for 5 min for OD_{540} test, while 500 μl of the bacterial culture in blank M9 medium was used for OD_{600} test. The hemolytic activity of each strain was calculated by dividing the HU value by the OD_{600} value (Watson and White 1979).

ELISA

The cell lysis was added into 96-well plate with 100 μl per well and incubated at 4 °C overnight, and followed by washing with PBST for three times and blocking with 1 % (*v*/*v*) BSA at 37 °C for 1.5 h. After washing, 100 μl of diluted specific antibody (1:5000) was added into each well, and the plate was incubated at 37 °C for another 1 h before chromogenic reaction with 100 μl of soluble TMB for 15 min. OD_{450} values of the wells were assayed after termination of the reaction with 100 μl of 2 M sulfuric acid.

ECP preparation and Western blotting analysis

Each strain was inoculated into 50 ml of DMEM medium and cultured at 30 °C for 24 h. The cell-free supernatant was collected with addition of protease inhibitor. The solution was filtered through a 0.22-μm low protein-binding Millex filter (Merck Millipore, Bedford, MA, USA) and then concentrated by Amicon Ultra-15 centrifugal filter devices with a 10-kDa molecular weight cut-off (Millipore). Extracellular proteins (ECP) concentration was determined by a Bradford protein assay kit and normalized to 1 mg/ml according to the manufacturer's instructions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the same loaded amount of ECP for the strains (Lv et al. 2012).

For Western blotting analysis, SDS-PAGE-separated proteins were transferred onto PVDF membrane (Milipore) and probed with anti-EvpC or anti-EsrB rabbit antisera (GL Biochem, Shanghai, China). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:2000 dilution as a secondary antibody. The bands were developed with the TMB substrate (Amresco, Solon, OH, USA).

Invasion efficacy of *Edwardsiella tarda* in macrophage *J774A.1*

Invasion efficacy of *Edwardsiella tarda* strains in macrophage *J774A.1* was assayed as previously described (Wang et al. 2009a, b). Macrophage *J774A.1* cells treated with 0.25 % (*v*/*v*) trypsin and 0.02 % (*w*/*v*) EDTA were seeded into 24-well plate incubating at 35 °C with 5 % (*v*/*v*) CO_2. Each strain was inoculated into LB medium and incubated at 30 °C to OD_{600} as 0.6. Then, the culture was spectrophotometrically adjusted to OD_{600} as 1 and sampled for plate counting. Each strain was added into the corresponding well with a multiplicity of infection (moi) of 10:1 and incubated for 2 h. Then, the

bacterial cells were killed with gentamicin at a final concentration of 100 μg/ml in the culture medium for 2 h (time point 0). For the bacterial time course assay, the infected monolayers were incubated for a further 0–20 h in the medium containing gentamicin. The cells were then harvested and treated with 500 μl of 1 % (*v*/*v*) Triton X-100 for 30 min. The lysate was serially diluted and plated onto TSA agar in triplicate containing Col. The bacterial count in each cell was calculated by dividing the mean of three wells in triplicate experiments by the cell count.

RFP-labeled strains were used to visualize the invasion capacity of each strain in macrophage. When adding bacterial culture into the well, arabinose was simultaneously added into the medium at a final concentration of 0.02 % (*w*/*v*). The photo of macrophages in bright field was merged with that of bacteria in fluorescence field correspondingly.

Median lethal dose (LD_{50}) assay in turbot

The LD_{50} values of all strains were determined in turbot (Han et al. 2006). Healthy turbot that weighed 30±3 g was obtained from a commercial fish farm. After acclimatized for more than 1 week at 16 °C, all fish were infected intramuscularly (i.m.) with different *Edwardsiella tarda* strains. The mortalities were recorded over a period of 4 weeks after infection. The LD_{50} values were calculated by the method described by Fernández et al. (1995).

Determination of infection dynamics of *Edwardsiella tarda* in turbot

For in vivo infection kinetics investigation (Ling et al. 2000), each strain was spectrophotometrically adjusted to OD_{600} as 1 and sampled for enumeration by plate-count method. The fish in treatment group were i.m. challenged with 100 μl of bacterial suspension (≈1×10^6 cfu/ml), while those in control group were injected with 100 μl PBS. Three fish from each group were then sampled at 0, 1, 3, 5, 7, 10, 14, 18, 22, and 28 days after inoculation. Liver, spleen, kidney, and intestine were aseptically removed and weighed to normalize the samples. Then, the organs were digested in trypsin and homogenized in 500 μl PBS. The homogenates were serially diluted and plated in triplicate onto DHL plates containing Col or Amp, and the plates were incubated at 30 °C for 48 h. The colonies that featured with black centers were counted.

Vaccination and challenge

Healthy turbot that weighted 30±3 g was randomly grouped with 30 fish per group. Fish were inoculated by intraperitoneal (i.p.) injection with doses of 10^6 colony-forming unit (cfu) in 0.1 ml of PBS or 0.1 ml PBS as control. For immersion vaccination, fish were bathed for 1 h in aerated seawater with the cell concentration at 1×10^7 cfu/ml or aerated seawater alone. Fish were kept in 100-l tanks supplied with aerated sand-filtered seawater at 16 °C. At 35 days postvaccination, the fish were evenly sub-grouped into ten fish per container and i.m. challenged with wild-type *Edwardsiella tarda* EIB202 at a dose of 2×10^4 cfu as determined by plate count method. The relative percent survival (RPS) was calculated according to the formula RPS=[1−(% mortality in vaccinated fish/% mortality in control fish)]×100 % (Xiao et al. 2013). Six experiments were performed. In the abovementioned animal experiments, microbiological methods were routinely used to confirm that no fish were carrying *Edwardsiella tarda* before infection, and all the fish deaths were caused by *Edwardsiella tarda*.

