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Functional characterization of *Edwardsiella tarda* twin-arginine translocation system and its potential use as biological containment in live attenuated vaccine of marine fish

Yamin Wang · Qiyao Wang · Weizheng Yang · Bing Liu · Yuanxing Zhang

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Abstract Bacterial twin-arginine translocation (Tat) system contributes to translocate folded proteins to the periplasm and plays pleiotropic roles in physiological fitness. Here, we showed that the fish pathogen *Edwardsiella tarda* Tat pathway was functional and was essential for H_2S production and hemolytic activity. *E. tarda* Tat mutant was more susceptible to diverse stresses such as high temperature, SDS, ethanol, and high-salt conditions. However, *E. tarda* Tat mutant displayed marginal in vivo virulence attenuation in fish models. Comparative proteomics analysis using two-dimensional gel electrophoresis (2-DGE) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry were performed to identify proteins undergoing changes in expression levels under high-salt conditons when the Tat pathway was mutilated. Of the 96 differently expressed proteins on the 2-DGE map, 15 proteins were successfully identified with a MASCOT score >45 ($p<0.05$) and fold change higher than 2. These significantly differentially expressed proteins were functionally related to basal metabolism and the biosynthesis of proteins and macromolecules. The results of plate counting further confirmed that the Tat mutant was high-salt-sensitive, indicating that Tat mutant merits as a novel salt-sensitive biological containment system for live attenuated vaccine (LAV) in marine fish vaccinology. To test this, we deleted the type III secretion system genes and cured endogenous plasmid pEIB202 to construct a LAV candidate in the context of Tat abrogation in *E. tarda*. The results indicated that the LAV candidate was highly attenuated when injected intraperitoneally and elicited significant protection against challenge of wild-type *E. tarda* in turbot while being rapidly eliminated in seawater.

Keywords Tat system · Biological containment · Marine fish vaccinology · *Edwardsiella tarda*

Y. Wang · Q. Wang (✉) · W. Yang · B. Liu · Y. Zhang
State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology,
130 Meilong Road,
Shanghai 200237, People's Republic of China
e-mail: oaiwqiyao@ecust.edu.cn

Introduction

Bacterial adaptation to a specific niche is often critically dependent upon various secretion systems to mediate the transport of molecules or enzymes out of their cells into the extracellular milieu or into the host cells. Some of these secretion systems rely on the Sec-dependent translocation of effector proteins across the cytoplasmic membrane, while some are established to be associated with the twin-arginine translocation (Tat) branch (Santini et al. 1998). The Tat secretion system is reported to translocate the folded substrate proteins, which carry signal peptides with featured consensus "twin-arginine" motif S/T-R-R-x-F-L-K, into the periplasmic space (Weiner et al. 1998). In *Escherichia coli*, TatA, TatB, TatC, and TatE are considered as essential components of the Tat pathway (Berks et al. 2003). Co-transcribed with *tatABC*, *tatD* seems to exert no obligated role in protein export in *E. coli* Tat pathway (Wexler et al. 2000). Gene *tatE* is regarded as a cryptic gene of *tatA*, which plays a more important role than *tatE* in *E. coli* (Jack et al. 2001). The Tat pathway is functionally related to the translocation of cofactor-containing enzymes involved in respiratory and electron transport chain (Berks et al. 2005). The Tat pathway plays important roles in virulence in some bacterial pathogens such as *Pseudomonas aeruginosa* (Ochsner et al. 2002) and *Vibrio cholerae* (Zhang et al. 2009). In halophilic archaea, Tat-mediated secretion of extracellular proteins plays major parts in their adaption to cope with the extreme environments (Rose et al. 2002). In

bacteria, however, there are very few reports regarding to the roles of the Tat pathway in response to osmotic stresses.

Edwardsiella tarda, a Gram-negative, facultative aerobic pathogen inhabits a broad range of hosts including fish, reptiles, amphibians, chickens, and humans (Abbott and Janda 2006). In worldwide aquaculture industries, outbreak of *E. tarda*-associated edwardsiellosis in various fish species has been increasingly reported (Mohanty and Sahoo 2007). At present, virulence factors including the type III secretion system (TTSS) contributing to the pathogenicity of *E. tarda* have been described (for a recent comprehensive review, see Leung et al. 2012). Recently, the genome sequence of *E. tarda* wild-type strain EIB202 isolated from diseased turbot has been determined to unravel the genetic properties for niche adaptation and virulence (Wang et al. 2009a). As noted in the genome of *E. tarda* EIB202, the Tat system might serve for the secretion of 33 putative substrates as well as their co-transported proteins (Wang et al. 2009a). The bacterium displays fast growth rates in a wide range of sodium chloride concentrations (0.5–5 %; Xiao et al. 2009a). It is highly invasive toward fish and closely clustered with other fish pathogenic strains as well as *Edwardsiella ictaluri* strains in terms of their phylogenomic relationship, which might facilitate the development of a highly cross-protective vaccine against edwardsiellosis caused by pathogenic *Edwardsiella* strains (Wang et al. 2011; Yang et al. 2012).

To elucidate the function of the Tat system in *E. tarda*, we here inactivated the *tatABCD* locus and revealed that the Tat pathway was functionally associated with stress responses, especially high salt tolerance in this bacterium. Proteomics approaches were used to investigate the roles of Tat in response to high-salt stress in this bacterium, which provided a platform for the construction of a novel biological containment for live attenuated vaccine (LAV) in marine fish vaccinology.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains (Table 1) were routinely grown in Luria–Bertani medium (LB; Oxoid, Basingstoke, UK) or

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Reference
Edwardsiella tarda		
EIB202	Col^r, Cm^r; wild type (CCTCC no. M208068)	Xiao et al. (2009a)
Δ*esrB*	Col^r, Cm^r, in-frame deletion of *esrB*	Wang et al. (2009b)
Δ*tatABCD*	Col^r, Cm^r; EIB202, in-frame deletion of *tatABCD* from 94 to 2,478 bp (CCTCC no. M2011338)	This study
tatABCD$^+$	Col^r, Cm^r, Km^r; Δ*tatABCD* carrying pMMBK-*tatABCD*	This study
WYM1	Col^r, Δ*tatABCD*, in-frame deletion of *eseB*, *eseC*, *escA*, and *eseD*, and curing of pEIB202 (CCTCC no. M2011339)	This study
Escherichia coli		
Top10F′	General cloning strain	Invitrogen
cc118 *λpir*	*λpir lysogen of* cc118 Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1*	Dennis and Zylstra (1998)
SM10 *λpir*	Km^r; *thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu*, *pirR6K*	Liang et al. (2003)
BL21 (GFP)	*E. coli* BL21 derivative with a pET28a plasmid expressing GFP	Lab collection
Plasmids		
pMD19-T	Amp^r; PCR cloning vector	TaKaRa
pDM4	Cm^r; *sacBR*, suicide vector, R6K, *pir* requiring	Wang et al. (2002)
pDMK	Cm^r, Km^r; pDM4 derivative with Km fragment inserted in *Sac*I site	Xiao et al. (2009b)
pMMB206	Cm^r ; IncQ lacI^q Δ*bla* $P_{tac\text{-}lac}$ *lacZα*	Morales et al. (1991)
p34S-Km	Km^r; provide the Km fragment	Dennis and Zylstra (1998)
pUTat	Amp^r; cloning vector, pUC18 derivative with P_{lac} and MCS deleted containing pAT153 replicon	Xiao et al. (2011b)
pDMK-Δ*tatABCD*	Cm^r, Km^r; pDMK derivative containing *tatABCD* 1–93 bp fused in-frame to 2,479–2,532 bp	This study
pMMBK-*tatABCD*	Cm^r, Km^r; pMMB206 derivative containing 3,896-bp fragment of intact *tatABCD*	This study
pUTat-ssTorA::GFP	Amp^r; pUTat derivative expressing signal sequence of TorA of *Ennn tarda* fused to GFP driven by promoter *evpP*	This study

