J. Gen. Appl. Microbiol., **55**, 57–61 (2009)

Short Communication

Immunoprotective analysis of two *Edwardsiella tarda* antigens

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(Received June 23, 2008; Accepted September 20, 2008)

Key Words——*Edwardsiella tarda*; immune protection; immunogen; RPS; vaccine

Edwardsiella tarda is a Gram-negative bacterium of the family *Enterobacteriaceae*; it is an opportunistic pathogen with a broad host range that includes humans and animals (Miyazaki and Kaige, 1985). The major concern raised by this bacterium is its ability to cause a severe systematic disease, edwardsiellosis, in many economically important species of fish including tilapia (Kubota et al., 1981), flounder (Nakatsugawa, 1983), carp (Sae-Oui et al., 1984), channel catfish (Meyer and Bullock, 1973), mullets (Kusuda et al., 1976), and eels (Wakabayashi and Egusa, 1973). Owing to the lack of effective means of control and prevention, especially the lack of effective vaccines, *E. tarda* infection has been the cause of heavy economic losses in aquaculture industries worldwide. To date a number of virulence systems and factors are found to exist in *E. tarda*, notably the type III secretion system (TTSS) (Tan et al., 2005), the type VI secretion system (T6SS) (Zheng and Leung, 2007), hemolysins (Hirono et al., 1997; Janda and Abbott, 1993), catalase (Srinivasa Rao et al., 2003), and dermatotoxins (Ullah and Arai, 1983), but only one (to our knowledge) protein immunogen with significant protective capacity has been reported in this bacterial species (Liu et al., 2005). Verjan et al. (2005) have recently identified the major *E. tarda* antigens, but the immunoprotective potentials of these proteins have not been investigated. In addition to subunit vaccines, immunization with lipopolysaccharides, ghost cells and attenuated *E. tarda* have also been tried to combat *E. tarda* infection (Kwon et al., 2006; Mo et al., 2007; Salati et al., 1984). With an aim to discover new *E. tarda* immunogens with protective properties, we examined nine *E. tarda* proteins that have been reported as virulence factors or antigens but have not been studied from the aspect of immunoprotectivity.

Of the nine proteins analyzed in this study, three (EseB, EseD, and ORF26) are TTSS components (Tan et al., 2005), one (EvpC) is a T6SS protein (Zheng and Leung, 2007), four (Et18, ET28, Et38, and Et49) are antigenic proteins that reacted with the rabbit anti-*E. tarda* serum (Verjan et al., 2005), and the remaining one (EtfA) is a fimbrial protein involved in hemagglutination (Sakai et al., 2003). These proteins were chosen based on the consideration that (i) they are exported proteins, with subcellular locations in the extracellular, outermembrane, or periplasm; (ii) they are known to be virulence-associated or antigenic and widely distributed in virulent *E. tarda* strains. The genes encoding EseB, EseD, ORF26, EvpC, Et18, Et28, Et38, Et49, and EtfA were cloned from TX1, a pathogenic *E. tarda* strain isolated from diseased fish at a fish farm in north China (Zhang et al., 2008), by PCR using the primer pairs EBF1/EBR1, EDF1/EDR1, 26F3/26R1, EPCF1/EPCR1, 18F1/18R1, 28F1/28R1, 38F1/38R1, 49F1/49R1, and 19F1/19R1, respectively. The PCR

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Table 1. Bacterial strains, plasmids, and primers used in this study.

Strain, plasmid or primer	Relevant characteristics	Source or reference
Bacillus sp.		
B187	Fish commensal isolate	This study
E. coli		
DH5α	Host strain for general cloning	TaKaRa (China)
BL21(DE3)	F^- *ompT hsd*S_B($r_B^-m_B^-$)*gal dcm*	Tiangen (China)
E. tarda		
TX1	Fish pathogen	Zhang et al., 2008
Plasmids		
pET258	Expression plasmid	Zhang and Sun, 2007
Primers	Sequences (5′→3′)[a]	
EBF1	CATATGACTGTCAATACAGACT (*Nde*I)	
EBR1	CTCGAGGCGGATATTCTGGGCGA (*Xho*I)	
EDF1	CATATGACGACTATCGACAGCG (*Nde*I)	
EDR1	CTCGAGGGACATGCGTACGCTGC (*Xho*I)	
26F3	CATATGCGTAAATGGGTTCTGCTG (*Nde*I)	
26R1	CTCGAGTGTGCGGGAGGACGCCGAA (*Xho*I)	
EPCF1	CATATGGCTTTTGATACTTATATC (*Nde*I)	
EPCR1	CTCGAGCTTTTTCTTGTTGGTAATAAG (*Xho*I)	
18F1	CATATGACGATGCGTTTTCCCT (*Nde*I)	
18F6	CTCGAGGGAGGAGGAGGAGGAGGAGGATGCGTCGCCGCCGT (*Xho*I)	
18R1	CTCGAGCTTCAGCAGCGAGAACG (*Xho*I)	
18R7	CTCGAGCCCGGGCTTCAGCAGCGAGAACGCG (*Xhol*)	
28F1	CATATGGGCTGTGGACAGGAAGAGA (*Nde*I)	
28R1	CTCGAGCCAGCCTTTTACTGCCCCG (*Xho*I)	
38F1	CATATGAAGATCGAAGAGGGTAAACTG (*Nde*I)	
38R1	CTCGAGTTTTTTGAGGATCCGCTCCTTG (*Xho*I)	
49F1	CATATGGCAGAGACCTCTTCCGCC (*Nde*I)	
49R1	CTCGAGCTGCATCAGCAGATACAGG (*Xho*I)	
19F1	CATATGGCTAACGGTAAAGTTGAGTT (*Nde*I)	
19R1	CTCGAGTTTATATTCGATGGTGAACGGG (*Xho*I)	