Homology modeling

A molecular model for the EsrB variant in YWZ47 strain ($EsrB_{YWZ47}$) was generated using the one-to-one threading protocol implemented in Phyre2 (Kelley and Sternberg 2009), based on the structure of a NarL family response regulator spr1814 from *Streptococcus pneumoniae* (PDB ID: 4HYE). The overall sequence identity between EsrB and spr1814 is 31 %, and the protein coverage is 93 % (199/214) with prediction confidence of 100 %. The resolved $SsrB_C$ (2JPC) structure (Carroll et al. 2009) was also used for the analysis for the C-terminal domain of EsrB and its variants.

Statistical analysis

All statistical analyses were performed with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). The unpaired *t* test (for RPS) and one-way ANOVA following post hoc Turkey test (for hemolytic activities, enzyme-linked immunosorbant assay (ELISA), and in vivo survival kinetics) were chosen to analyze the difference level of the data set with $P<0.01$ as statistical significance.

Nucleotide sequence accession numbers

The gene sequences encoding $EsrB_{YWZ15}$ and $EsrB_{YWZ47}$ were submitted to GenBank and were assigned accession numbers as KM267086 and KM267087, respectively.

Results

Screening for *esrB* variants

Here, error-prone PCR was used to introduce the random mutations in the *esrB* sequence with a rationale that some *esrB* variants might be able to produce higher hemolytic

activity but low or no T3SS and T6SS secretion in the background of Δ*esrB* mutant (Wang et al. 2010; Lv et al. 2012). Sequencing results of some mutated gene fragments showed that the error-prone PCR mutation rate was approximately 3.8 nucleotides per sequence with 1.01 % mutation frequency in each site which evenly distributed on the whole ORF of *esrB* gene (data not shown), indicating that the error-prone PCR protocol used in this work was efficient as compared to the previous report (Cadwell and Joyce 1994).

After cloning the pool of the mutated sequences into a stable plasmid pUTat (Xiao et al. 2011) and transforming into Δ*esrB* strain (Table 1), we screened the *esrB* variants generating high hemolytic activity but inefficient secretion of T3SS and T6SS (Lv et al. 2012). In this study, sequential runs of screening with qualitative hemolytic activity assay on agar plate and quantitative hemolytic activity assay were performed. The complementation of an intact wild-type *esrB* gene into Δ*esrB* mutant (*esrB*$^+$) could decrease the hemolytic activity of the strain as previously described (Wang et al. 2010). This complementation strain was used as a screening control in these assays. Out of 132 *esrB*-allele harboring positive clones, only eight strains possessed hemolytic activity different from those of EIB202 (data not shown), and two strains (YWZ15 and YWZ47) showed significantly heightened hemolytic activity as compared with Δ*esrB* strain (Fig. 1). Compared to the Δ*esrB*, YWZ15 and YWZ47 increased their hemolytic activities by 25–28 % in the qualitative assays and by 42–68 % in the quantitative assays (Fig. 1), suggesting that they might possess enhanced invasion capacities toward hosts (Hirono et al. 1997; Wang et al. 2010; Dong et al. 2013).

ELISA experiment was also performed with specific antibodies against EseB and EvpC to investigate the secretion capacities of T3SS and T6SS, respectively, in the selected 132 strains. The results indicated that most of the strains (108/132, 81.8 %) shared the same T3SS/T6SS secretion patterns as the wild-type EIB202, while minority of candidate strains (24/132, 18.2 %) including YWZ15 and YWZ47 displayed significant secretion deficiency of T3SS or T6SS (Fig. 1a). To confirm this, their ECP profiles were further investigated with SDS-PAGE (Fig. 1b) and Western blotting against EvpC (Fig. 1c) and EsrB (Fig. 1d). *Edwardsiella tarda* EIB202 yielded six major extracellular proteins of T3SS (EseB, EseC, and EseD) and T6SS (EvpI, EvpP, and EvpC) when cultivated in DMEM medium (Lv et al. 2012), and the corresponding protein bands could not be found in ΔT3SS, ΔT6SS, and ΔT3SS/ΔT6SS mutants (Fig. 1b). While YWZ47 displayed extracellular protein profiles identical to that of Δ*esrB*, YWZ15 intriguingly showed the missing or decreased secretion of T6SS or T3SS proteins, in particular the significant decrease of EvpC secretion (Fig. 1b, c). Western blotting experiment with antiserum against EsrB indicated that all the strains except for Δ*esrB* strain could express the comparable amount of EsrB or variant proteins. These results indicated that the EsrB variant in YWZ15 ($EsrB_{YWZ15}$) showed different capacities in regulating T3SS and T6SS protein production.

To sum up, YWZ15 and YWZ47 were the two strains with the enhanced hemolytic activity and the appropriately weakened production capacity of secreted T3SS or T6SS proteins.

Invasion efficacy of the mutants in *J774A.1* macrophage

Previous work has demonstrated that *Edwardsiella tarda* is capable of invading and replicating in murine macrophage (Wang et al. 2010; Okuda et al. 2006). When the RFP-labeled strains were used to infect the macrophage model *J774A.1*, three invasion patterns for these strains could generally be observed: (i) high invasion rate with high cell lysis rate for EIB202 and YWZ15 (Fig. 2a); (ii) significantly impaired invasion rate with low cell lysis rate for ΔT6SS, ΔT3SS, and ΔT3SS/ΔT6SS (Fig. 2b); and (iii) high invasion rate with low cell lysis rate for Δ*esrB* and YWZ47 (Fig. 2c). The intracellular bacteria with the passage of time also demonstrated that T3SS and T6SS mutants were inefficient to invade the macrophage, and the wild-type EIB202 and YWZ15 could efficiently infect the macrophage cells in the early stage of infection (8 h) although the intracellular bacterial counts dramatically decreased in the late stages of infection, which might be resulted from the rapid cell lysis and release of the bacteria from the cells and then being killed by the antibiotics in the medium (Fig. 2d). In contrast, Δ*esrB* and YWZ47 not only infected macrophage cells with high efficiency but also kept stable survival inside the cells (Fig. 2d). In addition, when compared to the wild-type EIB202 and Δ*esrB*, YWZ15 and YWZ47 showed significantly higher initial invasion capacities (as of time point 0 in Fig. 2d) (Fig. 2e). Taken together, YWZ15 and YWZ47 displayed different patterns of macrophage invasion capabilities.