CCTCC China Center for Type Culture Collection

deoxycholate hydrogen sulfide lactose agar (DHL; Nissui, Japan). In biological containment experiment, *E. tarda* strains were cultured in artificial sterile seawater (28.13 g NaCl, 0.77 g KCl, 1.60 g $CaCl_2{\cdot}2H_2O$, 4.80 g $MgCl_2{\cdot}6H_2O$, 0.11 g $NaHCO_3$, and 3.50 g $MgSO_4{\cdot}7H_2O$ in 1,000 mL distilled water; Thouand et al. 2003). *E. tarda* and *E. coli* strains were grown at 28 and 37 °C, respectively. When necessary, antibiotics or other agents (Sigma, St. Louis, MO, USA) were added at the following concentrations (in micrograms per milliliter): colistin (Col), 12.5; kanamycin (Km), 50; chloramphenicol (Cm), 30; ampicillin (Amp), 100; IPTG, 24; X-gal, 40.

Construction of in-frame deletion mutants and complement strains

For the construction of a *tatABCD* site-directed deletion mutant, PCR amplifications were performed to generate the upstream and downstream fragments of the *tatABCD* locus with primer pairs *tatABCD*-deupF/R and *tatABCD*-dedownF/R, respectively (Table 2). The product containing the in-frame deletion fragment from 94 to 2,478 bp in *tatABCD* was obtained by overlap PCR with primer pair *tatABCD*-deupF/dedownR and then cloned into the *Bgl*II/*Sph*I sites of a suicide plasmid pDMK (Table 1). The resulting plasmid pDMK-Δ*tatABCD* was transferred from *E. coli* SM10 *λpir* into *E. tarda* EIB202 by conjugation (Table 1). During a single crossover event, the exconjugants with plasmid pDMK-Δ*tatABCD* integrated into the chromosome by homologous recombination were selected as previously described (Xiao et al. 2009b) on LB agar containing Col, Km, and Cm. PCR with primers Sal-up/Sac-down (Table 2) were used to verify the correct integration of pDMK-Δ*tatABCD*. The recombinant strain was propagated in LB containing 10 % (*w*/*v*) sucrose to allow for a second crossover of allelic exchange. The targeted mutants were counter-selected on LB agar containing 10 % sucrose (Xiao et al. 2009b). The selected colonies were confirmed by PCR with the primer pair *tatABCD-de*F/R flanking the insertion sites (Table 2) and DNA sequencing. The confirmed clone was designated as Δ*tatABCD* (Table 1). The LAV candidate strain WYM1 (Table 1) was further constructed by in-frame deletion of the TTSS genes and curing endogenous R plasmid pEIB202 (Wang et al. 2009a) in Δ*tatABCD* with

Table 2 Primers used in this study

Primer	Sequence (5′–3′)[a]
For the construction of Δ*tatABCD*	
tatABCD-deupF	GGAAGATCTCGTCTACAGCACAGCATGGA
tatABCD-deupR	GCTTCAGCCAATCCGAACCCAGAGTACGCA
tatABCD-dedownF	GGGTTCGGATTGGCTGAAGCAGGTGACTGA
tatABCD-dedownR	ACATGCATGCATCGCTGCTGTACGCCTCTT
*tatABCD-de*F	CCTGTTCAATACCGCACGGCGTTTT
*tatABCD-de*R	TGCGGCCATCCATCATATCGCTCGG
Sal-up	GGTGCTCCAGTGGCTTCTGTTTCTA
Sac-down	CAGCAACTTAAATAGCCTCTAAGGT
For the construction of complement strain *tatABCD*$^+$	
tatABCD-comF	CCGGAATTCGCTGGGTGCCGCCGGATACCAATG
tatABCD-comR	ACGCGTCGACTCGCGGAACGGACCGTAGTAGCAAG
pMMB206-F	CCCCAGGCTTTACACTT
pMMB206-R	GCTTCTGCGTTCTGATTT
For the construction of TTSS genes deletion mutant	
TTSS-deupF	GGAAGATCTCGCCTTTCACACGTTACAGCAAGAG
TTSS-deupR	GCTGGGCATCCGATTAGCCACCTGCTGGGA
TTSS-downF	CAGGTGGCTAATCGGATGCCCAGCAAAAGA
TTSS-downR	ACATGCATGCCCTGCGACTGACGCGACATGTCATT
TTSS-deF	GCAACTGATGCAAGACCTGCAGCGG
TTSS-deR	AGGCCGTGCTGATATTCGGACAGCG
For curing of endogenous plasmid pEIB202	
catA-P1	GGAAGATCTTCTTTACCAAGCACAT
catA-P2	ACATGCATGCGCTCAGGTTAAATTAAAGGG
C6-P1	CGGCAGCTTCAATAACCA
C6-P2	GGAACTCCGTAACGTCGAA

[a]Restriction enzyme sites used for the cloning of PCR products were underlined

specific primer pairs (Table 2) following the aforementioned procedures (Xiao et al. 2009b).

To construct the complement strain *tatABCD*$^+$, an intact *tatABCD* gene containing the promoter region was amplified with primers *tatABCD*-comF/R (Table 2). The product was cloned into the *Eco*RI/*Sal*I sites of vector pMMB206 (Table 1) to construct pMMB206-*tatABCD*. The Km resistance gene fragment on p34S-Km (Table 1) was *Sal*I-digested followed by ligation to the similarly digested pMMB206-*tatABCD*. The derivative pMMBK-*tatABCD* plasmid was mated from *E. coli* SM10 *λpir* into Δ*tatABCD* by conjugation to produce *tatABCD*$^+$ (Table 1). This strain was confirmed by PCR with primers pMMB206-F/R (Table 2) and DNA sequencing.

Evaluation of Tat translocation function in *E. tarda*

To assess the function of the Tat pathway in *E. tarda*, plasmid pUTat-ssTorA::GFP (Table 1) expressing the signal sequence (ss) of trimethylamine N-oxide (TMAO) reductase (TorA) of *E. tarda* fused to green fluorescent protein (GFP) was constructed (Lavander et al. 2006). The expression of ssTorA::GFP was under the control of an *esrB*-regulated *evpP* promoter in *E. tarda* (Wang et al. 2009b). The constructed plasmid was introduced into the wild-type strain EIB202 and Δ*tatABCD* by electroporation, respectively. Δ*esrB* carrying plasmid pUTat-ssTorA::GFP (Table 1) was taken as a negative control in which the *evpP* promoter was depressed (Wang et al. 2009b). The whole cell lysate (WCL) of *E. coli* BL21(GFP) (Table 1) with a pET28a plasmid expressing GFP was selected as the positive control. The subcellular location of GFP in the cytoplasmic, periplasmic, and extracellular fractions were determined by Western blotting (Santini et al. 1998). RNA polymerase (RNAP) was used as the cytoplasmic protein marker to check fractionation purity.