[a]Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

products were ligated into pET258 between the *Nde*I/*Xho*I sites, resulting in plasmids pEseB, pEseD, pORF26, pEvpC, pEt18, pEt28, pEt38, pEt49, and pEtfA, respectively. Subsequent sequence analysis revealed that the amino acid sequences of the nine proteins derived from TX1 are 99−100% identical to those of their counterparts reported previously in *E. tarda* strains isolated from different geographical locations, suggesting that these proteins are probably highly conserved among virulent *E. tarda* isolates. The *E. coli* strain BL21(DE3) was transformed separately with pEseB, pEseD, pORF26, pEvpC, pEt18, pEt28, pEt38, pEt49, and pEtfA. The transformants were grown to mid-log phase and the expression of the recombinant proteins in the transformants were induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 μM. The His-tagged proteins were then purified with nickel-nitrilotriacetic acid (Ni-NTA) beads as described previously (Zhang and Sun, 2007). The purified proteins were analyzed by electrophoresis in 0.1% sodium dodecyl sulfate (SDS)/12% polyacrylamide gels and viewed after being stained with Coomassie blue (Fig.1).

To determine the immunoprotective potentials of the above purified proteins against *E. tarda*, vaccination tests were performed using Japanese flounder as animal models. For this purpose, B187, a fish commensal isolate of the *Bacillus* sp. used here as an adjuvant, was grown in LB medium to OD_{600} 0.6; the cells were washed and resuspended in phosphate-buffered saline (PBS) to 4×10^8 CFU/ml. The purified recombinant *E. tarda* proteins were each diluted in the B187-PBS

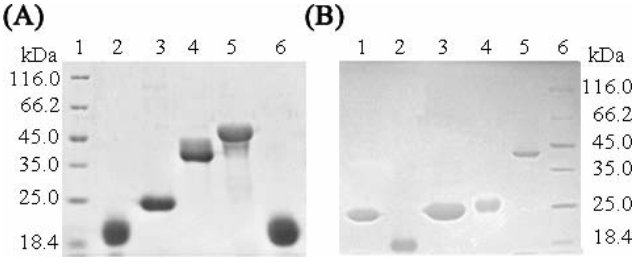

Fig. 1. Electrophoresis analysis of the purified recombinant *E. tarda* proteins.

The nine recombinant *E. tarda* proteins were purified with Ni-NTA beads and subjected to electrophoresis in 12% SDS-polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue. (A) Et18, Et28, Et38, Et49, and EtfA (Lanes 2–6). (B) EseD, EvpC, EseB, ORF26, and EEH (Lanes 1–5).

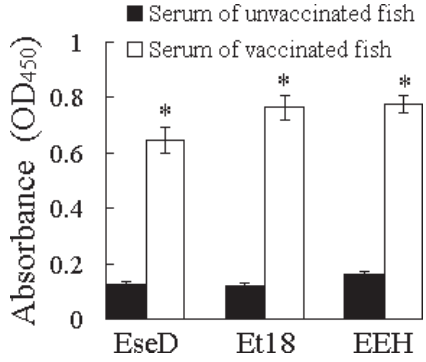

Fig. 2. Detection of specific antibodies in the vaccinated fish.

Antisera were taken from at least three vaccinated fish at the 5th (for EseD-vaccinated fish) and the 6th (for Et18- and EEH-vaccinated fish) week post immunization and used for ELISA. Data are the means of three independent assays. $^{*}p<0.01$ (Student's *t* test).

suspension to 350 μg/ml. Disease-free Japanese flounder (~16 g), purchased from a commercial fish farm in China, were divided randomly into ten groups (38 fish/group) named 1 to 10. The fish in groups 1 to 9 were, respectively, injected intraperitonealy (i.p.) with 100 μl of the following proteins diluted in B187-PBS: EseB, EseD, ORF26, EvpC, Et18, Et28, Et38, Et49, and EtfA. The fish in group 10 (the control group) were injected with 100 μl of B187-PBS. Twenty-five days later the fish in groups 1 to 9 were boosted with 35 μg of the same proteins used in the first immunization but this time the proteins were diluted in 100 μl PBS without B187; the fish in the control group were sham boosted with 100 μl PBS. At the 14th day post boost, the fish in all groups were challenged with 100 μl TX1 that had been grown in LB medium to OD_{600} 0.6, washed and resuspended in PBS to 2×10^7 CFU/ml. The vaccination experiments were repeated once and each time the fish were monitored for mortality over the 14 days post challenge. Relative percent of survival (RPS) was calculated according to the formula: RPS=$\{1-$(%mortality in vaccinated fish/%mortality in control fish)$\}\times100$ (Amend, 1981). The results showed that the mean accumulated mortality of the control fish was 94.7% while those of the fish vaccinated with Et18 and EseD were, respectively, 36.9 and 46.1%, which are significantly ($p<0.001$, chi-square test) lower than that of the control fish; hence the RPS of Et18 and EseD were 61 and 51.3% respectively. The RPS values of other seven proteins were less than 50%.