Pathogenicity and propagation dynamics of the mutants in turbot

Turbot model was further used to test the pathogenicity and the propagation ability of strains YWZ15 and YWZ47 (Fig. 3). LD_{50} assays through i.m. injection route indicated that EIB202 was highly virulent toward turbot with an LD_{50} value of 4.51×10^1 cfu/g, and ΔT3SS and ΔT6SS mutants showed 10^3- and 10^2-fold attenuation, respectively, indicating that T3SS contributed more to the pathogenesis in fish than T6SS (Table 3). In line with the T3SS and T6SS secretion patterns (Fig. 1b), Δ*esrB*, ΔT3SS/ΔT6SS, and YWZ47 were highly attenuated (over 10^5-fold). Intriguingly, YWZ15 was found to be highly pathogenic toward turbot with an LD_{50} value of 1.73×10^2 cfu/g (Table 3), suggesting that the $EsrB_{YWZ15}$ protein might be fully potentiated to activate the expression of T3SS besides T6SS in vivo in fish (Fig. 1b).

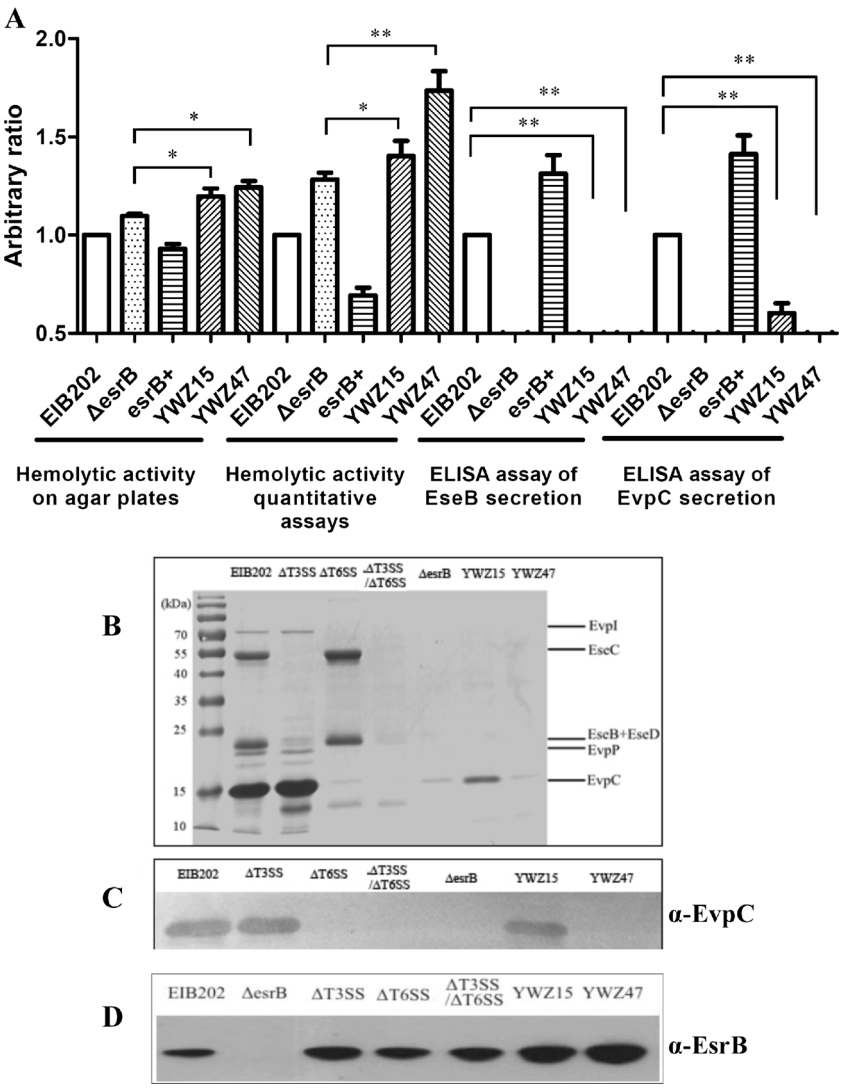

Fig. 1 **a** Hemolytic activity assays and ELISA screening of the *E. tarda* strains expressing wild-type or *esrB* variants. All the data of hemolytic activities on agar plats or quantitative hemolytic activity assays, as well as of the ELISA read-out, were normalized by wild-type EIB202, which was arbitrarily set as 1. For ELISA, specific antibodies anti-EseB and anti-EvpC diluted at the ratio of 1:4000 and 1:10,000 were used, and read-out was calculated by interpolation according to the corresponding OD_{450}. **b**, **c** Extracellular protein profiles of the *E. tarda* strains. T3SS and T6SS proteins were as indicated (Lv et al. 2012). Note that a weak non-EvpC band with similar molecular weight of EvpC appeared for the strains as compared to **c** Western blotting of the strains by using anti-EvpC specific antiserum. **d** Western blotting analysis of cell lysis of the strains with anti-EsrB specific antiserum. All the experiments were triplicated and recorded as mean±SD. **P*<0.01; ***P*<0.001 relative to wild-type EIB202 or Δ*esrB* as indicated