Growth assessment

Overnight cultures of the tested strains were adjusted with fresh LB medium to an optical density at 600 nm (OD_{600}) of 0.1. The cultures were inoculated 1:100 into 50 mL fresh LB medium, and 100 μL of the samples was taken every 4 h. Cell density was measured at 600 nm with an ND1000 spectrophotometer (NanoDrop Technologies, Delaware, USA).

Hemolytic activity assays

Hemolytic activity was tested according to the protocol described previously, with minor modifications (Wang et al. 2010). Briefly, sheep erythrocytes (Sangon, Shanghai, China) were washed three times with phosphate-buffered saline (PBS; pH 7.4) and then added into fresh LB medium at a final concentration of 5 % (*v*/*v*). Overnight cultures of the strains were washed twice and resuspended in PBS to an OD_{600} of 0.1 and then were inoculated 1:100 into fresh LB medium containing sheep erythrocytes. *E. coli* Top10F′ (Table 1) was taken as the negative control. The LB medium containing sheep erythrocytes was used as the blank control. Samples (100 μL) were taken every 3 h and the supernatant was measured at 540 nm. One hemolytic unit was arbitrarily defined as the amount of hemolysin that would produce 1 % hemolysis.

Stress response and sensitivity assay

Stress response was determined as previously described (Ize et al. 2003; Rajashekara et al. 2009). Briefly, overnight cultures of the strains were adjusted to an OD_{600} of 1 and then inoculated 1:100 into 50 mL LB medium containing different reagents or cultured at different conditions. For temperature stress, the tested bacteria were cultured at 28 and 42 °C, respectively. For salinity stress, each cell culture was inoculated into LB medium containing 0.5, 4.0, or 5.0 % NaCl (*w*/*v*), respectively. Cell densities were then measured at 600 nm at different time intervals. For SDS and ethanol sensitivity tests, the cultures were inoculated 1:100 into LB medium containing 0–2 % (*w*/*v*) SDS or 0–15 % (*v*/*v*) ethanol under continuous shaking at 200 rpm for 3 h; then, the samples were serially diluted with LB medium and plated on LB agar plates to determine the colony forming units (CFUs). The CFU of each culture without SDS or ethanol was defined as 100 % survival.

Median lethal dose (LD_{50}) assay

Healthy zebra fish (*Dario rerio*) of approx. 0.2 g were raised in reverse osmosis-purified water in a flow-through auto-controlled system at 25 °C. Healthy turbot (*Scophthalmus maximus*) of about 30 g were maintained in aerated tanks with a continuous flow of sand-filtered seawater at 14–16 °C. All the fish were acclimated for 2 weeks prior to infection and divided randomly into groups of ten fish. Fish were anesthetized in an aqueous solution with tricaine methanesulfonate (MS-222; Sigma-Aldrich) at a concentration of 80 mg/L before injection. For killing, fish were over-anesthetized with 200 mg/L MS-222. Fish were injected intramuscularly (i.m.) with each of tenfold diluted *E. tarda* strains ranging from 10^1 to 10^8 CFU in triplicate. The control group was injected with an equal volume of sterile saline water. Mortality was recorded over a period of 28 days, and the LD_{50} values were calculated by the method described previously (Santander et al. 2010). The mortality was the average of three paralleled experiments. Microbiological methods were conducted on all the dead

fish to confirm whether they were associated with *E. tarda* infection.

2-DGE and protein identification

Two-dimensional gel electrophoresis (2-DGE) of whole cell lysate protein (WCLP) was conducted according to the methods described previously (Daware et al. 2012; Srinivasa Rao et al. 2004; Wang et al. 2012). Briefly, *E. tarda* strains were cultured in LB medium containing 4 % NaCl and the cells harvested at the late exponential phase by centrifugation at 5,000×*g* for 10 min at 4 °C. Then, the pellets were resuspended in 10 mM Tris–HCl (pH 8.0) containing complete protease inhibitor cocktail (Roche, Mannheim, Germany) and lysed on ice with a sonicator. The supernatant was precipitated by TCA–acetone and the dried proteins resuspended in a sample solution buffer containing 8 M urea, 2 % (*w*/*v*) Chaps, 50 mM dithiothreitol, and 0.5 % (*v*/*v*) Pharmalyte (pH range 3–10). The protein concentration was measured by a 2D Quant Kit (GE Healthcare Bio-Sciences). Of each sample, 500 μg was used for protein separation.

First-dimension isoelectric focusing was performed in immobilized pH gradient strip with pH range 3–10 (Amersham Biosciences, Piscataway, NJ, USA) under the following conditions: 0 V for 8 h, 30 V for 8 h, 500 V for 2 h, 4,000 V for 1.5 h, and 8,000 V for 40,000 V h. The second dimension was run on 12 % SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue R250 (Sigma). Proteins were isolated in triplicate from separate cell cultures. Images were analyzed using PDQuest software package, version 8.0.1 (Bio-Rad Laboratories, Hercules, CA, USA). The reproducible spots of interest were excised and identified by a plus 4800 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) analyzer (Applied Biosystems, Foster City, CA, USA). The acquired MS spectra were submitted to MASCOT search engine for protein/peptide sequence identification using the NCBI and MSDB protein databases. The parameters were as follows: fragment mass tolerance, ±0.8 Da; one missed cleavage per peptide; fixed modifications of carbamidomethyl; peptide mass tolerance, 100 ppm; peptide charge state, 1+. A protein score more than 45 was considered a statistically significant match ($p<0.05$; Perkins et al. 1999).

Biological containment of *E. tarda* strains in different salt conditions

To determine the number of culturable cells in different salt conditions, overnight-cultured EIB202 and Δ*tatABCD* were inoculated into three paralleled 50 mL fresh artificial sterile seawater with different NaCl concentrations and cultured at various temperatures. Of the diluted cultures, 100 μL was sampled and plated on LB agar in triplicate and then incubated at 28 °C for 48 h. The bacteria were categorized to be non-culturable when there was less than one platable cell on LB agar. To determine the number of viable cells, the samples cultured for 2 months were stained with propidium iodide provided in the live/dead "BacLight bacterial viability" kit (Invitrogen, Carlsbad, CA, USA) and then assayed using FACS calibur flow cytometry (BD Biosciences, San Jose, CA, USA) as previously described (Shi et al. 2007).

Evaluation of protection efficiency and in vivo/in vitro survival of WYM1

Turbot were divided randomly into groups of 60 fish and vaccinated with LAV strain WYM1 in triplicate. Each turbot was intraperitoneally (i.p.) vaccinated with 5×10^3 CFU/g of WYM1, while the control group was i.p. injected with an equal volume of sterile saline water. All the fish were raised in aquaria supplied with a continuous flow of aerated sand-filtered seawater at 14–16 °C for 32 days and then were challenged by i.m. injection with 1×10^2 CFU/g of *E. tarda* EIB202. The death of fish was further observed and counted for 32 days. The relative percent survival (RPS) was calculated according to the following formula (Xiao et al. 2011a):

$$RPS = 100\% \times \left(1 - \frac{\%mortality\ in\ vaccinated\ fish}{\%mortality\ in\ control\ fish}\right)$$

For the determination of bacterial burden, turbot were raised as in the aforementioned methods with 40 fish in each group. Fish were i.p. challenged with a dose of 5×10^3 CFU/g EIB202 or WYM1 in triplicate. Three fish from each group were sampled at each time interval post-injection. All the samples were weighed and homogenized in 1 mL sterile saline water. The homogenates were serially diluted and plated onto DHL plates for bacteria counting in triplicate. To compare the survival ability between EIB202 and WYM1 in seawater, the two strains were inoculated into sand-filtered seawater in triplicate with an initial concentration of 10^8 CFU/mL, respectively. At days 1, 3, 4, 5, 7, 9, and 11 post-inoculation, 100 μL of cultures was sampled and the number of culturable cells measured.