To examine whether Et18 and EseD induced the production of specific antibodies in the vaccinated animals, the antisera were prepared from the blood taken from the vaccinated fish at 4, 5, and 6 weeks post vaccination. The presence of Et18- and EseD-specific antibodies in the antisera were determined by enzyme-linked immunosorbent assay (ELISA), which was carried out according to the method recommended by Aquatic (UK) using fish antisera as the first antibody, mouse anti-Japanese flounder IgM monoclonal antibody (Aquatic) as the second antibody, and HRP-conjugated goat anti-mouse IgG (Bios, China) as the third antibody. In addition, western immunoblotting was also performed by applying the purified recombinant Et18 and EseD into 0.1% SDS/12% polyacrylamide gels and, after electrophoresis, transferring the proteins to nitrocellulose membranes; subsequent immunoblotting was conducted as described by Kawai et al. (2004) using the antibodies described above for ELISA. The result of ELISA showed that the highest antibody titers were detected in the antisera taken at, respectively, the 6th and 5th week post vaccination from the Et18- and EseD-immunized fish (Fig. 2). The results of western immunoblotting showed that the Et18 and EseD antsera could react specifically with the respective proteins but not with each other (Fig. 3, A and B).

Zhang et al. (2007) have observed that fusion of the *Vibrio harveyi* antigens OmpK and GAPDH produced a synergistic immune response. We then wondered whether an organic hybrid consisting of EseD physically linked to Et18 would have better immunoprotective efficacy than either Et18 or EseD. To investigate this idea, the coding sequence of the processed Et18

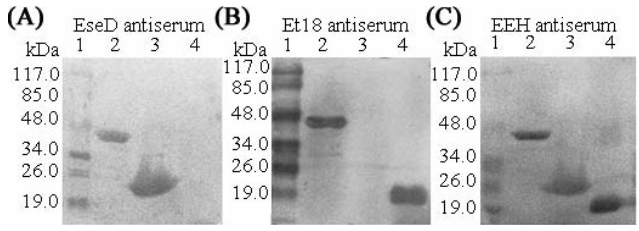

Fig. 3. Specific reaction of the EseD, Et18, and EEH antisera with the respective immunogens.

The purified recombinant EEH, EseD, and Et18 (Lanes 2, 3, and 4, respectively, in all panels) were electrophoresed in 12% SDS-polyacrylamide gels. After electrophoresis the proteins were transferred to nitrocellulose membranes and blotted with the EseD (A), Et18 (B), and EEH (C) antisera, respectively. Lane 1 in all panels, protein size markers.

was amplified by PCR with the primers 18F6 and 18R7, the former containing a sequence tag that encodes a linker (LEGGGGGGG), which was designed based on the linker used by Zhang et al. (2007). The PCR products were ligated into pEseD at the *Xho*I site, resulting in plasmid pEEH, in which *eseD* forms, through the linker, a translational fusion with *et18*. BL21(DE3) was transformed with pEEH and the hybrid protein, i.e. EseD-linker-Et18 (named EEH), was purified from BL21(DE3)/pEEH under native conditions as described above.

To examine the immunoprotective potential of EEH, vaccination experiments were performed with the purified EEH exactly as described above. The results showed that vaccination with EEH yielded a mean RPS of 71%. Specific antibodies as detected by ELISA peaked at the 6th week post vaccination in EEH-vaccinated fish (Fig. 2). Western immunoblotting analysis showed that the EEH antiserum could react not only with the EEH hybrid but also with Et18 and EseD (Fig. 3C), suggesting that the EEH antiserum contained antibodies to both Et18 and EseD. Since the Et18 antiseum failed to recognize EseD and vice versa, which implied that Et18 and EseD possess different epitopic structures, the co-reaction of Et18 and EseD with the EEH antiserum indicated that, though existing as one body in EEH, Et18 and EseD functioned as distinct immunogens and elicited distinct host immune responses. The enhanced immunoprotection observed with EEH suggested that the physical closeness of EseD and Et18 in EEH may have facilitated the interaction of the host immune system with one or both of the component antigens.

In conclusion, our study demonstrated that EseD, an effector protein of the virulence-associated TTSS system, and Et18, a major *E. tarda* antigen, could confer significant ($p < 0.001$) protection upon Japanese founder against *E. tarda* infection when administered as individual immunogens and that the genetically recombinant hybrid vaccine EEH is a more effective immunogen than either of the component antigens alone.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC) grant 40576071 and the 973 Project of China grant 2006CB101807.

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