The infection dynamics of the wild-type and mutant strains in turbot organs (liver, spleen, kidney, and intestine) were then determined (Fig. 4). During the 4-week experiment, high burden of bacteria in all the organs of turbot infected with EIB202 and YWZ15 was detected, and all the fish died on the 15 to 19 day postinoculation. While 46 and 72 % of turbot infected with ΔT3SS and ΔT6SS died at the end of the 28-day observation, respectively, no fish death was observed for the groups infected with Δ*esrB*, ΔT3SS/ΔT6SS, and YWZ47. Generally, all of the five strains (Δ*esrB*, YWZ47, ΔT3SS, ΔT6SS, and ΔT3SS/ΔT6SS) transiently replicated in the fish organs, and among them, ΔT6SS showed the highest overall in vivo replication activity. ΔT3SS, together with ΔT3SS/ΔT6SS, was inefficient in propagation in those organs. These data further indicated that T3SS, as compared to T6SS, was the major virulence determinant in *Edwardsiella tarda*. YWZ47 showed high colonization capacities in different organs (Fig. 4). It is noticeable that the overall replication rate for YWZ47 was significantly higher (*P*<0.0001) than Δ*esrB* in the major immune organs (liver, spleen, and kidney), and in the intestine, there was no significant difference in colonization rate for the two strains.

The symptoms in fish were also observed following the infection of the wild-type and mutant strains. When infected with the wild-type EIB202 and YWZ15, the fish displayed typical signs of edwardsiellosis, manifesting as lethargy, darkening skin, subcutaneous hemorrhages, white feces, swelling, and bleeding in the sites of injection (Xiao et al. 2013). In contrast, the strains Δ*esrB*, ΔT3SS/ΔT6SS, and YWZ47 could not cause any sign of edwardsiellosis and turbots behaved as the healthy control fish (data not shown). These data demonstrated that YZW47 strain exhibited very low virulence respective to YWZ15, T3SS, or T6SS single deletion mutants and significantly higher infection capability respective to Δ*esrB*.

Potential of YWZ47 as an LAV

To further verify the potential use of YWZ47 as an LAV candidate, healthy turbot were vaccinated with YWZ47,

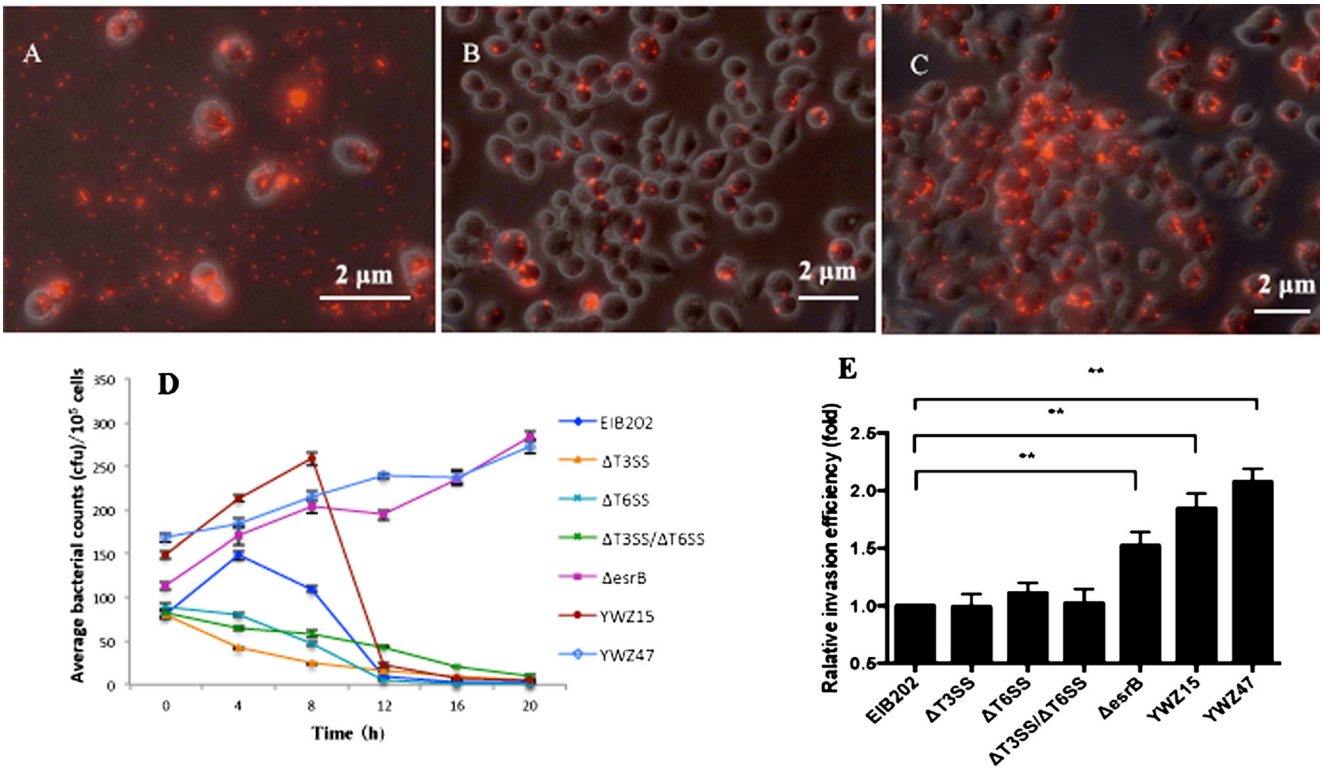

Fig. 2 Invasion capacities of *E. tarda* strains in macrophage *J774A.1*. The RFP-labeled strains were used to infect normalized macrophages at moi of 10:1 and incubated for 16 h, and the photos were taken by overlay of the photo in bright field and that in fluorescence field. The typical pictures showed high infection rates for EIB202, ΔT6SS, or YWZ15 (**a**), low invasion rates for ΔT3SS and ΔT3SS/ΔT6SS (**b**), and high infection rates of Δ*esrB* and YWZ47 (**c**) in macrophage. Note that the group of strains EIB202, ΔT6SS, or YWZ15 (**a**) caused higher cell lysis rate than that in ΔT3SS and ΔT3SS/ΔT6SS (**b**), and in Δ*esrB* and YWZ47 (**c**). **d** Invasion of *E. tarda* strains into *J774A.1* cells. Values are the mean±SD of three experiments with triplicate wells. The dynamic changes of average bacterial counts per 10^5 cells over time were shown. **e** Invasion efficacy of the strains at the time 0 (**d**) relative to wild-type EIB202