Results

Tat pathway is functional in ssTorA::GFP transportation in *E. tarda*

Based on the genomic sequence information, *tatA*, *tatB*, *tatC*, and *tatD* (ETAE_0150-0153) were located in a *tat* locus, while *tatE* (ETAE_2651) was located elsewhere on

the chromosome (Wang et al. 2009a). Blastp results demonstrated that TatA, TatB, TatC, TatD, and TatE in *E. tarda* EIB202 shared 56, 57, 77, 62, and 67 % amino acid identity with their counterparts in *E. coli* K-12, respectively. TatA and TatE shared 65 % amino acid sequence identity in *E. tarda* EIB202. To assess whether the Tat pathway was functional in *E. tarda*, we constructed plasmid pUTat-ssTorA::GFP for subcellular location detection in which the signal sequence of a putative Tat substrate, TMAO reductase (TorA), was fused to GFP and the expression of ss*torA-gfp* was driven by an *esrB*-regulated *evpP* promoter (Wang et al. 2009b). The expression and translocation of ssTorA::GFP was examined in wild-type strain, Δ*tatABCD*, and Δ*esrB* with GFP-specific antibody by Western blotting (Fig. 1a). The results herein indicated that the majority of expressed ssTorA::GFP was translocated into the periplasm in the wild-type strain as mature GFP while being confined in the cytoplasm in Δ*tatABCD* as an ssTorA::GFP precursor (Fig. 1a). As previously described, the results also indicated that GFP could not be detected in the extracellular products (ECP) (Meissner et al. 2007). The *evpP* promoter was strictly regulated by EsrB (Wang et al. 2009b) and, thus, did not produce ssTorA::GFP in the context of Δ*esrB* (Fig. 1a). These results demonstrated that Tat was competent for the export of ssTorA::GFP across the cytoplasmic membrane into the periplasm (Fig. 1a).

The Tat mutant is defective in the production of H_2S and hemolytic activity

We compared the phenotypes of the wild-type, Tat mutant, and the complement strains. The results of the API 20E assays indicated that the wild-type strain and the *tat* mutant had no differences in biochemical characteristics except for production of H_2S (data not shown). On DHL agar, typical colonies with a black center appeared, indicating the production of iron sulfide for EIB202 (Fig. 2a), while Δ*tatABCD* was negative for the production of iron sulfide and *tatABCD*$^+$ restored to generate iron sulfide. In addition, Δ*tatABCD* exhibited a dramatic defect in hemolytic activity compared with EIB202 and *tatABCD*$^+$ (Fig. 2b), which showed strong hemolytic activity, as previously described (Wang et al. 2010).

The Tat system plays important roles in pleiotropic stress responses

We further analyzed the sensitivity of the *tat* mutant to various stresses. The growth of EIB202 and Δ*tatABCD* was similar at 28 °C (data not shown). When cultured at 42 °C, Δ*tatABCD* displayed a defect in late log and the stationary phases compared with EIB202 (Fig. 3a), suggesting that the Tat pathway contributed to high-temperature stress response. When treated with various concentrations of SDS (0–2.0 %) and ethanol (0-12 %), Δ*tatABCD* showed a significantly less survival rate than that of EIB202 and *tatABCD*$^+$ (Fig. 3b, c). EIB202 and Δ*tatABCD* displayed a similar growth rate when cultured at 0.5 % NaCl (Fig. 3d). When cultured at a higher salinity, Δ*tatABCD* exhibited a significantly lagged and defective growth as compared with EIB202 and *tatABCD*$^+$, and the tendency became more evident when salinity increased (Fig. 3e, f). These data revealed that the Tat pathway played an important part in diverse stress responses in *E. tarda*.

The Tat system is not essential for in vivo infection and virulence

The infection and virulence of the *E. tarda* strains was investigated with zebra fish and turbot. EIB202 exhibited

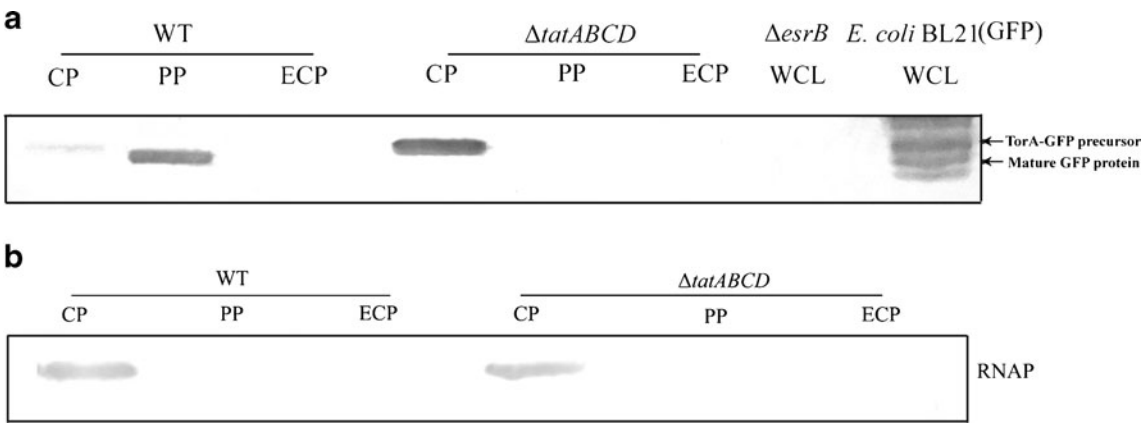

Fig. 1 Functional characterization of Tat translocation in *E. tarda*. **a** Western blotting analysis of cellular and secreted GFP. The expression of ssTorA::GFP was driven by an *esrB*-regulated *evpP* promoter in the wild-type strain and Δ*tatABCD*, respectively. *CP* cytoplasmic proteins, *PP* periplasmic proteins, *ECP* extracellular proteins, *WCL* whole cell lysate, *WT* wild-type strain. **b** Western blotting analysis using RNAP as a control for the purification of cellular fractions

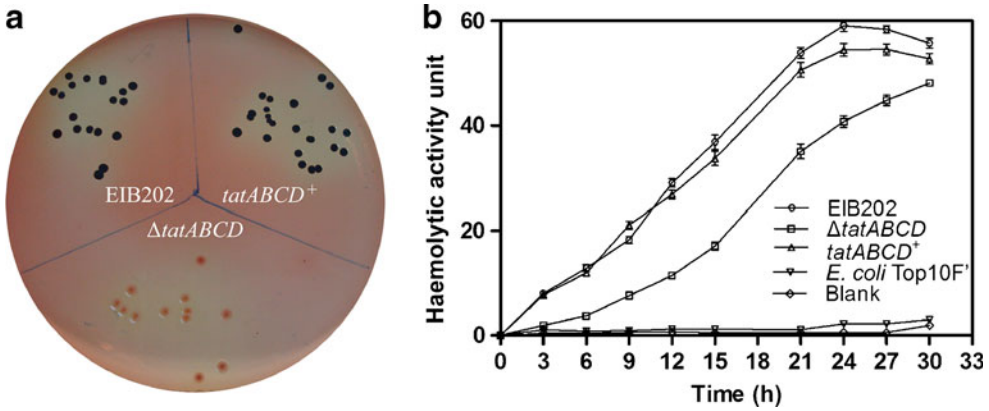

Fig. 2 Morphology characterization of the *tatABCD* mutant. **a** H_2S production analysis of EIB202, Δ*tatABCD*, and *tatABCD*$^+$ cultured on DHL plate. H_2S production was featured with the clone with a black center appearing on DHL agar. **b** Hemolytic activity analysis of EIB202, Δ*tatABCD*, and *tatABCD*$^+$. The data were representative of three replicated experiments yielding similar results. *Error bars* indicated standard deviations of three parallels

an LD_{50} of 2.5×10^2 CFU/g in zebra fish and 4.7×10^2 CFU/g in turbot via i.m. challenge. When i.m. infected to zebra fish and turbot, Δ*tatABCD* displayed marginal virulence attenuation by 4.3- and 5.2-fold, respectively, compared with EIB202. The complement strain *tatABCD*$^+$ shared the comparable virulence level with that of EIB202. Δ*tatABCD* caused diseases in zebra fish and turbot with the same symptoms as that of EIB202 and *tatABCD*$^+$, i.e., lethargy, darkening skin, subcutaneous hemorrhages, eye tumefaction, and swelling abdomen and, finally, leading to the death of fish with a high load of this bacterium in organs including kidney, liver, spleen, and intestine (Mohanty and Sahoo 2007). These results indicated that *tatABCD* was not essential for its in vivo virulence toward zebra fish and turbot in *E. tarda*.

Characterization of the high-salt-stressed proteome affected by Tat abrogation

To identify proteins involved in high-salt stress response and affected by the Tat pathway, a comparison of WCLP between EIB202 and Δ*tatABCD* cultured in 4 % NaCl LB

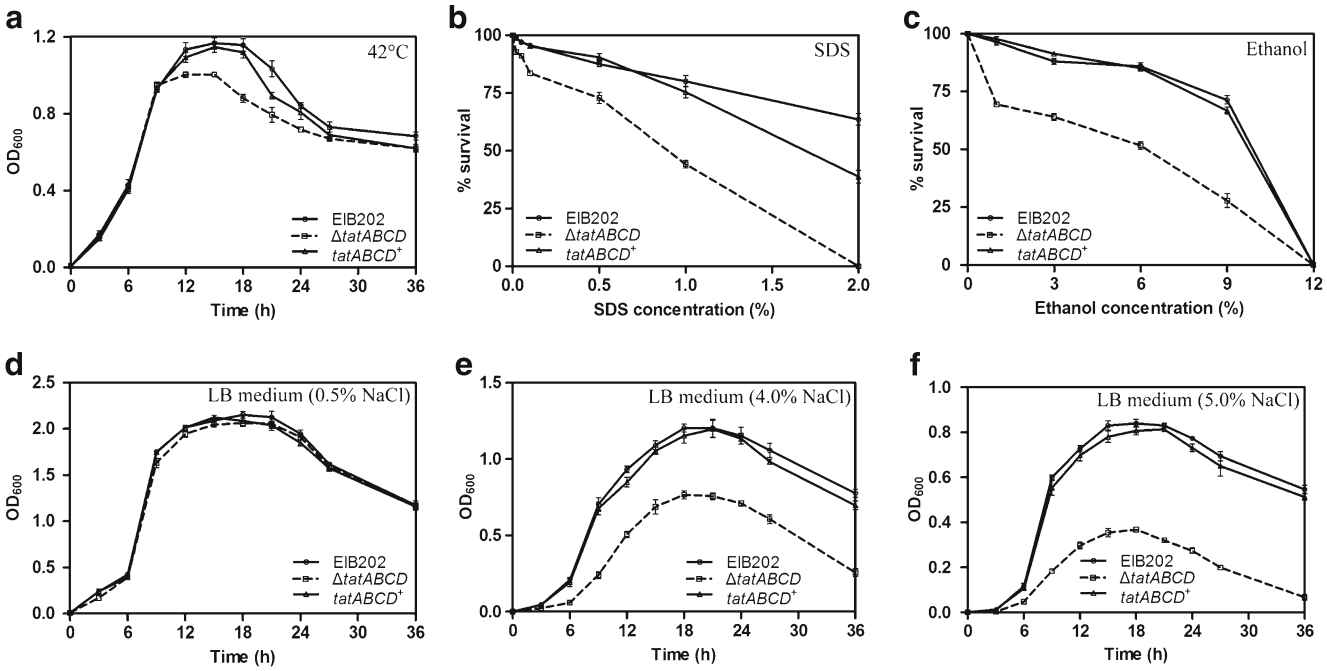

Fig. 3 The Tat pathway played important roles in stress responses in *E. tarda*. Stress sensitivities such as high temperature 42 °C (**a**), SDS (**b**), and ethanol (**c**) were assayed in EIB202, Δ*tatABCD*, and *tatABCD*$^+$. Salt tolerance was analyzed by culturing the stains in LB medium with the addition of different concentrations of NaCl, i.e., 0.5 % (**d**), 4.0 % (**e**), and 5.0 % (**f**). The data were representative of three replicated experiments yielding similar results. *Error bars* indicated standard deviations of three parallels

medium was performed using proteomics approach. The WCLP of wild-type and *tat* mutant strains were resolved by 2-DGE (Fig. 4), and 15 spots (Table 3) were identified with the MALDI-TOF/TOF method. The results indicated that proteins identified to be affected by Tat inactivation under high-salt condition were classified to be mainly involved in energy production and conversion (C, 3/15), amino acid transport and metabolism (E, 3/15), coenzyme transport and metabolism (H, 1/15), carbohydrate transport and metabolism (G, 1/15), and inorganic ion transport and metabolism (P, 2/15; Table 3). Four proteins (DsbA, ETAE_1482, GlmU, and ETAE_1187) putatively involved in cellular processes and signaling (posttranslational modification, protein turnover, chaperones—O; cell wall/membrane/envelope biogenesis—M; and cell cycle control, cell division, chromosome partitioning—D) were also identified (Table 3). Protein ETAE_2758, classified to be involved in translation, ribosomal structure, and biogenesis (J), was spotted to be significantly affected by Tat abrogation in *E. tarda*. These results suggested that the Tat pathway might be extensively involved in many facets of cellular function underlying high-salt resistance in *E. tarda*.