Δ*esrB*, or PBS via i.p. injection or immersion and incubated for 5 weeks followed by i.m. challenging with the wild-type EIB202. The results showed 100 % accumulated mortalities for the control fish (Fig. 4a, b). The mortality rates for the fish vaccinated with YWZ47 via i.p. injection and immersion were 22.2 and 35.5 % with the corresponding RPS of 77.8 and 64.4 %, respectively (Fig. 4c). Accordingly, the RPS of the fish vaccinated with Δ*esrB* via i.p. injection and immersion were 80 and 51.1 %, respectively (Fig. 4c). While there was no significant variation in the RPS of i.p. inoculated groups of YWZ47 and Δ*esrB* strains, significant difference ($P<0.001$) existed in the RPS between the immersion vaccinated groups of these two strains. These results further demonstrated that YWZ47 was featured with high invasion capability and low virulence and showed the potential to be a promising LAV. High level of protection could be achieved with a single dose of immersion or injection inoculation of YWZ47.

Homology modeling of the wild-type and EsrB variant

EsrB is a protein of 214 aa and belongs to NarL/FixJ superfamily proteins and contains two conserved domains, i.e., N-terminal REC superfamily domain and C-terminal LuxR-C-like domain (Fig. 5a). REC domain might be required for the dimerization and activation by phosphorylation with the existence of dimerization sites (L63, P64, M66, G68), dimerization interface (K110-S112), and a phosphorylation site (D60) (Fig. 5a). The putative three-dimensional structure of EsrB was predicted by homology modeling (Fig. 5b). Four alpha-helixes in C-terminal and six alpha-helixes in N-terminal as well as six beta-sheets were predicted inside the model. The structure of C-terminal (L152-R208) was highly similar to that of SsrB (Carroll et al. 2009), with four alpha-helixes (H1-H4) adjacent to each other to form the helix-turn-helix (H-T-H) DNA-binding domain. There were five substituted sites in EsrB alleles of YWZ15 ($EsrB_{YWZ15}$) and two in YWZ47 ($EsrB_{YWZ47}$) (Table 4). Most of the mutations took place in the loop structure of the EsrB sequences. In particular, both of the two substituted amino acids in $EsrB_{YWZ47}$ were located on loops between two helixes (helix 1–2 for H197L, helix 3–4 for K167M) in the C-terminal domain. In the two sequences, no significant modifications were observed except that YWZ15 lost one β-sheet structure because of the nearby mutation of P133S. However, the minor modifications might actually fine-tune the regulatory roles of EsrB on the virulence determinants in *Edwardsiella tarda*.

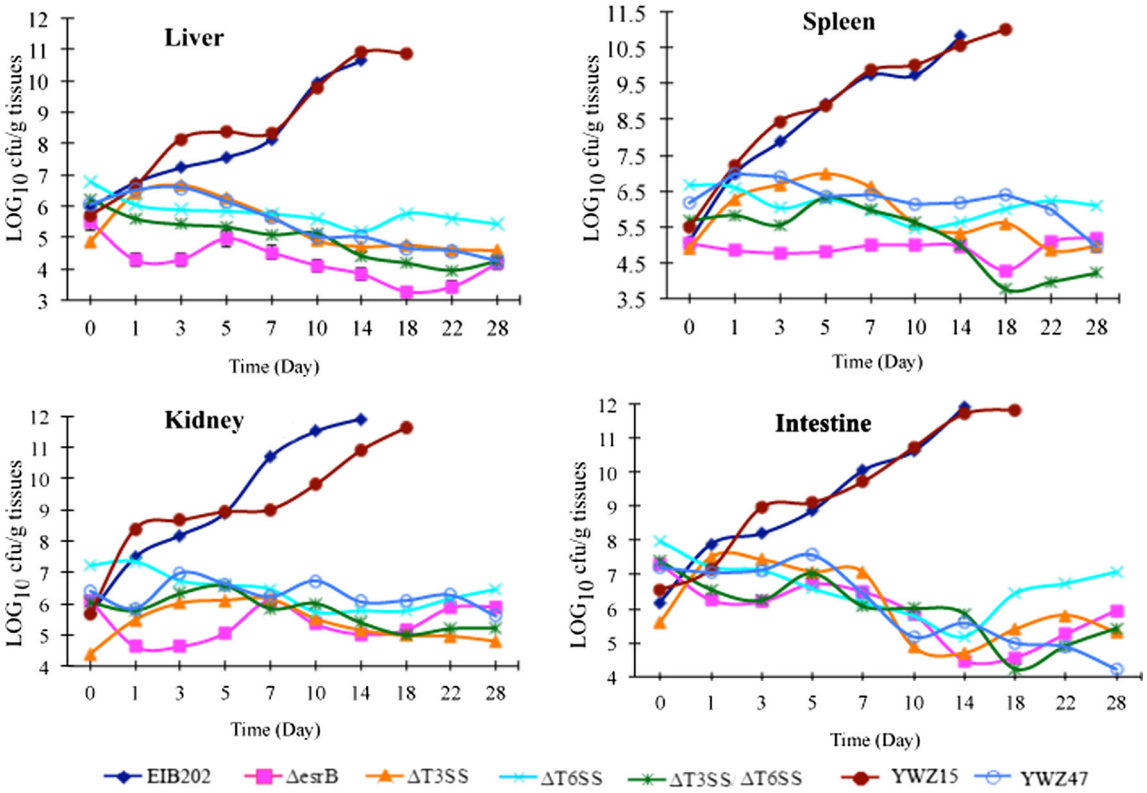

Fig. 3 Infection kinetics of *E. tarda* strains in turbot. Following i.m. infection with corresponding strains at a dose of 1.5×10^5 cfu/fish, the fish were sampled in triplicate at 0, 1, 3, 5, 7, 10, 14, 18, 22, and 28 days. The organs (liver, spleen, kidney, and intestine) from three fish were mixed and homogenized. The serial dilutions were plated on DHL agar plates. Bacterial count was normalized by dividing the mean counts (n=3) with the weight of the mixed samples