Mutation in the Tat pathway confers biological containment at high-salt conditions

The containment of EIB202 and Δ*tatABCD* cultured in different salt conditions (2.5, 3, 3.5, and 4 %) as well as temperatures (10, 16, and 28 °C) was further investigated with an initial inoculating concentration of 10^8 CFU/mL (Fig. 5). In comparison with EIB202, Δ*tatABCD* displayed a drastically decreased growth rate when inoculated into different salt conditions at three different temperatures in the initial 2 days (Fig. 5a–c). In the following 30–35 days of experiments, EIB202 and Δ*tatABCD* exhibited different rates of decreased growth in the various salt and temperature conditions (Fig. 5a–c). Compared with Δ*tatABCD*, EIB202 displayed 3.5–5, 5–15, and 4–25 days of lag before being totally inhibited in growth at 28, 16, and 10 °C, respectively, indicating a tight growth containment for Δ*tatABCD* in high-salt conditions (Fig. 5a–c). Furthermore, the difference in growth inhibition between EIB202 and Δ*tatABCD* cultured at 10 °C was more evident than that cultured at 28 or 16 °C, suggesting that temperature was a factor involved in the biological containment of Δ*tatABCD* in high-salt conditions. To further determine whether the non-culturable cells were entering into a so-called viable but not culturable state (Du et al. 2007), a live/dead "BacLight bacterial viability" kit (Invitrogen) was utilized; the results indicated that the cells were 100 % dead at the time when no single bacterial cell was cultured on the plates (data not shown). Taken together, our data provided evidence that *tatABCD* played essential roles in high-salt stress response and that Δ*tatABCD* could be employed as a strain for salt-dependent biological containment, which is active in low-salt conditions in fish and contained in high-salt conditions in seawater.

Use of the Tat system as a target for the construction of a live attenuated vaccine

To further test the feasibility of using the Tat system as a biological containment in marine fish vaccinology, we deleted the TTSS genes, *eseBCD* and *escA*, which were

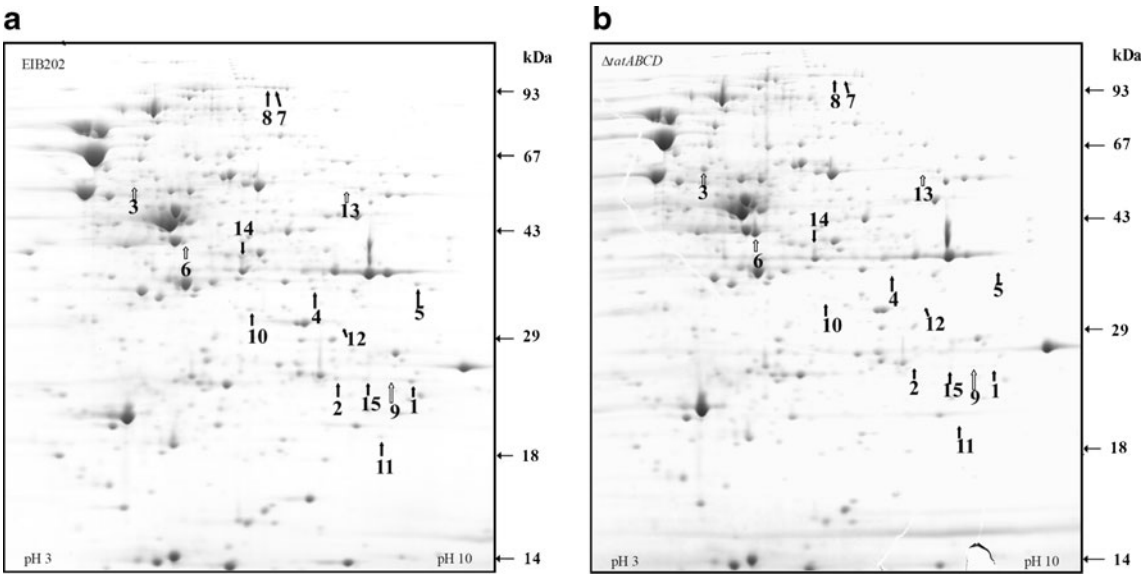

Fig. 4 2-DGE separation of WCLP in *E. tarda* EIB202 (**a**) and Δ*tatABCD* (**b**) cultured in LB medium containing 4 % NaCl. Three parallel gels were repeatedly run for three times; representative gels were exhibited. *Arrows* indicate the corresponding proteins in Table 3 analyzed by MALDI-TOF/TOF. Upregulated proteins in Δ*tatABCD* were indicated by *open arrows*, while downregulated proteins were indicated by *solid arrows*

Table 3 List of altered WCLP under high-salt condition

Spot no.[a]	Name	Accession no.	Subcellular location	COG[b]	Character description	MW (kDa)/ p*I*	Moscow score	Coverage (%)	Pep. count	Fold change[c]
Metabolism										
1		ETAE_1540	Periplasmic	E	Amino-acid ABC transporter periplasmic component	28/8.9	651	61	14	0.30
2	PepE	ETAE_0156	Cytoplasmic	E	Peptidase E	27/6.2	433	54	9	0
3		ETAE_2022	Cytoplasmic	E	Zinc metalloproteinase aureolysin	51/5.2	160	32	11	3.25
4	PstS	ETAE_3538	Periplasmic	P	ABC-type phosphate transport system, periplasmic component	36/7.7	429	46	15	0.39
5		ETAE_2768	Periplasmic	P	ABC transporter, substrate binding protein	38/8.4	251	37	8	0.44
6	HemB	ETAE_0154	Cytoplasmic	H	Delta-aminolevulinic acid dehydratase	38/5.4	189	42	24	6.62
7	NuoG	ETAE_2380	Cytoplasmic	C	NADH dehydrogenase/NADH: ubiquinone oxidoreductase 75-kDa subunit (chain G)	102/5.9	140	20	18	0.47
8	NuoG	ETAE_2380	Cytoplasmic	C	NADH dehydrogenase/NADH: ubiquinone oxidoreductase 75-kDa subunit (chain G)	102/5.9	67	19	15	0.50
9	SdhB	ETAE_2585	Cytoplasmic membrane	C	Succinate dehydrogenase iron–sulfur subunit	27.2/7.5	86	31	11	∞[d]
10		ETAE_3014	Periplasmic	G	Hypothetical protein ETAE_3014	35/6.8	64	35	7	0.49
Cellular processes and signaling										
11	DsbA	ETAE_1062	Periplasmic	O	Periplasmic protein disulfide isomerase I	23/8.4	45	11	3	0
12		ETAE_1482	Unknown	O	Putative aldose 1-epimerase	33/6.3	52	29	7	0
13	GlmU	ETAE_3535	Cytoplasmic	M	*N*-acetylglucosamine-1-phosphate uridyltransferase	47/6.2	285	48	17	2.35
14		ETAE_1187	Cytoplasmic membrane	D	Putative ATPase	40/5.8	304	18	4	0
Information storage and processing										
15		ETAE_2758	Unknown	J	Putative ribonuclease, T2 family	28/7.6	69	21	5	0

[a] Spot no. was consistent with the number in Fig. 4

[b] For details of the COG catalog, see the related context

[c] Fold change was considered as proteins expressed in Δ*tatABCD*/EIB202

[d] ∞ indicates that protein was absent in EIB202, in contrast to Δ*tatABCD*

established to be essential for bacterial virulence (Xiao et al. 2011a) in the context of Tat abrogation along with pEIB202 cured in *E. tarda* to generate a LAV candidate WYM1 (Table 1). The abilities of EIB202 and WYM1 to proliferate in vivo in turbot were assayed. Fish were i.p. injected with the same dose of the bacteria, and the infection dynamics was examined in different organ samples over 11 days post-injection. By day 1, EIB202 and WYM1 emerged in the organ samples of injected fish, such as liver, kidney, spleen, and intestine (Fig. 6a). EIB202 proliferated in these organs in the following 4 days and reached the highest levels at day 5 post-injection and then kept high levels in the following days of observation, whereas the fish injected with WYM1 displayed gradually decreased bacterial burdens over all the days post-infection (Fig. 6a). Death in fish infected with EIB202 was observed after day 3 post-injection; all the fish died during the following 11 days. However, only 6.7 % fish died when infected with WYM1. The ability of EIB202 and WYM1 to sustain in filter-sterilized seawater was also tested. The results indicated that WYM1 exhibited a significantly more rapid elimination rate ($p<0.001$) than EIB202 in filter-sterilized seawater and could only be sustained in seawater for about 1 week before being totally eliminated (Fig. 6b).

To evaluate the virulence and immunoprotective potential of WYM1, healthy turbot were i.p. injected with EIB202 or WYM1. For virulence test, a 5×10^3-CFU/g dose of EIB202 or WYM1 was employed. After a 32-day observation, the fish in the control group injected with sterile saline water showed no death (data not shown). The accumulated mortalities of EIB202-challenged and WYM1-vaccinated groups were 100 and 6.67 %, respectively (Fig. 6c). Then, the fish alive inoculated with WYM1 were i.m. challenged with EIB202 at a dose of 1×10^2 CFU/g. The results indicated that the accumulated mortalities of the WYM1-vaccinated group and the sterile saline water-injected group were 18.19 and 100 %, respectively (Fig. 6d). Hence, the RPS of turbot vaccinated with WYM1 via i.m. injection was 81.81 %,

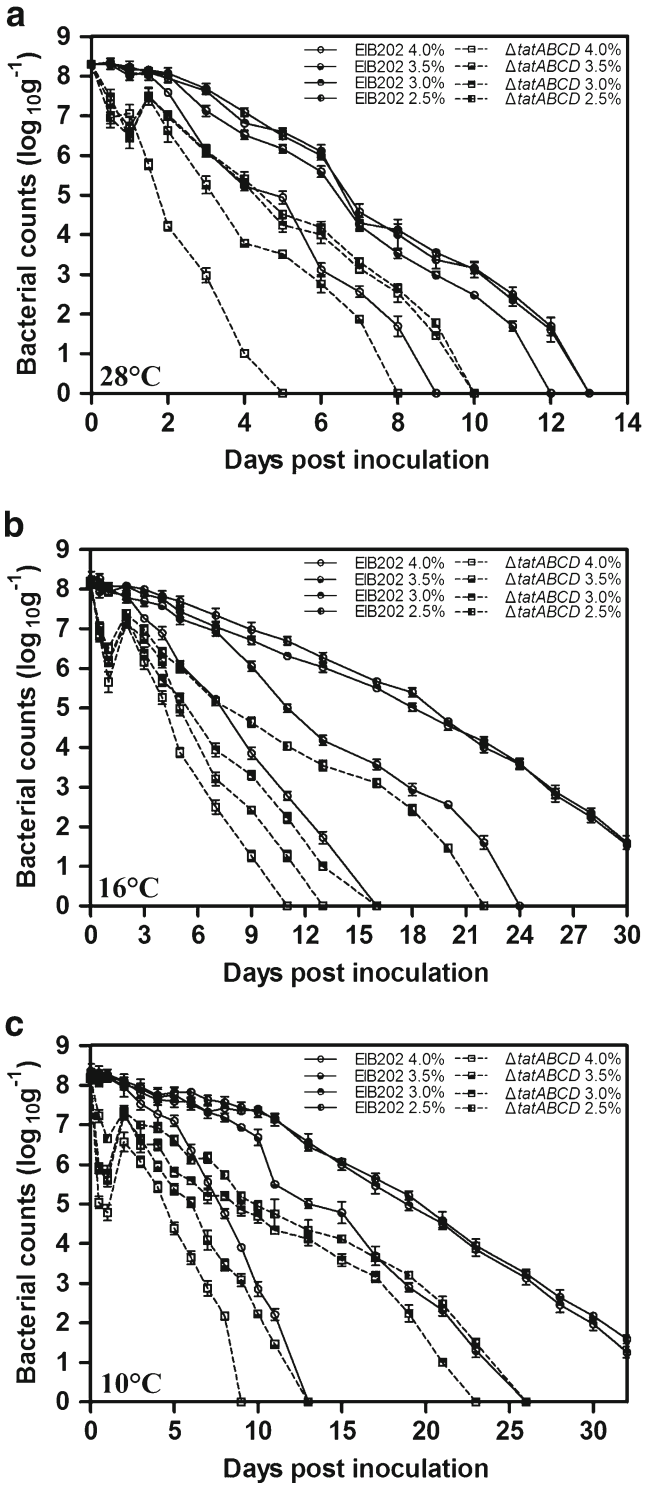

Fig. 5 Containment of *E. tarda* strains in different salt conditions and temperatures. Growth of EIB202 and Δ*tatABCD* cultured at 28 °C (**a**), 16 °C (**b**), and 10 °C (**c**) in artificial sterile seawater with different NaCl concentrations of 4.0, 3.5, 3.0, and 2.5 %. The data were representative of two replicated experiments yielding similar results. *Error bars* indicate standard deviations of four technical replicates

indicating that it has merits as a potential LAV candidate against edwardsiellosis. Taken together, the Tat system plays essential roles in response to high-salt stress and could be used as a novel biological containment for LAV in marine fish vaccinology.

Discussion

As a versatile fish pathogen, *E. tarda* possesses the large genetic properties devoted to fitness under diverse conditions, including intracellular niches, as revealed by the whole-genome sequencing (Wang et al. 2009a). Regarding osmotic stress, EIB202 has developed the ability to tolerate high concentrations of NaCl (up to 5 %) while lacks the genes responsible for the synthesis and uptake of several compatible solutes as well as osmolarity responding proteins, which supports the idea that EIB202 might utilize unusual mechanisms to cope with the osmotic challenges (Wang et al. 2009a). Here, we demonstrated that the Tat system was functional for protein export carrying ssTorA across the cytoplasmic membrane to the periplasm (Fig. 1) and various stress responses (Fig. 3), and could be used as a novel biological containment for LAV development for marine fish or fish cultured in seawater.

The Tat system has been found to be involved in several important processes like the biogenesis of the cell envelope (Bernhardt and De Boer 2003), biofilm formation (Ochsner et al. 2002), stress adaptation (Lavander et al. 2006), and the secretion of virulence factors (De Buck et al. 2008). The situation seems to be different to some extent in *E. tarda*, where Δ*tatABCD* displayed no apparent growth defects during in vitro growth in normal rich medium (Fig. 3d). The results also indicated that the Tat mutant displayed no chain-forming colony morphology under electron microscopy or cell envelope and biofilm formation defects in *E. tarda* (data not shown). The Tat mutant secreted comparable amounts of TTSS and T6SS proteins into the extracellular medium to EIB202 when cultured in DMEM medium (data not shown), caused disease in fish, and showed marginal virulence attenuation in vivo in fish models. Thus, Tat seems to play elusive roles in the physiological and virulence mechanism of *E. tarda*. Phenotypic analysis indicating that Δ*tatABCD* was deficient in H_2S production (Fig. 2a), hemolytic activity (Fig. 2b), and increased susceptibility to stress response, including high salinity (Fig. 3d–f), confirmed the above notion.

It is intriguing to disclose that Tat was involved in the high-salt tolerance in *E. tarda*. In halophilic archaeon, Tat systems have evolved to play essential parts in adaptation to niches with high osmotic stresses (Bolhuis 2002). In bacteria, there are very few reports regarding to the roles of the Tat pathway in response to osmotic stresses, except the evidences provided in *P. aeruginosa* PAO1 (Ochsner et al. 2002) and *Campylobacter jejuni* (Rajashekara et al. 2009).