Discussion

Edwardsiella tarda is an important zoonotic pathogen causing diseases in a wide range of hosts including the farmed fishes such as turbot (Xu and Zhang 2014). Its pathogenesis includes two important steps, i.e., adhesion and invasion of the epithelial cells as well as survival and colonization inside the macrophage cells. It is believed that hemolysin EthA (Hirono et al. 1997; Wang et al. 2010) and other adhesins (Dong et al. 2013) are important for the primary stage of infection, while T3SS and T6SS are essential for the later stages of infection (Tan et al. 2005; Zheng and Leung 2007; Zheng et al. 2005). Previous studies have revealed that TCS EsrA-EsrB is essential for the coordinated regulation of these virulence determinants (Wang et al. 2010). Illumination of the molecular basis of these processes will facilitate our understanding of the pathogenesis of the pathogen and contribute to designing a promising vaccine against the bacterium. In this study, error-prone PCR mutagenesis was used to successfully isolate EsrB variants that generated high host-invasion-associated hemolytic activity but low T3SS and T6SS expressions in *Edwardsiella tarda*.

Table 3 LD_{50} values of *E. tarda* strains in turbot

Strain	LD_{50} (cfu/g)	Attenuation fold as compared to EIB202
EIB202	4.51×10^1	Not applicable
Δ*esrB*	$>4.03\times10^7$	$>8.8\times10^5$
ΔT3SS	2.63×10^5	5.8×10^3
ΔT6SS	3.42×10^4	7.5×10^2
ΔT3SS/ΔT6SS	$>2.17\times10^7$	$>4.8\times10^5$
YWZ15	1.73×10^2	3.8
YWZ47	$>2.30\times10^7$	$>5.09\times10^5$

Virulence attenuation has always been the major concern in designing an LAV. First of all, it is intriguing to investigate and clarify the relative contribution of T3SS and T6SS in pathogenesis of *Edwardsiella tarda*. T3SS and/or T6SS mutants showed significantly impaired cell invasion capabilities (Fig. 2), as well as in vivo propagation capability (Fig. 3). LD_{50} values indicated that T3SS might play a more important role in the in vivo infection process than T6SS (Table 3). Indeed, T3SS was chosen as an ideal candidate gene superior to T6SS for construction of LAV candidates (Xiao et al. 2013; Xu and Zhang 2014). As previously described (Lv et al. 2012; Zheng et al. 2005), Δ*esrB* was unable to activate T3SS and T6SS expression and secretion, resulting in an extremely