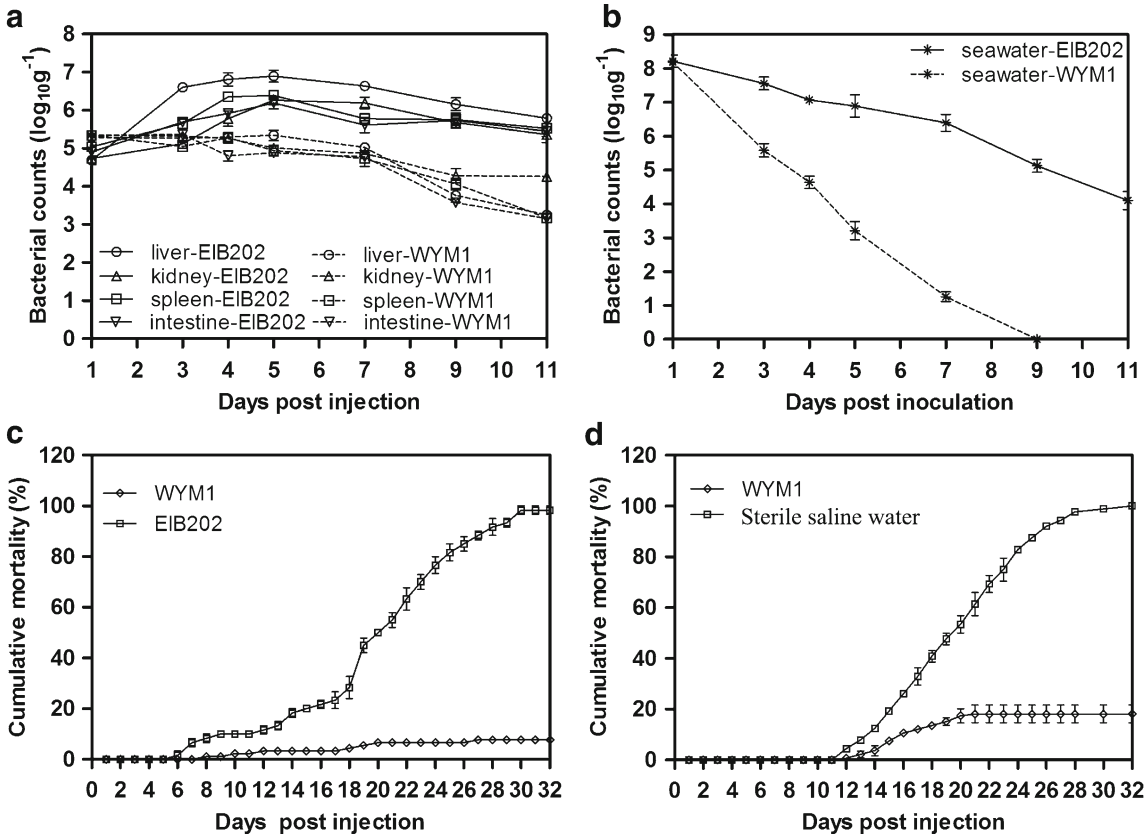

Fig. 6 Evaluation of in vivo/in vitro survival, virulence, and vaccine efficiency of WYM1 in turbot. Propagation of EIB202 and WYM1 in turbot organs following i.p. injection (**a**) and in seawater (**b**). **c** The cumulative mortality of fish after i.p. injection with EIB202 and WYM1 at a dose of 5×10^3 CFU/g was recorded over 32 days, respectively. **d** The RPS of the vaccinated fish after challenging with EIB202 was recorded for 32 days. The results are presented as the mean ± standard deviation (n=3)

However, a *Yersinia pseudotuberculosis* mutant strain with a disrupted Tat system was not affected in in vitro growth or more susceptible to high osmolarity (2.4 M NaCl; Lavander et al. 2006). We further tested the high-salt-stressed proteome significantly affected by Tat inactivation in *E. tarda* (Fig. 4). In the list of identified significantly altered WCLP under the high-salt condition (Table 3), HemB displayed a significant 6.62-fold overexpression in Δ*tatABCD*, which might be resulted from the polar effect of deletion in *tatABCD* destroying the stem-loop between *tatC* and *tatD* (data not shown) and activate the transcription of the direct downstream *hemB* gene (Lavander et al. 2006; Wexler et al. 2000). The proteomics analysis of Δ*tatABCD* cultured in LB medium also spotted the upregulated HemB protein with similar fold changes (5.05-fold, our unpublished data). Interestingly, a protein (ETAE_2022) encoding zinc metalloproteinase aureolysin was also observed to be upregulated by about 3.3-fold, which suggested that the high-salt tolerance might be, to some extent, related to the resistance of cationic antimicrobial peptides in *E. tarda* (Nizet 2006). DsbA was an oxidizing disulfide catalyst for folded proteins in the periplasm (Masip et al. 2004). The disappearance of DsbA strongly underlies the weakened salt tolerance as well as other stress responses in Δ*tatABCD*. In Δ*tatABCD*, the abrogation of ETAE_1187 possessing a putative interaction with PotD (ETAE_1886; http://string.embl.de/), which is a membrane-associated surface protein and binds polyamines playing an important role in stress response (Shah et al. 2008), might further underpin the defects of osmotic response in this bacterium.

In the development and application of LAV or other genetically modified organisms, biological containment systems are required to prevent the organisms from dissipating into the natural environments. Regulated replicon gene ParB (Gerdes et al. 1986) as well as toxin–antitoxin systems such as lysis protein E were often used (Guan et al. 2011) in biological containment. Such kinds of biological containment systems include the use of complex regulatory circuits and are subject to two major drawbacks: (1) the specificity and efficiency of the killing elements and (2) the arising of mutations in the killing circuits resulting in the killing escape of mutants (Haidinger et al. 2003). Other types of biological systems beyond the so-called active biological containment system include the use of auxotroph mutants in the genes for the synthesis of aromatic amino acids

(*aroA*, *aroC*, or *aroD*; Bumann et al. 2000; Priebe et al. 2002), thymidylate (*thyA*; Morona et al. 1991), purine (*purA* and *pure*; Cheville et al. 1996), guanine (*guaA*; Kotloff et al. 2004), and aspartate-β-semialdehyde (*asd*; Santander et al. 2010). However, the biological containment systems based on these targets will render too much fundamental effects on the live vaccines to limit their vaccination potentials, such as issue or organ targeting, longer persistence, as well as triggering cellular immune reactions (Bumann et al. 2000). In this study, our data indicated that the inactivation of Tat elevated the sensitivity to salinity in *E. tarda*. We further described a mutant strain WYM1 which was constructed based on the abrogation of Tat and the TTSS as well as the curing of the endogenous R plasmid pEIB202. The bacterium was characterized to be drastically attenuated by a higher than 10^5-fold and a RPS of 81.81 % as evaluated on the turbot model. Furthermore, when released into seawater, Δ*tatABCD* could be eliminated significantly earlier than EIB202, which would enhance the environmental safety. In summary, the data herein described the essential roles of the Tat pathway in stress response, especially in high-salt tolerance in *E. tarda*, which has merits as a novel biological containment for live attenuated vaccine in marine fish vaccinology.

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