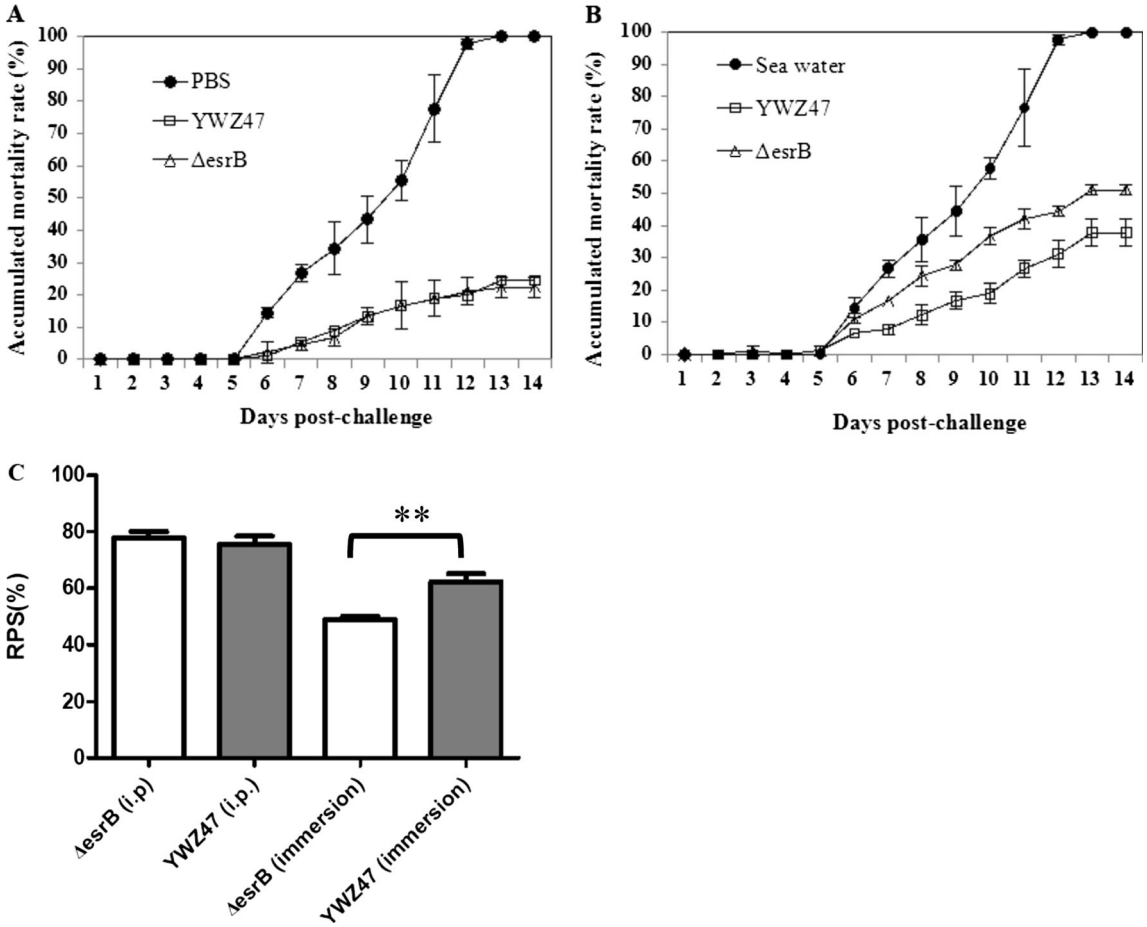

Fig. 4 Evaluation of the vaccine efficacy of the strain YWZ47 and Δ*esrB* in turbot. Healthy turbots were inoculated with i.p. injection (**a**) or immersion (**b**) methods. Five weeks after inoculation, fish were i.m. challenged with wild-type *E. tarda* EIB202 at a dose of 2×10^4 cfu/fish, and the cumulative mortalities of turbot were recorded for 28 days. No mortality was observed after 14 days of observation. **c** The relative survival percentage was calculated. $^{**}P<0.001$

higher LD_{50} than EIB202. This confirmed the importance of EsrB in the regulation network of pathogenesis in *Edwardsiella tarda*. Although the isolated strain YWZ15 significantly decreased the production of extracellular T6SS proteins and abolished T3SS secretion under in vitro culture condition, the strain was apparently not suitable to be a vaccine candidate because of its high virulence toward fish with only 3.8-fold attenuation (Table 3). The in vivo colonization dynamics of the strain also demonstrated its full virulence potential as the wild-type EIB202 (Fig. 3). Thus, it will be intriguing to test how this EsrB variant differentially regulates T3SS and/or T6SS in vivo and in vitro (Fig. 1) in the future. However, the strain YWZ47 was completely impaired in its capacity to cause disease or lethality toward fish with an LD_{50} value comparable to that of Δ*esrB* and ΔT3SS/ΔT6SS strains (Table 3 and Fig. 3), which featured an LAV candidate with low residual virulence toward host. Without T3SS and T6SS proteins secreted into the medium (Fig. 1b, c), this strain was assumed not to activate the T3SS and T6SS in vivo in fish. qRT-PCR targeting the T3SS and T6SS genes also showed that no T3SS and T6SS genes were expressed in both the fish and the DMEM medium (data not shown), further supporting that the $EsrB_{YWZ47}$ was not functional in activating the virulence.

In our previous experiences of vaccine development, we noticed that, in comparison with i.p. injection inoculation, the vaccination of a vaccine through immersion route could only evoke a low protection rate in turbot, which might be mainly resulted from the physical (i.e., thick skin) or immunological (i.e., mucosal immune system) barrier of turbot to prevent the entry of the vaccine. Thus, the design of genetically modified recombinant LAV strains to display an invasive phenotype would be helpful to improve host entry and host cell internalization via immersion administration, a welcomed labor-saving and economic vaccination route used in aquaculture industry. For this purpose, we firstly investigated the cell invasion ability and in vivo colonization dynamics of YWZ47, and the expected phenotypes of strong invasion toward macrophages (Fig. 2) and high colonization rate in the fish immune tissues (liver, spleen, and kidney)

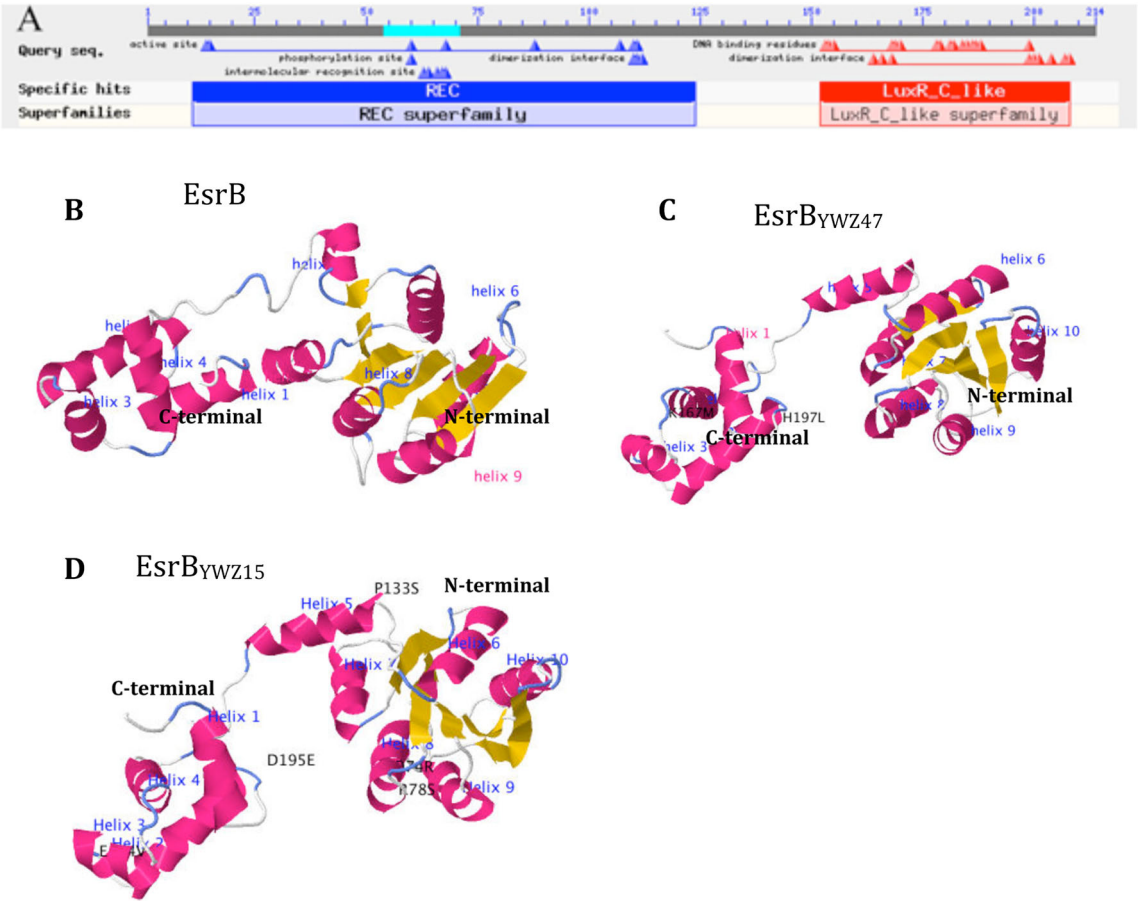

Fig. 5 Structural analysis of EsrB and its variants. **a** EsrB has two conserved domains, N-terminal REC superfamily domain and C-terminal LuxR-C like H-T-H domain. **b**–**d** The model of wild-type (WT) and variant EsrB from strains EIB202 (**b**), YWZ47 (**c**), and YWZ15 (**d**) established by homology modeling. The α-helix, β-sheet, and loop structures were as depicted in colors. The mutated amino acid residuals (Table 4) were also shown as labeled

demonstrated that the strain was better than Δ*esrB* strain in these respects (Fig. 3). Just before the challenge with the wild-type EIB202 strain, no YWZ47 cells could be detected except for individual fish after inoculation (data not shown), suggesting that YWZ47 could only transiently colonize in the tissues for 4–5 weeks. When inoculated in turbot through i.p. injection route, YWZ47 could trigger a high protection rate comparable to Δ*esrB* strain. However, significantly higher protective rate was achieved for YWZ47 than Δ*esrB* strain when administered through immersion inoculation route. In this work, a stable plasmid (Xiao et al. 2011) was used to express EsrB variants with native *esrB* promoter. Even when the plasmid was lost, YWZ47 would still be kept as Δ*esrB* strain and will be highly attenuated (Table 3). Thus, the safety

Table 4 Mutation sites in the EsrB variants of the isolated typical strains

Strain	Base	Base substitution	Position in codon	Amino acid	Amino acid substitution	Mutation in the structure
YWZ15	221	A–>G	2	74	Q–>R	α-Helix 8
	232	C–>A	1	78	R–>S	α-Helix 8
	397	C–>T	1	133	P–>S	Loop
	510	G–>T	3	170	Silent	–
	521	A–>T	2	174	E–>V	α-Helix 3
	585	T–>A	3	195	D–>E	Loop
YWZ47	500	A–>T	2	167	K–>M	Loop
	590	A–>T	2	197	H–>L	Loop

of this vaccine would not be a major concern for practical use. As a result, YWZ47 was a promising LAV used for turbot aquaculture.

By far, little was known for the structural basis of EsrB regulation of T3SS, T6SS, and hemolytic activity in *Edwardsiella tarda*. With error-prone PCR mutagenesis, two interesting EsrB variants were screened in this study in respective of low or no secretion of T3SS and T6SS but high hemolytic activity as compared with the wild-type EsrB. The promoter region of *esrB* gene was not subjected to mutagenesis although it was also a potential target to modify the regulation patterns in vivo and in vitro. However, we were more interested in the protein per se in the differential regulation of virulence-associated factors in *Edwardsiella tarda* to potentially illuminate its regulation mechanisms. At the same time, here, no attention was paid to the direct or indirect regulation of EsrB to the abovementioned virulence factors that we are investigating in a separate work. For $EsrB_{YWZ15}$ variant, five aa residual substitutions were found to locate on the helices or loop structures, and it was difficult to assign their specific contributions to the related functional alterations in this mutant strain. For $EsrB_{YWZ47}$ variant, it was notable that two substituted aa residues (K167M, H197L) were non-conserved or essential residues in $SsrB_C$ and were mapped to the loops between helices (Fig. 5c). Moreover, the two aa residues (K167 and H197) were positively charged and might be also required for the supplemental interaction with the bounded DNA molecules as analyzed in the $SsrB_C$ structural investigation (Carroll et al. 2009). The replacement of the two aa residues might individually or collectively alter the binding affinity and specificity of the EsrB variant toward the promoter regions of T3SS, T6SS, EthA, or other virulence determinants in *Edwardsiella tarda*. A more detailed understanding of the specific mechanism with which EsrB activates or inhibits gene transcription is required to distinguish between these possibilities with the *bona fide* structures of full-length or partial EsrB protein and the variants. However, as $EsrB_{YWZ15}$ variant could produce high levels of hemolytic activity (Fig. 1a), infectivity in macrophages at certain stage (0–8 h in Fig. 2d, e), and persistence in vivo of turbot, it will be intriguing to test the protective efficacy of recombinant mutagenesis of T3SS, T6SS, and $EsrB_{YWZ47}$ in the context of YWZ15 strain in the future.

In summary, error-prone PCR mutagenesis allowed us to screen EsrB variants which were incapable of activating T3SS and T6SS expression but efficiently enhancing the yields of hemolytic activity in Δ*esrB* mutant. The characterization of the isolated strain YWZ47 confirmed its highly attenuated virulence and significantly increased host invasion ability toward fish, as well as its high protection efficiency against the challenge of wild-type *Edwardsiella tarda* when inoculated by both immersion and i.p. injection routes. Compared to the established LAV candidate Δ*esrB* mutant, YWZ47 exhibited higher protection rate when administered by immersion route, meriting as an ideal LAV candidate conveniently used in aquaculture industry. The molecular modeling of the wild-type and variant EsrB will facilitate the illustration of the possible structural basis for the differential regulation of virulence determinants in *Edwardsiella tarda*.

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